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(54) Title: HUMAN NEURAL PROGENITOR DERIVED DOPAMINERGIC PROGENITORS AND NEURONS

(57) Abstract: The present invention relates to a method for producing mature dopaminergic neurons from human neural progenitor cells and/or from dopaminergic progenitor cells. The invention provides these cells from human embryonic stem cell (hESC) derived human neural progenitor (hNP) cells or from dopaminergic progenitor cells as otherwise described herein. A dopaminergic progenitor cell, which can lead to the differentiation/production of mature dopaminergic in high yield, is an additional aspect of the present invention.



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Human Neural Progenitor Derived Dopaminergic Progenitors and Neurons

Field of the Invention

The present invention relates to a method for producing dopaminergic progenitor cells and mature dopaminergic neurons from human neural progenitor cells and/or from human dopaminergic progenitor cells. The invention provides these cells from human neural progenitor (hNP) cells or from human dopaminergic progenitor cells as otherwise described herein. A novel dopaminergic progenitor cell, which can lead to the differentiation/production of mature dopaminergic in high yield, is an additional aspect of the present invention. The cells that are produced are feeder cell free and consequently, are more readily used in therapeutic applications as otherwise described herein because of the absence of contaminating cells (feeder cell free).

Related Applications and Grant Support

This application claims the benefit of priority of provisional application no. US61/352,585, filed June 8, 2010, entitled "Human Neuronal Progenitor Derived Dopaminergic Progenitors and Neurons", the entire contents of which application is incorporated by reference in its entirety herein.

This invention was made with government support under contract no. N000140810989 awarded by the Department of Defense. Consequently, the government has certain rights in the invention.

Background of the Invention

Glial cell-line derived neurotrophic factor (GDNF) was discovered in 1993 for its role in promoting dopamine uptake into midbrain dopaminergic neurons [1]. The potential of GDNF as a treatment for Parkinson's disease (PD) was evident due to the link between the motor symptoms in PD being caused by the degeneration of the dopaminergic neurons in the substantia nigra (SN) [2]. At the time of GDNF's discovery,

current PD treatment options did not protect the remaining dopaminergic neurons from degenerating leading to progressive increase in symptoms and further quality of life decline [3]. Since its discovery, GDNF has been tested in vitro and in vivo as a treatment for PD without having reached a definitive treatment option. This is due in part to lack of understanding as to how GDNF protects dopamine neurons. GDNF was first tested for recovery of dopaminergic neurons in PD rat models with success demonstrated by increased TH expression and behavioral recovery [4]. From there, GDNF was used as a preventative measure in rats [5] and tested in non-human primates with positive results [6]. This led to human trials which failed due to side effects, lack of long term recovery, and difficulty with route of administration [7, 8].

Human neural progenitors (hNPs) derived from human embryonic stem cells (hESCs) provide a suitable model system for studying the pathway through which dopaminergic neurons can be protected or differentiated with GDNF. Our lab has previously derived hNPs from hESCs that are maintained in a stable, adherent monolayer culture system [9]. These hNPs remain continually proliferative for many passages and maintain a stable karyotype in addition to being able to differentiate into the three main types of cells found in the nervous system, neurons, oligodendrites and astrocytes [9]. Our lab has successfully enriched a population of the hNPs to become dopaminergic neurons with the addition of GDNF to the differentiation media. Currently, approximately 50% of the differentiated neurons were tyrosine hydroxylase (TH) positive [10]. Understanding the mechanism through which GDNF enhances the differentiation of the hNPs to dopaminergic neurons would allow for elucidating a potential mechanism through which to increase the percentage of dopaminergic neurons obtained. Additionally, it would allow for understanding of the basic science behind GDNF's mechanism of action for its protection of midbrain dopaminergic neurons allowing for research in potential mechanisms for designing future treatments for PD.

Currently, the actions of Src protein tyrosine kinases and the c-Jun N-terminal kinase (JNK) pathways in GDNF's neuroprotective role in midbrain dopamine neurons have been elucidated while the mitogen-activated protein kinase (MAPK) and

phosphatidylinositol 3-kinase (PI3K) pathways have been implicated in GDNF's role but not fully understood. Src has known roles in cell growth, differentiation and survival [11]. When GDNF binds to its co-receptor GDNF family receptor alpha 1 (GFR α 1), it activates rearranged in transcription (RET) receptors which are brought to the cellular membrane with the assistance of Src [12]. When GDNF is present and active, Src causes the up regulation of RET receptors to lipid rafts allowing for increased binding of the GDNF-GFR α 1 complex to the RET receptor [13, 14]. The JNK pathway is a subfamily of the MAPK pathway. When GDNF and its co-receptor GFR α 1 bind to the RET receptor, JNK modulates dopaminergic neurite outgrowth as well as initiating a delay at G2/M to allow for actin reorganization within the neuron [15, 16].

The mechanisms through which the MAPK/ERK and PI3K pathway promote dopaminergic neuronal survival and differentiation are elusive. Rat cortical cells have shown that GDNF increases neurite outgrowth through the MAPK/ERK pathway [17]. In the rat dopaminergic cell line MD90, inhibiting the PI3K pathway prevents GDNF from protecting the dopaminergic neurons from 6-hydroxydopamine induced death [18]. The objective of this study was to establish the involvement of the MAPK and PI3K pathway in dopaminergic enrichment of the hNPs as previously reported and to evaluate the mechanisms through which this enrichment occurred. Inhibiting both the MAPK and the PI3K pathway prevented the establishment of dopaminergic neurons in the differentiated hNP population when differentiated with GDNF. Additionally, RAC genes involved in neurite extension were up regulated in dopaminergic-like neurons relative to differentiated hNPs. Inhibition of the MEK, ERK and p38 affected the dopaminergic-like neurons while the GSK3 β pathway was unaffected. The data in this study provides insight into the changes that occur when GDNF enhances dopaminergic-like neuron differentiation.

Brief Description of the Invention

The present invention is directed to a method of producing mature dopaminergic neuron cells (hDNCs) from human pluripotent stem cells, especially including human

embryonic stem cells (hESCs), human neural progenitor cells (hNPs) and/or human dopaminergic progenitor cells (hDPCs). The present invention also is directed to methods of producing human dopaminergic progenitor cells (hDPCs) from human neural progenitor cells and cells produced according to these methods as isolated human dopaminergic progenitor cells (hDPCs). The hDPCs may be differentiated into dopaminergic neuron cells (hDNCs) in high yield, or alternatively, isolated and/or cryopreserved for further differentiation into dopaminergic neuron cells and other neuron cells.

