TITLE

METHODS FOR TREATING AND/OR REVERSING NEURODEGENERATIVE DISEASES AND/OR DISORDERS

BACKGROUND

[0001] A stroke is the sudden death of brain cells in a localized area due to inadequate blood flow. A stroke occurs when blood flow is interrupted to part of the brain. Without blood to supply oxygen and nutrients and to remove waste products, brain cells quickly begin to die. Depending on the region of the brain affected, a stroke may cause paralysis, speech impairment, loss of memory and reasoning ability, coma, or death.

SUMMARY

[0002] The present disclosure relates to methods for treating and/or reversing neurological diseases or disorders associated with neuronal cell loss (*e.g.*, cell death caused by cerebral ischemic stroke, hemorrhagic stroke, cerebral palsy, or traumatic brain injury) in a brain. Such methods may be useful for treating symptoms resulting from stroke or other brain injuries.

[0003] The present disclosure also provides methods for treating a disease or disorder associated with neuronal cell loss in a brain including, for example, for treating the symptoms resulting from stroke or other brain injuries, by obtaining an expanded neural stem cell population and introducing a therapeutically effective amount of the expanded neural stem cell population to at least one area of the brain of a subject, wherein the neural stem cell population differentiates into neurons that integrate *in vivo* into the brain of the subject. Without wishing to be bound by a theory of the invention, it is believed that the integrated neurons then promote repair of the injured tissue by replacing the dead neurons, regenerating the host neurons, and/or inducing plasticity of the host circuitry to re-establish the damaged neuronal connections that include the donor neurons.

[0004] In some embodiments, introducing the therapeutically effective amount of the expanded population includes injecting at least a portion of the therapeutically effective amount into one to a plurality of areas of the brain of the subject.

[0005] In some embodiments, expanding the at least one neural stem cell includes culturing the neural stem cell in the absence of serum.

[0006] In some embodiments, expanding the at least one neural stem cell includes exposing the at least one neural stem cells to at least one growth factor. In some embodiments, the growth factor is selected from the group consisting of bFGF, EGF, TGF-alpha, aFGF and combinations thereof.

[0007] In some embodiments, at least 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the expanded stem cell population is capable of generating neurons in brain tissue of the subject.

[0008] In some embodiments, introducing the therapeutically effective amount of the expanded stem cell population includes injecting at least a portion of the therapeutically effective amount into a plurality of areas of brain tissue of the subject.

[0009] In some embodiments, the areas of the brain involve the motor pathway—the neuronal fibers that conduct electrical signals to and from the brain, brainstem, and spinal cord that govern muscle contraction. Such areas include the motor cortex, striatum, internal capsule, thalamus, midbrain, brainstem, and cerebellum. Due to stroke or trauma, a discrete area or areas within this pathway may be damaged, resulting in symptoms of paralysis, spasticity, rigidity, and other motor dysfuctions.

[0010] In another embodiment, a suspension of the spinal cord-derived human neural stem cells are injected into, near, or around the affected area(s). The injected cells differentiate into neurons and glia which integrate into the affected area to promote repair of motor pathway that results in motor recovery and/or amelioration of the motor dysfunction.

[0011] In some embodiments, the subject had an event that caused cerebral ischemia such as a heart attack or stroke.

[0012] The present disclosure also provides methods for treating stroke in a subject in need thereof comprising isolating at least one neural stem cell from spinal cord tissue of a human; expanding *in vitro* said neural stem cell to form an expanded population; concentrating the expanded population; and introducing a therapeutically effective amount of said expanded population to at least one area of the subject brain.

[0013] In other embodiments, the expanded neural stem cell population is derived from a spinal cord tissue including, for example, a post-mortem fetal spinal cord tissue of a human.

[0014] In some embodiments, the gestational age of the post-mortum fetus is between about 5 and about 20 weeks.

[0015] In some embodiments, expanding the at least one neural stem cell includes culturing the neural stem cell in the absence of serum.

[0016] In some embodiments, expanding the at least one neural stem cell includes exposing the at least one neural stem cells to at least one growth factor. In some embodiments, the growth factor is selected from the group consisting of bFGF, EGF, TGF-alpha, aFGF and combinations thereof.