In a method of producing dopaminergic neuron cells (hDNCs), dopaminergic progenitor cells are first produced by differentiating hNPs (including optionally, hNPs which have been continually propagated in effective amounts of LIF and bFGF) leukaemia inhibition factor (LIF) or glial cell-line derived neurotrophic factor (GDNF) or mixtures thereof (preferably both, in the absence of basic fibroblast growth factor, or bFGF) in a cellular differentiation medium, preferably a neurobasal medium, containing components as otherwise disclosed herein for a period of time (about 3-21 days, about 7-21 days, about 7-14 days, about 14-21 days) such that the cells become human dopaminergic progenitor cells (or a mixture of dopaminergic progenitor cells and dopaminergic neuron cells the longer the hNPs are differentiated in the GDNF) as determined by expression of the biomarker Nurr1 and preferably one or more of EN1 or the biomarkers tyrosine hydroxylase (TH) and PITX3. These human dopaminergic progenitor cells may be isolated and cryopreserved for further use, including neuronal transplant, or alternatively, further differentiated in glial cell-line derived neurotrophic factor (GDNF) to produce human dopaminergic neuron cells (hDNCs). The dopaminergic neuron cells may be used in bioassays and for therapy, among a number of other uses. Prior to differentiating the hNPs into dopaminergic progenitor cells and/or dopaminergic neuronal cells, the hNPs may be propagated in cellular nutrient medium comprising leukaemia inhibition factor (LIF) and

In a preferred method according to the present invention, feeder free hNPs which have been propagated are exposed to a growth medium which comprises both LIF and

bFGF for a period of 1-3 days, followed by exposure of those cells to LIF and GDNF in a neural differentiation medium for a period ranging from about 3 days about 21 days, 7 days to 14 days, 7 days to 21 days, 14 days to 21 days to provide dopaminergic progenitor cells and/or dopaminergic neuronal cells which may be isolated using standard separation methods available in the art which after isolation, may be cryopreserved to provide storage stable human dopaminergic progenitor cells or dopaminergic neuronal cells, preferably cells which are feeder cell free.

In a further alternative embodiment, hNPs, preferably propagated hNPs as otherwise described herein are exposed to a differentiation medium comprising an effective amount of Sonic Hedgehog and FGFR8 and optionally, BDNF and/or TGF β 3 to produce human dopaminergic progenitor cells and/or human dopaminergic neurons as otherwise described herein. In this method, hNPs are preferably propagated for a period of about 3 days or more with an effective amount of LIF and optionally, an effective amount of bFGF as otherwise described herein or alternatively and preferably, with both LIF (e.g., about 2 ng/ml to about 25 ng/ml, about 5 to 20 ng/ml, about 5 to 15 ng/ml, about 8-12 ng/ml, preferably about 10 ng/ml) and bFGF (e.g., about 5 ng/ml to about 50 ng/ml, about 10 ng/ml to about 40 ng/ml, about 15 ng/ml to about 30 ng/ml, about 15 to about 25 ng/ml, about 20 ng/ml) for a period of between about 10 to 40 passages, preferably about 20 and 30 passages with each passage occurring on day 3-4, preferably day 4). The propagated hNPs produced as described above, are exposed to a cellular differentiation medium as otherwise described herein to produce human dopaminergic progenitors and/or human dopaminergic, wherein the cellular differentiation medium comprises an effective amount of Sonic Hedgehog (about 50-500 ng/ml, about 100-400 ng/ml, about 150-300 ng/ml, about 200 ng/ml.) and an effective amount of fibroblast growth factor 8 (FGF8) (about 25ng/ml to about 400 ng/ml, about 50 ng/ml to about 300 ng/ml, about 75 ng/ml to about 200 ng/ml, about 100 ng/ml and optionally, effective amounts of brain-derived neurotrophic factor (BDNF) (about 2 ng/ml to about 50 ng/ml, about 5 ng/ml to about 35 ng/ml, about 10 ng/ml to about 20 ng/ml) and/or transforming growth factor type β 3 (TGF β 3) (about 0.1 ng/ml to about 20 ng/ml, about 0.25 ng/ml to about 10 ng/ml, about 0.5 to about 5 ng/ml, about 1-2 ng/ml, about 1 ng/ml) for a period

ranging from about 3 days to about 21 days, about 3 days to about 18 days, about 3 days to about 14 days, about 7 days to about 14 days, about 3 days to about 7 days (preferably, without passaging and with the media including differentiation factors being changed about every 1-4 days, preferably every other day) to produce human dopaminergic progenitor cells and/or human dopaminergic neuron cells, which are optionally separated and cryopreserved. In this method, the cellular differentiation medium comprising Sonic Hedgehog and FGF8 may further comprise GDNF and/or LIF in effective amounts as otherwise described herein, in addition to, or as a substitute for the BDNF and/or the TGF β 3 to produce greater concentrations of human dopaminergic progenitor cells and/or dopaminergic neuron cells.

Methods of transplanting human dopaminergic progenitor cells and/or dopaminergic neuronal cells, preferably feeder cell free progenitor cells and/or neuronal cells are another aspect of the present invention. In this method, an effective number of cells are administered to a patient in need in order to treat one or more neurodegenerative diseases including one or more of Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis (Lou Gehrig's disease), Tay Sachs disease, other genetic diseases, and multiple sclerosis, as well as lysosomal storage disease and stroke. In addition, methods according to the present invention may be used to repair damage to a patient's central nervous system, treat a neurodegenerative disease as discussed above, treat nerve damage caused by stroke, cardiovascular disease, a heart attack, physical injury or trauma, genetic damage or environmental insult to the brain and/or spinal cord caused by an accident or other activity.

Brief Description of the Figures

Figure 1 shows the developmental timeline of dopaminergic progenitor cells (dopaminergic progenitor stage) and mature dopamine neurons from neural progenitor cells and biomarkers expressed for each type of cell during the different stages of development.

Figure 2 shows certain implications of GDNF for dopaminergic differentiation.

Figure 3 shows a progression of methodology of neural progenitor cell differentiation from proliferation to differentiation to analysis of the cells.

Figure 4 shows that NP cells can be induced and specified in the presence or absence of GDNF.

Figure 5 shows that NP cells can differentiate to dopaminergic progenitor cells in the presence or absence of GDNF.

Figure 6 shows that mature dopaminergic neuron cells express functional markers DAT and VMAT2 as indicated.

Figure 7 shows that GDNF is sufficient to enhance differentiation of neural progenitors to a dopaminergic phenotype.

Figure 8 shows dopaminergic differentiation in vivo and in vitro. In vitro development shown in this paper differs from in vivo mouse development in earlier expression of Nurr1 (A). In vitro cells progress from hNP cells to a neural induction stage to a dopaminergic specification stage to mature dopaminergic neurons (A). While mesenchymal stem cells (B) do not express the RET receptor for GDNF, hNP cells (C), differentiated neurons (D), and dopamine progenitors (E) do express RET as a active site for GDNF shown here in red with DAPI in blue. TH - tyrosine hydroxylase; EN 1 - engrailed I ; SHH - sonic hedgehog; FGF8 - fibroblast growth factor 8; RET - REarranged in Transfection; GDNF - glial cell-line derived neurotropic factor; Pitx3 - paired-like homeodomain trans,cription factor 3; Nurr1 - Nuclear receptor related 1; TGF - Transforming growth factor; Tuj – Beta III Tubulin; Scale bars 10 μ M.