[0017] In some embodiments, at least 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the expanded stem cell population is capable of generating neurons in brain tissue of the subject.

[0018] In some embodiments, introducing the therapeutically effective amount of the expanded stem cell population includes injecting at least a portion of the therapeutically effective amount into a plurality of areas of brain tissue of the subject.

[0019] These and other embodiments of the invention are described in further detail herein below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] 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.

[0021] Figure 1 shows that transplantation of human spinal-cord derived neural stem cells (HSSC) improved neurological behavior after cerebral ischemia. Neurological behavior measurement protocols were used to assess neurological function before and after MCA ligation in HSSC-implanted rats (n = 10) and buffer-injected control rats (n = 10). HSSC rats exhibited significantly reduced body asymmetry in comparison with control rats in a body swing test (Panel A). Locomotor activity was examined before and after cerebral ischemia in all animals. Vertical activity, vertical movement time, and the number of vertical movements showed a significant increase after cerebral ischemia in rats receiving HSSC transplantation in comparison with control rats (Panels B-D). Furthermore, measurement of grip strength was performed to examine the forelimb strength of all experimental rats after each of the two treatments (Panel E). The results revealed a higher ratio of grip strength in the HSSC group than in the control groups.

[0022] Figures 2A-B shows images obtained from an immunohistochemical (IHC) analysis of exogenously transplanted HSSC engrafted in the ischemic rat brain. The penumbra of HSSC-treated ischemic rat brains were immunostained with antibodies for

human specific nuclear antibody (HuNu) (Figure 2A) and neuronal specific enolase (NSE) (Figure 2B).

DETAILED DESCRIPTION

[0023] The disclosed methods are related to the treatment of neurological diseases or disorders associated with neuronal cell loss in the brain. It has now been discovered that a human spinal cord stem cell line (HSSC), when transplanted into a peri-infarct area in the brain, was able to survive and differentiate en mass into neurons in the damaged tissue. Notably, the differentiated neurons integrated (i.e. established interconnectivity with other neurons) into the surrounding brain tissue and reversed motor deficits. As such, the methods of the disclosure may be used to treat including, reverse, neurodegenerative diseases including, for example, diseases of the brain. In particular, the methods of the disclosure may be used to treat any motor symptom (*e.g.*, paresis, paralysis, spasticity or rigidity) arising from cerebral ischemia. The motor symptoms may be in acute, subacute, or chronic phases of the disease (*e.g.*, in chronic stroke).

[0024] The present disclosure provides methods for treating and/or reversing a neurological or neurodegenerative disease or disorder associated with neuronal cell loss (*e.g.*, cell death) in a brain (*e.g.*, a brain tissue such as cerebral hemispheres, cerebral cortex, subcortex motor cortex, striatum, internal capsule, thalamus, hypothalamus, hippocampus, midbrain, brainstem, and cerebellum) by obtaining an expanded neuronal stem cell population (*e.g.*, a human spinal cord stem cell line) and introducing a therapeutically effective amount of the expanded neuronal stem cell population to one to a plurality of areas of the brain of a subject, wherein the neuronal stem cell population is capable of differentiating into neurons that integrate *in vivo* into the brain of the subject.

[0025] The present disclosure also provides methods of treating and/or reversing paresis, paralysis, spasticity, rigidity or muscular hyperactivity conditions associated with cerebral ishcemia in a subject in need thereof by isolating at least one neural stem cell (*e.g.*, a human spinal cord stem cell line) from a mammal; expanding *in vitro* said neural stem cell to an expanded population; concentrating the expanded population; and introducing a therapeutically effective amount of said expanded population to at least one area of the subject brain (*e.g.*, a brain tissue such as cerebral hemispheres, cerebral cortex, subcortex motor cortex, striatum, internal capsule, thalamus, hypothalamus, hippocampus, midbrain,

brainstem, and cerebellum). In one embodiment, at least 20% of the expanded population is capable of generating neurons in the subject brain.