Figure 9 shows hNP cells can be induced and specified hNP cells express NURR1 (A), shown here in red with DAPI in blue, and continue to express NURR1 through 21

days of differentiation with GDNF (B). Immunocytochemistry demonstrates no EN1 expression in hNP cells (D); however, EN1 expression with 21 days of differentiation with GDNF (E) shown here in green with DAPI in blue. The NURR1 and EN1 expression becomes significantly different at day 7 with GDNF differentiation (C, F) suggesting a progression to the dopamine progenitor stage. Flow cytometry analysis demonstrates a population of cells positive for Nurrl and EN1 with GDNF differentiation (G-J). Nurrl - Nuclear receptor related 1; EN1 - engrailed; GDNF - glial cell-line derived neurotrophic factor; Scale bars 10 μ M. # Significantly different from differentiated neurons without GDNF; * significantly different from day 0; + significantly different from day 3; t significantly different from day 7.

Figure 10 - hNP cells can be differentiated to dopaminergic progenitors. Differentiation of hNP cells with GDNF demonstrates expression of TH shown in red with DAPI in blue and Tuj in green (B) and PITX3 shown in red with DAPI in blue and Tuj in green (E). After 14 days of GDNF differentiation, PITX3 expression increases significantly demonstrating a progression to a dopamine progenitor stage (F). This is further confirmed by the significant expression of TH at day 21 with GDNF differentiation (C). Flow cytometry demonstrates a population of TH and Pitx3 positive neurons with GDNF differentiation (G-J). TH - tyrosine hydroxylase; Pitx3 - paired-like homeodomain transcription factor 3; GDNF - glial cell-line derived neurotrophic factor; Scale bars 10 μ M; # significantly different from differentiated neurons without GDNF; * significantly different from day 0; t significantly different from day 7.

Figure 11 shows that mature dopaminergic neurons express functional markers. Dopamine progenitors progress to mature dopaminergic neurons as immunocytochemistry for DAT shown in green with DAPI in blue (B) and VMAT2 shown in pink with DAPI in blue (E) demonstrates at day 21 of GDNF differentiation compared to the lack of staining in hNP cells for DAT (A) and VMAT2 (D). VMAT2 expression (F) increases significantly at day 21 with or without GDNF and DAT expression (C) increases significantly with GDNF at day 14. Flow cytometry demonstrates a population of cells that express VMAT2 (I, J) and DAT (G, H). DAT -

dopamine transporter; VMAT2 - vesicular monoamine transporter 2; GDNF - glial cell-line derived neurotrophic factor; Scale bars 10 μ M; # significantly different from differentiated neurons without GDNF; * significantly different from day 0; + significantly different from day 3; † significantly different from day 7; ++ significantly different from day 14

Figure 12 Evoked differentiated hNP cells release dopamine PCR for the 5 dopamine receptors (A) demonstrates expression of the D1, D4, and D5 receptors in hNP cells, differentiated neurons, and neurons differentiated with GDNF. HPLC demonstrates increased expression of dopamine (B) and L-dopa (C) with GDNF exposure. DRI - dopamine receptor 1; DR2 - dopamine receptor 2; DR3 - dopamine receptor 3; DR4 - dopamine receptor 4; DR5 - dopamine receptor 5; GDNF - glial cellline derived neurotrophic factor; Scale bars 10 μ M; * significantly different from hNP cells; # significantly different from neurons differentiated without GDNF.

Figure 13 indicates that PCR for markers of other neural cell types showed no expression for G protein-coupled inwardly rectifying potassium channel (GIRK) or tryptophan hydroxylase 1 (TPH1), with expression in differentiated neurons for choline acetyltransferase (ChAT). Neurons differentiated with GDNF also expressed ChAT, glutamate decarboxylase (GAD), phenylethanolamine-N-methyl transferase (PMNT), and dopamine- β -hydroxylase (DBH). DBH was expressed in hNP cells, differentiated neurons, and neurons differentiated in the presence of GDNF (Figure 13). To confirm that the cells that were reactive for TH were not also reactive for DBH, immunocytochemistry was performed on hNP cells differentiated for 14 days with GDNF. Separate populations, one that expressed DBH only, one that expressed TH only, and one that expressed DBH and TH, were found within the differentiated cultures (Figure 13).

Figure 14 shows that the dopaminergic progenitor cells expressed the biomarker LRRK2 (Leucine Rich Repeat Kinase 2 gene) in each instance. This is a common autosomal dominant missense mutation, in this gene has previously been identified 0.6%-

1.6% and 2% to 8% of sporadic and familial PD cases. Histological studies of postmortem brain tissue from PD patients, including those with the p.G2019S LRRK2 mutation, showed cell loss in the substantia nigra and formation of Lewy bodies, protein aggregates containing α -synuclein. Antibodies were stored in 4°C as supplied. Cells were differentiated for 7 and 14 days and then fixed in 2%PFA. Cells were washed 3 times for 5 minutes each in permeabilization buffer containing 20 μ L of Tween 20/50mL of high salt buffer. Cells were blocked in 6% donkey serum and then stained with primary antibody for one hour at 1:25. Cells were washed 4 times for 5 minutes each in high salt buffer and then AlexaFluor secondary antibody was added for one hour. DAPI and Prolong Gold were added after removal of secondary washing of cells in PBS+/-.

Figure 15 shows that GDNF enhancement of differentiation of dopaminergic-like neurons was blocked with inhibitors to MAPK and PI3K. Inhibiting GDNF with an antibody that binds all GDNF found in the cell decreased TH expression significantly ($p < .05$) at 1ng/ml and 10ng/ml and prevented TH expression completely at 100ng/ml (A). Immunocytochemistry images show the difference in differentiation without inhibitor (B) and with inhibitor (C). Blocking the RET receptor also decreased TH expression with 1ng/ml but completely killed all cells at 10ng/ml and 100ng/ml (D). Immunocytochemistry images show dopaminergic-like neurons without inhibitor (E) and with (F). Examination of the PI3K pathway with an inhibitor to prevent its activation killed all cells at 10ng/ml and 100ng/ml but only slightly decreased TH expression with 1ng/ml (G). Similar immunocytochemistry images are shown without inhibitor (H) and with (I). Inhibition of MEK at a dosage of 1ng/ml and 10ng/ml significantly reduced TH expression while 100ng/ml killed all cells (J). Immunocytochemistry showed TH expression without inhibitor (K) and no TH expression with (L). Scale bars = 10 μ M.

Figure 16 shows that changes in the MAPK Pathway with GDNF enhance dopamine-like neurons relative to differentiated hNPs, dopaminergic-like neurons express higher levels of *Creb1*, *Mapk8*, *Mapk13* and *Mef2c* aspects of the ERK and p38 pathways (A). Inhibition of the MEK pathway lead to a greater decrease in apoptosis in differentiated hNPs than in dopaminergic-like neurons with no significant change in

proliferation (B). These results were similar to those found with inhibition of ERK pathway (C). Inhibiting the p38 pathway leads to a greater decrease in apoptosis in differentiated hNPs relative to dopaminergic-like neurons and a decrease in proliferation in dopaminergic-like neurons (D). The mechanisms for the GDNF activation in the ERK pathway are outlined in (E) and those for the p38 pathway are outlined in (F). hNP – human neural progenitors; MEK – mitogen activated kinase kinase; ERK – extracellular signal related kinase; RET – rearranged in transcription; GFR α 1 – glial cell-line derived neurotrophic factor family receptor alpha 1; RFU – relative fluorescence unit; DA – dopamine; * significant ($p > 0.05$) relative to differentiated hNPs, # significant ($p > 0.05$) relative to culture with no inhibitor.

Figure 17 shows the role of the Rac1 Pathway in Dopaminergic-like Neurons. Dopamine-like neurons to express higher levels of *Pak1* and *Rac1* relative to differentiated hNPs with no change in CDC42 expression (A). Apoptosis in dopaminergic-like neurons cultured with inhibitor to RAC1 decreased significantly ($p > 0.05$) relative to dopaminergic-like neurons without inhibitor while proliferation in differentiated hNPs with inhibitor increased significantly ($p > 0.05$) relative to differentiated hNPs without inhibitor with no significant changes in apoptosis or proliferation between differentiated hNPs and dopaminergic-like neurons (B). The mechanism through which GDNF activates the RAC1 pathway is outlined in (C). RET – rearranged in transcription; GFR α 1 – glial cell-line derived neurotrophic factor family receptor alpha 1; RFU – relative fluorescence unit; DA – dopamine; # significant ($p > 0.05$) relative to culture with no inhibitor

Figure 18 shows that PI3K sub-pathway Effects on Dopaminergic-like Neurons. *Gsk3 β* and *Apc* expression is not changed in dopaminergic-like neurons relative to differentiated hNPs (A). There was no change in apoptosis and proliferation when differentiation hNPs or dopaminergic-like neurons when cultured with inhibitor (B). The mechanism of action of GSK3 β is shown in (C). *Pten* expression is up regulated in dopaminergic-like neurons relative to differentiated hNPs while *Tsc1* and *Tsc2* expression is down regulated in dopaminergic-like neurons relative to differentiated hNPs

(D). There is a significant increase in apoptosis in dopaminergic-like neurons inhibited with an mTOR inhibitor relative to differentiated hNPs inhibited (B) with an mTOR inhibitor suggesting the role of mTOR in dopaminergic-like neuron enhancement with GDNF is through changes in transcription factors as outlined in (C). RET – rearranged in transcription; GFR α 1 – glial cell-line derived neurotrophic factor family receptor alpha 1; RFU – relative fluorescence unit; mTOR – mammalian target of rapamycin; GSK3 β – glycogen synthase kinase 3 beta; DA – dopamine; * significant ($p > 0.05$) relative to differentiated hNPs.

Figure 19 shows the changes in cellular functions with inhibitors to PI3K pathway targets *Igf1* and *Shc* expression decreased in dopamine-like neurons relative to differentiated hNPs (A). The mechanism through which SHC acts to modulate IGFR insertion in the membrane is outlined in (B). Cell cycle genes are up regulated in hNPs relative to H9s while cell cycle genes are down regulated in differentiated hNPs relative to hNPs and in dopaminergic-like neurons relative to hNPs (C). Inhibition of H9 (D) and hNP (E) and cell cycle increases the number of cells in G1. Inhibition of differentiated hNP (F) and dopaminergic-like neuron (G) cell cycle had no effect on the G1 stage and the cell cycle inhibits that of post-mitotic neurons, hNPs – human neural progenitors; IGF1 – insulin growth factor 1; DA – dopamine.

Figure 20 shows the GDNF Pathway in Dopaminergic Enhancement. Src binds to the GFR α 1 co-receptor when GDNF binds to its co-receptor and helps to bring RET to the membrane to allow for further cell signaling (A). GDNF binding to the RET receptor activates the MAPK pathway (B). The ERK pathway activation leads to dopamine neural survival, p38 activation leads to dopamine neural survival and RAC1 activation leads to axon regulation (B). Activation of the PI3K pathway with GDNF leads to the activation of the mTOR pathway and dopamine neural survival and synaptic plasticity through the eukaryotic initiation factors (C). The GSK3 β pathway is not involved in GDNF enhancement of dopamine neurons (C). GDNF – glial cell-line derived neurotrophic factor; GFR α 1 – glial cell-line derived neurotrophic factor family receptor alpha 1.

Detailed Description of the Invention

The following terms are used within context to describe the present invention. Note that terms used to generally describe the present invention are used in a manner keeping with its common meaning as understood by one of ordinary skill in the art.

Unless otherwise noted, the terms used herein are to be understood according to conventional usage by those of ordinary skill in the relevant art within the context of the use of the term. In addition to the definitions of terms provided below, definitions of common terms in molecular biology may also be found in Rieger *et al.*, 1991 Glossary of genetics: classical and molecular, 5th Ed., Berlin: Springer-Verlag; and in Current Protocols in Molecular Biology, F.M. Ausubel *et al.*, Eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1998 Supplement). It is to be understood that as used in the specification and in the claims, "a" or "an" can mean one or more, depending upon the context in which it is used. Thus, for example, reference to "a cell" can mean that at least one cell can be utilized.

The present invention may be understood more readily by reference to the following detailed description of the preferred embodiments of the invention and the Examples included herein. However, before the present compositions and methods are disclosed and described, it is to be understood that this invention is not limited to specific conditions, or specific methods, etc., as such may, of course, vary, and the numerous modifications and variations therein will be apparent to those skilled in the art.

Standard techniques for growing cells, separating cells, and where relevant, cloning, DNA isolation, amplification and purification, for enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like, and various separation techniques are those known and commonly employed by those skilled in the art. A number of standard techniques are described in Sambrook *et al.*, 1989 Molecular Cloning, Second Edition, Cold Spring Harbor Laboratory, Plainview, New York; Maniatis *et al.*, 1982 Molecular Cloning, Cold Spring Harbor Laboratory, Plainview,

New York; Wu (Ed.) 1993 Meth. Enzymol. 218, Part I; Wu (Ed.) 1979 Meth. Enzymol. 68; Wu et al., (Eds.) 1983 Meth. Enzymol. 100 and 101; Grossman and Moldave (Eds.) 1980 Meth. Enzymol. 65; Miller (ed.) 1972 Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Old and Primrose, 1981 Principles of Gene Manipulation, University of California Press, Berkeley; Schleif and Wensink, 1982 Practical Methods in Molecular Biology; Glover (Ed.) 1985 DNA Cloning Vol. I and II, IRL Press, Oxford, UK; Hames and Higgins (Eds.) 1985 Nucleic Acid Hybridization, IRL Press, Oxford, UK; and Setlow and Hollaender 1979 Genetic Engineering: Principles and Methods, Vols. 1-4, Plenum Press, New York.

Abbreviations and nomenclature, where employed, are deemed standard in the field and commonly used in professional journals such as those cited herein.