[0026] The disclosed methods include the use of NSCs to ameliorate a neurodegenerative condition. As used herein, the term, "NSCs" can also refer to neural or neuronal progenitors, or neuroepithelial precursors. NSCs can be functionally defined according to their capacity to differentiate into each of the three major cell types of the CNS: neurons, astrocytes, and oligodendrocytes.

[0027] In some embodiments, "treating" or "treatment" of a disease, disorder, or condition 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.

[0028] In some embodiments, "effective amount," as used herein, refers to the amount of an active composition that is required to confer a therapeutic effect on the subject. A "therapeutically effective amount," as used herein, refers to a sufficient amount of an agent or a compound 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 composition including a compound as disclosed herein 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 a compound disclosed herein, such as a compound of Formula (A) or Formula (I), 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.

[0029] In one embodiment, the NSCs are multipotential such that each cell has the capacity to differentiate into a neuron, astrocyte or oligodendrocyte. In another embodiment, the NSCs are bipotential 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 bipotential cells generating both neurons and astrocytes *in vitro* and include at least unipotential cells generating neurons *in vivo*.

[0030] 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 bipotential 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. In one embodiment, the ratio of neurons to astrocytes is about 20:80 to about 80:20, for example about 50:50.

[0031] In one embodiment of the disclosed methods, the cells to be transplanted into subjects are derived from brain tissue. In another embodiment, NSCs are isolated from human fetal brain tissue at gestational ages of between about 5 to about 20 weeks. It should be appreciated that the proportion of the isolatable neural stem cell population can vary with the age of the donor. Expansion capacity of the cell populations can also vary with the age of the donor. Such regional and temporal specificity of NSCs indicates that NSCs behave as fate-restricted progenitors and not as blank cells or a single population of cells.

[0032] 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 gluamate- and GABA-producing neurons. Therefore, in an embodiment, the disclosed methods include obtaining NSCs from the ventral midbrain to treat conditions ameliorated or attenuated, at least in part, by the implantation of tyrosine-hydroxylase-expressing dopaminergic neurons.

[0033] 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.

[0034] 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 the same neurodegenerative conditions.

[0035] 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.

[0036] 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.

[0037] 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 forth in U.S. 7,691,629, U.S. 5,753,506, U.S. 6,040,180, or U.S. 7,544,511, the entireties of which are incorporated by reference herein.

[0038] 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.

[0039] 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 neurodegenerative status of the injection site, the minimum dose necessary for a beneficial effect, and toxicity side-effect considerations. In an embodiment, the disclosed methods include injecting NSCs at a cell density of about 5,000 to about 50,000 cells per microliter.

[0040] 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.

[0041] 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.

[0042] 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 200,000 cells 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 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 neurodegenerative conditions 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.

[0043] 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 a flexible tubing or any other suitable transfer device.

[0044] In one embodiment, the desired injection site for treatment of a neurodegenerative condition includes at least one area of the brain. In another embodiment, the cells are implanted into at least one specific segment or region of the brain such as the cerebral cortex, cerebral hemispheres, thalamus, hypothalamus, midbrain, cerebellum, pons, or medulla oblongata.

[0045] In another embodiment, the cells are injected at between about 5 and about 50 sites in the brain. In an embodiment, the cells are injected at between about 10 to about 30 sites in the brain. 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 brain 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 in humans can be extrapolated from results in animal models.

[0046] 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 mileu 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 neurodegenerative conditions may be caused by or result in missing elements including neuron-derived elements. Therefore, subjects suffering from degeneration of CNS tissues due to lack of such neuron-derived elements, such as growth factors, enzymes and other proteins, can be treated effectively by the disclosed methods.

[0047] The disclosed methods can also be applied to treat paresis, paralysis, spasticity, rigidity or any other motor, speech, or cognitive symptoms arising from cerebral ischemia. Cerebral ischemia can occur as a result of a stroke event in the brain or from a heart attack in which the blood circulation to the brain is interrupted for a significant period of time. It is, thus, analogous to the spinal cord ischemia described above. Some stroke subjects develop seizures of central origin as well as other deficits such as memory loss, paralysis, or paresis. These deficits from cerebral ischemia are also likely due to selective loss of inhibitory interneurons in hippocampus and/or other brain areas. Thus, the disclosed methods can be applied to treat stroke subjects suffering from paresis, paralysis, spasticity, or other motor, speech, and cognitive symptoms.

[0048] 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 Spinal Cord Neural Stem/Progenitor Cells

[0049] 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.

[0050] 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 *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, O4 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

[0051] 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, fasciculated 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: Transplantation of Spinal-Cord Derived Neural Stem Cells to the Brain

[0052] A neuronal stem cell may be isolated from a mammal expanded *in vitro* and then introduced (*e.g.*, transplanted) to one or more areas in a subject (*e.g.*, a subject's brain) afflicted with a neurodegenerative disease and/or disorder.

[0053] In an exemplary method for treatment of a neurodegenerative disorder, cerebral ischemia (*e.g.*, a stroke) was induced in adult male Sprague-Dawley rats (weighing 250-300 g) on day 0 by subjecting the rats to three-vessel ligation by modified methods described previously (see, Chen *et al.*, 1986; Shyu *et al.* 2004). Briefly, rats were anesthetized with chloral hydrate (0.4 g/kg, ip) and bilateral CCAs were clamped with non-traumatic arterial clips. Next, using a surgical microscope, a 2 x 2 mm craniotomy was drilled where the zygoma fuses to the squamosal bone. The right MCA was then ligated with an l0-O nylon suture. Cortical blood flow was measured continuously with a laser Doppler flowmeter (PF-5010, Periflux system, Perimed AB, Stockholm, Sweden) in anesthetized rats. Next, a burr hole (l-mm diameter) was made in the right frontoparietal region to allow

placement of photodetectors. A probe (0.45 mm in diameter) was then stereotaxically placed in the cortex (l.3 mm posterior, 2.8 mm lateral to the bregma, and l.0 mm below the dura). After 90 minutes of ligation, the suture on the MCA and arterial clips on CCAs were then removed to allow reperfusion. While rats were under anesthesia, their core body temperature was monitored with a thermistor probe and maintained at 37°C with a heating pad during anesthesia. After recovery from anesthesia, body temperature was maintained at 37°C with a heat lamp.

[0054] Next, the rats subjected to three-vessel ligation procedure were divided into two groups. The first group ("transplantation group") received NSI-566RSC cells (a human spinal stem cell line, "HSSC" line; clinical lot, Neuralstem, Inc., USA) and the second group ("control group") received a vehicle-control. The NSI-566RSC stem cell line was derived from a fetal spinal cord tissue. Stem cells from a cGMP clinical lot (passage 12) were used in this study. On the day of surgery, one cryopreserved vial of the cells was thawed, washed, and concentrated according to Neuralstem's protocol. All animals in the study received a once daily injection of FK506 (or Prograf) at 1mg/kg i.p. to suppress their immune system.

[0055] NSI-566RSC cells or control vehicle was administered on day 7 to the transplantation and control group, respectively. Rats in the transplantation group were injected stereotaxically with approximately 2 x 10^5 NSI-566RSC cells in a 5 μl suspension through a 30-gauge Hamilton syringe into 3 subcortical areas, 3.0 to 5.0 mm below the dura. The approximate coordinates for these sites were l.0 to 2.0 mm anterior to the bregma and 3.5 to 4.0mm lateral to the midline, 0.5 to l.5mm posterior to the bregma and 4.0 to 4.5 mm lateral to the midline, and 3.0 to 4.0 mm posterior to the bregma and 4.5 to 5.0 mm lateral to the midline. The needle was retained in place for 5 minutes after each injection and a piece of bone wax is applied to the skull defects to prevent leakage of the injected solution. Experimental rats in the vehicle-control group were treated stereotaxically with the suspension buffer only without the cells.