The term “patient” or “subject” is used throughout the specification within context to describe an animal, generally a mammal and preferably a human, to whom treatment, including prophylactic treatment (prophylaxis), with the cellular compositions according to the present invention is provided. For treatment of those infections, conditions or disease states which are specific for a specific animal such as a human patient, the term patient refers to that specific animal.

The terms “treat”, “treating”, and “treatment”, etc., as used herein, refer to any action providing a benefit to a patient at risk for or afflicted by a disease state, condition or deficiency which may be improved using cellular compositions according to the present invention. Treating a condition includes improving the condition through lessening or suppression of at least one symptom, delay in progression of the effects of the disease state or condition, including the prevention or delay in the onset of effects of the disease state or condition, etc.. Treatment, as used herein, encompasses both prophylactic and therapeutic treatment.

The term “primate Pluripotent Stem Cells”, of which “human Embryonic Stem Cells” or hESCs and human induced pluripotent stem cells or hiPSCs are a subset, are derived from pre-embryonic, embryonic, fetal tissue or adult stem cells (in the case of

human induced pluripotent stem cells) at any time after fertilization, and have the characteristic of being capable under appropriate conditions of producing progeny of several different cell types that are derivatives of all of the three germinal layers (endoderm, mesoderm and ectoderm), according to a standard art-accepted test, such as the ability to form teratomas in 8-12 week old SCID mice. The term includes both established lines of stem cells of various kinds, and cells obtained from primary tissue that are pluripotent in the manner described.

Included in the definition of pluripotent or pPS cells (pPSCs) are embryonic cells of various primate types, especially including human embryonic stem cells (hESCs), described by Thomson et al. (Science 282: 1145, 1998); as well as embryonic stem cells from other primates, such as Rhesus stem cells (Thomson et al., Proc. Natl Acad. Sci. USA 92: 7844, 1995). Other types of pluripotent cells are also included in the term. Human Pluripotent Stem Cells includes stem cells which may be obtained from human umbilical cord or placental blood as well as human placental tissue and/or amniotic fluid. Any cells of primate origin that are capable of producing progeny that are derivatives of all three germinal layers are included, regardless of whether they were derived from embryonic tissue, fetal, or other sources. The pPSCs are preferably not derived from a malignant source. It is desirable (but not always necessary) that the cells be karyotypically normal.

pPSC cultures are described as "undifferentiated" when a substantial proportion of stem cells and their derivatives in the population display morphological characteristics of undifferentiated cells, clearly distinguishing them from differentiated cells of embryo or adult origin. Undifferentiated pPSC are easily recognized by those skilled in the art, and typically appear in the two dimensions of a microscopic view in colonies of cells with high nuclear/cytoplasmic ratios and prominent nucleoli. It is understood that colonies of undifferentiated cells in the population will often be surrounded by neighboring cells that are differentiated.

Pluripotent stem cells may express one or more of the stage-specific embryonic antigens (SSEA) 3 and 4, and markers detectable using antibodies designated Tra-1-60 and Tra-1-81 (Thomson et al., Science 282:1145, 1998). Differentiation of pluripotent stem cells in vitro results in the loss of SSEA-4, Tra-1-60, and Tra-1-81 expression (if present) and increased expression of SSEA-1. Undifferentiated pluripotent stem cells typically have alkaline phosphatase activity, which can be detected by fixing the cells with 4% paraformaldehyde, and then developing with Vector Red as a substrate, as described by the manufacturer (Vector Laboratories, Burlingame Calif.) Undifferentiated pluripotent stem cells also typically express Oct-4 and TERT, as detected by RT-PCR.

Another desirable phenotype of propagated pluripotent stem cells is a potential to differentiate into cells of all three germinal layers: endoderm, mesoderm, and ectoderm tissues. Pluripotency of pluripotent stem cells can be confirmed, for example, by injecting cells into severe combined immunodeficient (SCID) mice, fixing the teratomas that form using 4% paraformaldehyde, and then examining them histologically for evidence of cell types from the three germ layers. Alternatively, pluripotency may be determined by the creation of embryoid bodies and assessing the embryoid bodies for the presence of markers associated with the three germinal layers.

Propagated pluripotent stem cell lines may be karyotyped using a standard G-banding technique and compared to published karyotypes of the corresponding primate species. It is desirable to obtain cells that have a "normal karyotype," which means that the cells are euploid, wherein all human chromosomes are present and not noticeably altered.

The types of pluripotent stem cells that may be used include established lines of pluripotent cells derived from tissue formed after gestation, including pre-embryonic tissue (such as, for example, a blastocyst), embryonic tissue, or fetal tissue taken any time during gestation, typically but not necessarily before approximately 10-12 weeks gestation. Non-limiting examples are established lines of human embryonic stem cells or human embryonic germ cells, such as, for example the human embryonic stem cell lines

WA01, WA07, and WA099 (WiCell). Also contemplated is use of the compositions of this disclosure during the initial establishment or stabilization of such cells, in which case the source cells would be primary pluripotent cells taken directly from the source tissues. Also suitable are cells taken from a pluripotent stem cell population already cultured in the absence of feeder cells. Also suitable are mutant human embryonic stem cell lines, such as, for example, BG01v (BresaGen, Athens, Ga.), as well as normal human embryonic stem cell lines such as WA01, WA07, WA09 (WiCell) and BG01, BG02 (BresaGen, Athens, Ga.).

Epiblast stem cells (EpiScs) and induced pluripotent stem cells (iPSCs), especially human induced pluripotent stem cells (hiPSCs) fall within the broad definition of pluripotent cells hereunder and in concept, the technology described in the present application applies to these and other pluripotent cell types (ie, primate pluripotent cells) as set forth above. EpiScs are isolated from early post-implantation stage embryos. They express Oct4 and are pluripotent. See, Tesar et al, *Nature*, Vol 448, p.196 12 July 2007. iPS cells are made by dedifferentiating adult somatic cells back to a pluripotent state by retroviral transduction of four genes (c-myc, Klf4, Sox2, Oct4). See, Takahashi and Yamanaka, *Cell* 126, 663–676, August 25, 2006.

Human embryonic stem cells (hESCs) may be prepared by methods which are described in the present invention as well as in the art as described for example, by Thomson et al. (U.S. Pat. No. 5,843,780; *Science* 282:1145, 1998; *Curr. Top. Dev. Biol.* 38:133 ff., 1998; *Proc. Natl. Acad. Sci. U.S.A.* 92:7844, 1995).