[0056] Neurological behavioral assessments were performed 3 days before cerebral ischemia, and day 1, 7, 14, 21 and 28 after treatment. The tests measured (a) body asymmetry, (b) locomotor activity and (c) grip strength. Pre-treatment scores were recorded in order to normalize those taken after cerebral ischemia. For body asymmetry, the elevated body swing test (EBST) was used to assess body asymmetry after MCA ligation and evaluated quantitatively, as previous described (Borlongan *et al.*, 1998). Initially, animals were examined for lateral movement, with their bodies suspended by their tails. The

frequency of initial head swing contra-lateral to the ischemic side was counted in twenty continuous tests and normalized, as previously described (Chang *et al.*, 2003) (Figure 1, Panel A). For locomotor activity, rats were subjected to VersaMax Animal Activity monitoring (Accuscan Instruments, Inc., Columbus, OH) for about 2 hours for behavioral recording of each of the experimental rats. The VersaMax Animal Activity monitor contains 16 horizontal and 8 vertical infrared sensors spaced 87 cm apart. The vertical sensors are situated 10 cm from the floor of the chamber. Locomotor activity was counted as the number of beams broken by a rat's movement in the chamber. Three vertical parameters defined in the manufacturer's menu option were calculated over 2 hours at night: (i) vertical activity, (ii) vertical time, and (iii) number of vertical movements (Figure 1, Panels B-D). For grip strength, rats were analyzed using a Grip Strength Meter (TSE-Systems, Germany) as previously described, with modification (Dunnett *et al.*, 1998). In brief, the grip strength ratio of each forelimb was measured separately and calculated as the ratio between the mean strength out of 20 pulls of the side contralateral to the ischemia and that of ipsilateral side. In addition, the ratio of grip strength post-treatment and prior to treatment were also calculated and changes were presented as a percentage of the pre-treatment value (Figure 1, Panel E). Overall, the investigators were blinded when measuring behavioral changes in the cell-treated and control group.

[0057] Approximately twenty-seven days after the rats were subjected to the three-vessel ligation procedure, rats were anesthetized with chloral hydrate (0.4 g/kg, ip) and their brains fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde, before being removed and embedded in 30% sucrose. A series of adjacent 20-μm-thick sections were cut from each brain in the coronal plane, stained with H&E and observed by light microscopy (Nikon, E600, Japan). Human grafted cells were then identified by various human-specific antibodies (HuNu, synptophysin, hNSE, hNF, and others) (*see*, Figures 2A-2B). For BrdU immunostaining, DNA is first denatured by incubating each section in 50% formamide of 2X standard saline citrate at 65°C for 2 hours, then in 2 N HCl at 37°C for 30 minutes, and finally rinsed in 0.1 M boric acid with pH 8.5. Next, sections were rinsed with Tris buffer and treated with 1% H_2O_2 to block endogenous peroxidase. The immunostaining procedure was then performed using the labeled streptavidin-biotin (LSAB) method (DAKO LASB-2 Kit, Peroxidase, DAKO, USA). Brain tissue, on a silane-coated slide, was then placed in boiling citrate buffer (pH 6, ChemMate, DAKO, USA) twice for 5 minutes in a microwave oven at 750 W, after deparaffinization and

rehydration. Next, tissues were incubated with the appropriate diluted primary antibodies to BrdU (for nuclear identification, dilution 1:400, Sigma, USA), at room temperature for 1 hour. After washing with Tris-buffered saline, containing 0.1% Tween-20 (TBS-T), the specimens were sequentially incubated for 10 to 30 minutes with biotinylated anti-rabbit and anti-mouse (1:200, R&D Systems, USA) immunoglobulins and peroxidase-labeled streptavidin. Staining was performed after a 10 minute incubation with a freshly prepared substrate-chromogen solution, containing 3% 3-amino-9-ethylcarbazole and hydrogen peroxide. Finally, the slides were lightly counterstained with hematoxylin, washed with water, and then mounted. Negative control sections were stained with identical preparations of brain tissue specimen, except that primary antibodies were omitted. Quantification of BrdU immunoreactive cells were counted digitally using a 60X objective lens (Carl Zeiss LSM510, Germany) via a computer imaging analysis system (Imaging Research, Canada).

[0058] Additionally, cell type-specific markers co-localized with exogenous transplanted NPCs and endogenous homing stem cells (BrdU immunoreactive), may be identified by immunofluorescent colocalization analysis by laser-scanning confocal microscopy. In immuno-fluorescent colocalization studies, each coronal section is treated with cell-specific antibodies, for example, glial fibrillary acidic protein (GFAP for astrocyte, 1:400, Sigma, USA), Von-Willebrand factor (vWF for endothelial cell, 1:400, Sigma, USA), neuronal nuclear antigen (Neu-N for neuronal nuclei, 1:200, Chemicon, CA), Nestin (for neuronal dendrites, 1:200, Chemicon, CA), microtubule-associated protein 2 (MAP-2 for neuronal dendrites, 1:200; BM, Germany), stromal cell-derived factor 1 (SDF-1, 1:200) and CXC receptor type 4 (CXCR4, 1:200) from Chemokine, and PrP^C (1:300; M20, Santa Cruz). with Cy3 (Jackson Immunoresearch PA USA, 1:500, USA) staining.

[0059] As shown in Figure 1, animals treated with NSI-566RSC cells exhibited improvements in body swing, vertical movement, vertical activity, vertical movement time, and grip strength compared to animals treated with control vehicle.

[0060] Aspects, including embodiments, of the present subject matter described above may be beneficial alone or in combination, with one or more other aspects or embodiments. Without limiting the foregoing description, in accordance with one aspect of the subject matter herein, there is provided methods for treating a disease or disorder associated with neuronal cell loss in a brain, the methods comprising: obtaining at least one stem cell from spinal cord tissue of a human; expanding the at least one stem cell to form an

expanded stem cell population; and introducing a therapeutically effective amount of the expanded stem cell population to at least one area of the brain of a subject.

[0061] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, the expanded stem cell population differentiates into neurons that integrate *in vivo* into the brain of the subject.

[0062] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, the disease or disorder is caused by cerebral ischemia, hemorrhage, and trauma.

[0063] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, the disease or disorder involves paralysis, speech impairment, loss of memory, or reasoning ability.

[0064] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, the disease or disorder involves motor dysfunction or cognitive deficit.

[0065] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, the human has a gestational age of about 5 to about 20 weeks.

[0066] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, expanding the at least one neural stem cell includes culturing the neural stem cell in absence of serum.

[0067] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, expanding the at least one neural stem cell includes exposing the at least one neural stem cell to at least one growth factor.

[0068] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, the growth factor is selected from the group consisting of bFGF, EGF, TGF-alpha, aFGF and combinations thereof.

[0069] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, at least 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the expanded stem cell population is capable of generating neurons in brain tissue of the subject.

[0070] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, at least 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the expanded stem cell population is capable of differentiating into neurons *in vitro*.

[0071] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, introducing the therapeutically effective amount of the expanded stem cell population includes injecting at least a portion of the therapeutically effective amount into a plurality of areas of brain tissue of the subject.

[0072] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, the areas include cerebral hemispheres, cerebral cortex, subcortex motor cortex, striatum, internal capsule, thalamus, hypothalamus, hippocampus, midbrain, brainstem, and cerebellum.

[0073] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, the subject experienced an event that caused cerebral ischemia.

[0074] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, the event is a stroke.

[0075] Without limiting the foregoing description, in accordance with another aspect of the subject matter herein, there is provided methods of treating stroke in a subject in need thereof, the methods comprising: isolating at least one neural stem cell from spinal cord tissue of a human; expanding *in vitro* said neural stem cell to form an expanded population; concentrating the expanded population; and introducing a therapeutically effective amount of said expanded population to at least one area of the subject brain.

[0076] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, the spinal cord is from a gestation age of about 5 to about 20 weeks.

[0077] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, expanding the at least one neural stem cell includes culturing the neural stem cell in absence of serum.

[0078] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, expanding the at least one neural stem cell includes exposing the at least one neural stem cell to at least one growth factor.

[0079] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, the growth factor is selected from the group consisting of bFGF, EGF, TGF-alpha, aFGF and combinations thereof.