The term “embryonic stem cell” refers to pluripotent cells, preferably of primates, including humans, which are isolated from the blastocyst stage embryo. Human embryonic stem cell refers to a stem cell from a human and are preferably used in certain aspects of the present invention to produce human neural progenitor cells which are used in the present invention. The following phenotype markers are expressed by human embryonic stem cells:

SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, GCTM-2, TG343, TG30, CD9, Alkaline phosphatase, Oct 4, Nanog, TRex 1, Sox2, TERT and Vimentin . See Ginis, et al., *Dev. Biol.*, 269(2), 360-380 (2004); Draper, et al., *J. Anat.*, 200(Pt. 3), 249-258, (2002); Carpenter, et al., *Cloning Stem Cells*, 5(1), 79-88 (2003); Cooper, et al., *J. Anat.*, 200(Pt.3), 259-265 (2002); Oka, et al., *Mol. Biol. Cell*, 13(4), 1274-81 (2002); and Carpenter, et al., *Dev. Dyn.*, 229(2), 243-258 (2004). While any human stem cell can be used in the present methods to produce human dopaminergic and/or neuronal cells, preferred human embryonic stem cells for use in the present invention include stem cells from the cell lines BG01 and BG02, where available, as well as numerous other available stem cell lines.

The term “neuroprogenitor cells” or “neural progenitor cells” is used to describe cells which are the earliest multipotent neural stem cells and the cells from which dopaminergic progenitor cells and/or dopaminergic neuronal cells may be produced. A discussion of these cells and their production from pluripotent stem cells, including human embryonic stem cells, human induced stem cells, umbilical cord blood stem cells (which may include placental blood and/or tissue stem cells, as well as amniotic tissue stem cells) and related stem cells may be found in US patent no. 7,531,354 and international patent publication WO2006.044204, relevant portions of which are incorporated by reference herein, among others. These are self renewing cells that can differentiate into neurons, oligodendrocytes and astrocytes and in the present invention, dopaminergic progenitor cells and dopaminergic neurons. Neuroprogenitor cells (NP) or (NEP) according to the present invention may be further delineated into “early neuroprogenitor cells” and “late neuroprogenitor cells”. Early neuroprogenitor cells are neuroprogenitor cells which are freshly isolated without further propagation. Late neuroprogenitor cells are neuroprogenitor cells which have been propagated for at least about three months. In general, the present invention does not distinguish between early and late neuroprogenitor cells except with respect to the age of the cells, not their function or the production of dopaminergic progenitor cells and/or neuronal cells. Neuroprogenitor cells (hNPs) which are used in the present invention express markers associated with the earliest multipotent neural stem cells, including Nestin, Musashi-1,

Sox1, Sox2 and Sox3. It is noted that although feeder cell free neural progenitor cells may be used to produce dopaminergic progenitor cells and/or dopaminergic neuron cells according to the present invention, any neuroprogenitor cell as otherwise described herein may be used in the present invention. Preferred neuroprogenitor cells which are used in the present invention are produced according to the methods which are presented in U.S. patent no. 7,531,354 are adherent feeder cell free as well as free from embryoid bodies. Further preferred neuroprogenitor cells are those which have been propagated in a growth medium further comprising LIF and bFGF (e.g. about 3 days or considerably longer e.g. about 10-40 passages, about 20-30 passages) as otherwise described herein.

The term "differentiation protein" is used to describe a protein which optionally may be included in cell media used to grow cells to promote differentiation (also preferably attachment) of a primate embryonic stem cell, in particular, a human embryonic stem cell into a neuroprogenitor cell according to the present invention or differentiation of a neuroprogenitor cell into a dopaminergic progenitor cell or dopaminergic neuron cell. Embryonic stem cell differentiation proteins include for example, an extracellular matrix protein, which is a protein found in the extracellular matrix, such as laminin, tenascin, thrombospondin, and mixtures thereof, which exhibit growth promoting and contain domains with homology to epidermal growth factor (EGF) and exhibit growth promoting and differentiation activity. Other embryonic stem cell differentiation proteins which may be used in the present invention include for example, collagen, fibronectin, vitronectin, polylysine, polyornithine and mixtures thereof. In addition, gels and other materials which contain effective concentrations of one or more of these embryonic stem cell differentiation proteins may also be used. Exemplary embryonic stem cell differentiation proteins or materials which include these differentiation proteins include, for example, BD Cell-Tak™ Cell and Tissue Adhesive, BD™ FIBROGEN Human Recombinant Collagen I, BD™ FIBROGEN Human Recombinant Collagen III, BD Matrigel™ Basement Membrane Matrix, BD Matrigel™ Basement Membrane Matrix High Concentration (HC), BD™ PuraMatrix™ Peptide Hydrogel, Collagen I, Collagen I High Concentration (HC), Collagen II (Bovine), Collagen III, Collagen IV, Collagen V, and Collagen VI, among others. The preferred

embryonic stem cell differentiation protein for use in the present invention includes laminin.

In certain embodiments, a composition/material which contains one or more differentiation proteins is BD Matrigel™ Basement Membrane Matrix. This is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in ECM proteins. Its major component is laminin, followed by collagen IV, heparan sulfate, proteoglycans, entactin and nidogen.

The term "effective amount" is used throughout the specification to describe concentrations or amounts of components such as pluripotent stem cells, including embryonic stem cells or neuroprogenitor differentiation proteins, neuroprogenitor cells, dopaminergic progenitor cells or dopaminergic neuronal cells, components of cell media or other agents which are effective for producing an intended result within the context of practicing one or more aspects of the present invention. Effective amounts are those which are generally known to those of ordinary skill in the art and are typically used when growing embryonic stem cells, neuroprogenitor cells, dopaminergic progenitor and/or dopaminergic neuronal cells as otherwise described herein.

The term "dopaminergic progenitor cells" are progenitor cells which are capable of being differentiated to dopaminergic neuron cells in a differentiation medium which includes GDNF and optionally LIF, and express in one instance, Nurr1 and EN1, but not TH and Pitx3, in another instance, Nurr 1, EN1 and PITX3, but not TH and in a further instance, Nurr 1, EN1, PITX3 and TH, but not VMAT2 or DAT. These cells also express LRRK2 (see figure 15). A preferred dopaminergic progenitor cell is that which expresses Nurr 1, EN1, PITX3, TH and LRRK2, but not VMAT2 or DAT. Each of these cells may be isolated during differentiation from neural progenitor cells by standard methods in the art (flow cytometry, antibody capture, etc. specific for the expressed biomarkers all of which are well known in the art) and cryopreserved for storage, whereupon they can be later thawed and used in therapies as otherwise described herein.

The term "dopaminergic neuronal cells" are postmitotic dopaminergic neuronal cells which exhibit the presence of dopamine and dopamine receptors (1, 2 and/or 5) and have a neuronal phenotype which expresses the following biomarkers (as evidenced by the +): VMAT2+, DAT+, TH+, Pitx3+, EN1+ and Nurr1+. These cells may be obtained from neuroprogenitor cells according to the present invention, indirectly from pluripotent stem cells including embryonic stem cells according to the present invention or from dopaminergic progenitor cells as otherwise described herein.