[0080] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, at least 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the expanded population is capable of generating neurons in brain tissue of the subject.

[0081] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, introducing the therapeutically effective amount of the expanded population includes injecting at least a portion of the therapeutically effective amount into one to a plurality of areas of the recipient brain.

[0082] In accordance with another aspect which may be used or combined with any of the preceding or following aspects, the areas of the recipient brain include cerebral hemispheres, cerebral cortex, subcortex motor cortex, striatum, internal capsule, thalamus, hypothalamus, hippocampus, midbrain, brainstem, and cerebellum.

[0083] 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 for treating a disease or disorder associated with neuronal cell loss in a brain, said method comprising:

a) obtaining at least one stem cell from spinal cord tissue of a human;

b) expanding the at least one stem cell to form an expanded stem cell population; and

c) introducing a therapeutically effective amount of the expanded stem cell population to at least one area of the brain of a subject.

2. The method of claim 1 wherein the expanded stem cell population differentiates into neurons that integrate *in vivo* into the brain of the subject.

3. The method of claim 1, wherein the disease or disorder is caused by cerebral ischemia, hemorrhage, and trauma.

4. The method of claim 3, wherein the disease or disorder involves paralysis, speech impairment, loss of memory, or reasoning ability.

5. The method of claim 3, wherein the disease or disorder involves motor dysfunction or cognitive deficit.

6. The method of claim 1, wherein the human has a gestational age of about 5 to about 20 weeks.

7. The method of claim 1, wherein expanding the at least one neural stem cell includes culturing the neural stem cell in absence of serum.

8. The method of claim 1, wherein expanding the at least one neural stem cell includes exposing the at least one neural stem cell to at least one growth factor.

9. The method of 8, wherein the growth factor is selected from the group consisting of bFGF, EGF, TGF-alpha, aFGF and combinations thereof.

10. The method of claim 1, wherein at least 30% of the expanded stem cell population is capable of generating neurons in brain tissue of the subject.

11. The method of claim 1, wherein at least 50% of the expanded stem cell population is capable of differentiating into neurons *in vitro*.

12. The method of claim 1, wherein introducing the therapeutically effective amount of the expanded stem cell population includes injecting at least a portion of the therapeutically effective amount into a plurality of areas of brain tissue of the subject.

13. The method of 12, wherein the areas include cerebral hemispheres, cerebral cortex, subcortex motor cortex, striatum, internal capsule, thalamus, hypothalamus, hippocampus, midbrain, brainstem, and cerebellum.

14. The method of claim 1, wherein the subject experienced an event that caused cerebral ischemia.

15. The method of claim 14, wherein the event is a stroke.

16. A method of treating stroke in a subject in need thereof, said method comprising:

a) isolating at least one neural stem cell from spinal cord tissue of a human;

b) expanding *in vitro* said neural stem cell to form an expanded population;

c) concentrating the expanded population; and

d) introducing a therapeutically effective amount of said expanded population to at least one area of the subject brain.

17. The method of claim 16 wherein at least 20% of the expanded population is capable of generating neurons in brain tissue of the subject.

18. The method of claim 16, wherein the spinal cord is from a gestation age of about 5 to about 20 weeks.

19. The method of claim 16, wherein expanding the at least one neural stem cell includes culturing the neural stem cell in absence of serum.

20. The method of claim 16, wherein expanding the at least one neural stem cell includes exposing the at least one neural stem cell to at least one growth factor.

21. The method of claim 20, wherein the growth factor is selected from the group consisting of bFGF, EGF, TGF-alpha, aFGF and combinations thereof.

22. The method of claim 16, wherein at least 30%, of the expanded population is capable of generating neurons in brain tissue of the subject.

23. The method of claim 16, wherein introducing the therapeutically effective amount of the expanded population includes injecting at least a portion of the therapeutically effective amount into one to a plurality of areas of the recipient brain.

24. The method of 23, wherein the areas of the recipient brain include cerebral hemispheres, cerebral cortex, subcortex motor cortex, striatum, internal capsule, thalamus, hypothalamus, hippocampus, midbrain, brainstem, and cerebellum.