The term "administration" or "administering" is used throughout the specification to describe the process by which dopaminergic progenitor cells and/or dopaminergic neuronal cells according to the present invention are delivered to a patient for treatment purposes. Dopaminergic progenitor cells and/or dopaminergic neuronal cells may be administered a number of ways including parenteral (such term referring to intravenous and intraarterial as well as other appropriate parenteral routes), intrathecal, intraventricular, intraparenchymal (especially including into the spinal cord, brainstem or motor cortex), intracisternal, intracranial, intrastriatal, and intranigral, among others which term allows the cells to migrate to the site where needed. Administration will often depend upon the disease or condition treated and may preferably be via a parenteral route, for example, intravenously, by administration into the cerebral spinal fluid or by direct administration into the affected tissue in the brain. For example, in the case of Alzheimer's disease, Huntington's disease and Parkinson's disease, the preferred route of administration will be a transplant directly into the striatum (caudate putamen) or directly into the substantia nigra (Parkinson's disease). In the case of amyotrophic lateral sclerosis (Lou Gehrig's disease) and multiple sclerosis, the preferred administration is through the cerebrospinal fluid. In the case of lysosomal storage disease, the preferred route of administration is via an intravenous route or through the cerebrospinal fluid. In the case of stroke, the preferred route of administration will depend upon where the stroke is, but will often be directly into the affected tissue (which may be readily determined using MRI or other imaging techniques).

The terms "grafting" and "transplanting" and "graft" and "transplantation" are

used throughout the specification synonymously to describe the process by which progenitor and/or neuronal cells according to the present invention are delivered to the site within the nervous system where the cells are intended to exhibit a favorable effect, such as repairing damage to a patient's central nervous system, treating a neurodegenerative disease or treating the effects of nerve damage caused by stroke, cardiovascular disease, a heart attack or physical injury or trauma or genetic damage or environmental insult to the brain and/or spinal cord, caused by, for example, an accident or other activity. Progenitor and/or neuronal cells for use in the present invention may also be delivered in a remote area of the body by any mode of administration as described above, relying on cellular migration to the appropriate area in the central nervous system to effect transplantation.

The term "non-tumorigenic" refers to the fact that the cells do not give rise to a neoplasm or tumor. Progenitor and/or neuronal cells for use in the present invention are generally free from neoplasia and cancer.

The term "cryopreserved" refers to a population of cells which has been cryopreserved using methods which are well-known in the art. Cryoprotectants such as dimethylsulfoxide (DMSO), acetamide, dimethylacetamide, ethylene glycol, propylene glycol and glycerol among others are preferably added to the mixture of cells to be cryopreserved in order to limit cell damage principally during the cryopreservation step(s). Dopaminergic progenitor cells and/or dopaminergic neuronal cells may be cryopreserved as an optional step to the methods otherwise disclosed herein.

The term "cell medium" or "cell media" is used to describe a cellular growth medium in which pluripotent stem cells including embryonic stem cells, neuroprogenitor cells and/or dopaminergic progenitor cells and/or dopaminergic neuronal cells are grown. Cellular media are well known in the art and comprise at least a minimum essential medium plus effective amounts of optional agents such as growth factors, including fibroblast growth factor, preferably basic fibroblast growth factor (bFGF), leukaemia inhibition factor (LIF), glucose, non-essential amino acids, glutamine, insulin, transferrin,

beta mercaptoethanol, and other agents well known in the art. Preferably, cell media used in the present invention (to differentiate neural progenitor cells to dopaminergic progenitor cells and/or dopaminergic neuronal cells) also includes glial cell-line derived neurotrophic factor (GDNF). Preferred media include commercially available media such as DMEM/F12 (1:1) or neurobasal media, each of which may be supplemented with any one or more of L-glutamine, knockout serum replacement (KSR), fetal bovine serum (FBS), non-essential amino acids, leukemia inhibitory factor (LIF), beta-mercaptoethanol, basic fibroblast growth factor (bFGF), glial cell-line derived neurotrophic factor (GDNF) and an antibiotic, B27 medium supplement and/or N2 medium supplement. Cell media useful in the present invention are commercially available and can be supplemented with commercially available components, available from Invitrogen Corp. (GIBCO) and Biological Industries, Beth HaEmek, Israel, among numerous other commercial sources. In preferred embodiments at least one differentiation agent is added to the cell media in which a stem cell or neuroprogenitor cell is grown in order to promote differentiation of the stem cells into neuroprogenitor cells and the neuroprogenitor cells into motor neuron cells. One of ordinary skill in the art will be able to readily modify the cell media to produce neuroprogenitor or motor neuron cells pursuant to the present invention.

The term "laminin" refers to a specific differentiation protein used in the present invention which includes multidomain glycoproteins which are the major noncollagenous components of basement membranes. Laminin has numerous biological activities including promotion of cell adhesion, migration, growth, and differentiation, including neurite outgrowth. Laminin can be used as a thin coating on tissue-culture surfaces or as a soluble additive to culture medium. Laminin has been shown in culture to stimulate neurite outgrowth, promote cell attachment, chemotaxis, and cell differentiation.

The term "highly pure", "high purity" or "high yield" is used to describe a population of cells (whether dopaminergic progenitor cells or dopaminergic neuron cells) which have been differentiated are at least about 50% pure (i.e., about 50% or more of the cells within the population are of a particular type of cell), including in the absence of

further separation. In certain aspects of the invention, cells of high purity are at least about 55% pure, at least about 60% pure, at least about 65% pure, at least about 75% pure, at least about 85% pure or at least 90+% pure, at least about 95+% pure, at least about 99+% pure.

The term “neurodegenerative disease” is used throughout the specification to describe a disease which is caused by damage to the central nervous system and which damage can be reduced and/or alleviated through transplantation of dopaminergic progenitor cells and/or neuronal according to the present invention to damaged areas of the brain and/or spinal cord of the patient. Exemplary neurodegenerative diseases which may be treated using the neural cells and methods according to the present invention include for example, Parkinson’s disease, Huntington’s disease, amyotrophic lateral sclerosis (Lou Gehrig’s disease), Alzheimer’s disease, lysosomal storage disease (“white matter disease” or glial/demyelination disease, as described, for example by Folkerth, *J. Neuropath. Exp. Neuro.*, 58, 9, September, 1999), Tay Sachs disease (beta hexosaminidase deficiency), other genetic diseases, multiple sclerosis, brain injury or trauma caused by ischemia, accidents, environmental insult, etc., spinal cord damage, ataxia and alcoholism. In addition, the present invention may be used to reduce and/or eliminate the effects on the central nervous system of a stroke or a heart attack in a patient, which is otherwise caused by lack of blood flow or ischemia to a site in the brain of said patient or which has occurred from physical injury to the brain and/or spinal cord. The term neurodegenerative diseases also includes neurodevelopmental disorders including for example, autism and related neurological diseases such as schizophrenia, among numerous others.

Dopaminergic progenitor cells and/or neuronal cells which result from differentiation of neuroprogenitor cells according to the present invention may be used for *in vitro/in vivo* studies or for therapeutic intervention. For example, these cells may be used to conduct studies on treatments for patients, in cellular based assay systems (immunocytochemistry, high throughput and content assays, etc.) to identify drugs or other agents which may be useful to treat neurological conditions, to effect a

transplantation of the neuronal cells within a composition to produce a favorable change in the brain or spinal cord, or in the disease or condition treated, whether that change is an improvement (such as stopping or reversing the degeneration of a disease or condition, reducing a neurological deficit or improving a neurological response) or a complete cure of the disease or condition treated.

Dopaminergic progenitor cells and/or neuronal cells which result from differentiation of neuroprogenitor cells according to the present invention may be used for detection of toxins for homeland defense and in general neurotoxicity. Neurons are directly exposed to the substance of interest, thus potentially providing greater sensitivity to threats than that resulting from oral ingestion. The use of neurons is advantageous because they can provide not only a measure of the potential toxicity of a substance but also a warning that a substance has the capacity to influence neurobehavior.

The present invention may also be used in diagnostic/drug discovery applications, as well as the toxicology assays described above. In general, the present invention may be used to provide diagnostic assays for drug discovery, diagnostics for neurological diseases. In particular, the dopaminergic progenitor cells and/or dopaminergic neuron cells according to the present invention may be used to determine whether or not a suspect agent is a neurotoxin (for example, by looking at cell death or the effect on the health of the cells).

Dopaminergic progenitor cells and/or dopaminergic neuronal cells according to the present invention, which are free from contaminating feeder cells, may be used for treating a neurodegenerative disorder or a brain or spinal cord injury or neurological deficit comprising administering to (preferably, transplanting in) a patient suffering from such injury, a neurodegenerative disorder or neurological deficit an effective amount of dopaminergic progenitor cells and/or neuronal cells according to the present invention. Neurodegenerative disorders which may be treated using the method according to the present invention include, for example, Parkinson's disease, Huntington's disease, multiple sclerosis (MS), Alzheimer's disease, Tay Sachs's disease (beta hexosaminidase

deficiency), lysosomal storage disease, brain and/or spinal cord injury occurring due to ischemia, spinal cord and brain damage/injury, ataxia and alcoholism, among others, including a number which are otherwise described herein.

Dopaminergic progenitor cells and/or neuronal cells according to the present invention may be used for treating neurological damage in the brain or spinal cord which occurs as a consequence of genetic defect, physical injury, environmental insult or damage from a stroke, heart attack or cardiovascular disease (most often due to ischemia) in a patient, the method comprising administering (including transplanting), an effective number or amount of neural cells obtained from umbilical cord blood to said patient, including directly into the affected tissue of the patient's brain or spinal cord. Administering cells according to the present invention to a patient and allowing the cells to migrate to the appropriate site within the central nervous system is another aspect of the present invention.

Pharmaceutical compositions comprising effective amounts of dopaminergic progenitor and/or neuron cells are also contemplated by the present invention. These compositions comprise an effective number of dopaminergic progenitor cells and/or neuron cells, optionally in combination with a pharmaceutically acceptable carrier, additive or excipient. In certain aspects of the present invention, cells are administered to the patient in need of a transplant in sterile saline. In other aspects of the present invention, the cells are administered in Hanks Balanced Salt Solution (HBSS) or Isolyte S, pH 7.4. Other approaches may also be used, including the use of cellular media as otherwise described herein, preferably in the absence of growth factors. Such compositions, therefore, comprise effective amounts or numbers of dopaminergic progenitor or neuron cells in sterile saline. These may be obtained directly by using fresh or cryopreserved cells.

Pharmaceutical compositions according to the present invention preferably comprise an effective number within the range of about 1.0×10^2 progenitor and/or

neuron cells to about 5.0×10^7 cells, more preferably about 1×10^4 to about 9×10^6 cells, even more preferably about 1×10^6 to about 8×10^6 cells generally in solution, optionally in combination with a pharmaceutically acceptable carrier, additive or excipient. Effective numbers of progenitor and/or neuronal cells, either within a sample of other cells or preferably, as concentrated or isolated cells, may range from as few as several hundred or fewer to several million or more, preferably at least about one thousand cells within this range. In aspects of the present invention whereby the cells are injected in proximity to the brain or spinal cord tissue to be treated, the number of cells may be reduced as compared to aspects of the present invention which rely on parenteral administration (including intravenous and/or intraarterial administration).

In using compositions according to the present invention, fresh or cryopreserved progenitor and/or neuron cells may be used without treatment with a differentiation agent or progenitor cells may be used with or without an effective amount of a differentiation agent prior to being used in a neuronal transplant.

In a preferred aspect of the present invention, human neuroprogenitor cells are grown in standard cellular media (preferably, at least a minimum essential medium, preferably DMEM/F12 or neurobasal media) supplemented with effective amounts of at least one growth factor selected from the group consisting of glial cell-line derived neurotrophic factor (GDNF), leukaemia inhibition factor (LIF), basic Fibroblast Growth Factor (bFGF), and mixtures thereof, preferably at least GDNF, more preferably GDNF and LIF (preferably in the absence of bFGF), as well as additional components including one or more of L-glutamine, at least one antibiotic and optionally, one or more of non-essential amino acids, beta-mercaptoethanol, fetal bovine serum, (FBS), knockout serum replacement (KSR), for a period of at least about 3 days, 4 days, 5 days, 6 days, 7 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days 20 days and 21 days or more to provide dopaminergic progenitor cells (in preferred aspects, in high yield) as otherwise described herein. Depending upon the growth factors added to the media and the length of time neural progenitor cells are grown in the medium and whether one includes GDNF alone or in combination with LIF (preferably in the absence

of bFGF) in the media in which those progenitor cells are grown, dopaminergic progenitor cells (expressing at least Nurr+ and EN1+ and in addition, TH+ and PITX3+) may be isolated, purified and cryopreserved. It is noted that if dopaminergic progenitor cells which express Nurr+ and EN1+ are desired, differentiation for periods of 3 days to about 14 days are preferred, if progenitor cells which express Nurr+, EN1+ and PITX3+ (w/out TH+) are desired, differentiation for periods of up to about 14-18 or more days are preferred and if progenitor cells which express Nurr+, EN1+, PITX3+ and TH+ are desired, differentiation for periods of about 14-21 days, preferably closer to 21 days, are preferred. Each of these progenitor cells also generally expresses the biomarker LRRK2. Preferably, GDNF is included in the medium in which differentiation occurs (See figures 4-6), preferably along with LIF and preferably w/o bFGF. Progenitor cells may be isolated from the other cells within the medium by FACS, antibody capture or other methods standard in the art and thereafter cryopreserved for storage and subsequently thawed for therapy using standard methods. The progenitor cells so isolated are free from contaminating feeder cells. Dopaminergic neuronal cells (expressing Nurr+, EN1+, PITX3+, TH+, VMAT2+ and DAT+) may also be isolated from the GDNF containing media after approximately 21 days using FACS or other method known in the art.

The amount of growth factor used in the present invention, is an effective amount. In the case of leukaemia inhibition factor (LIF), the concentration when used generally ranges from about 1 to about 20 ng/ml or more, about 5 to about 15 ng/ml, about 10 ng/ml. In the case of basic fibroblast growth factor (bFGF), the concentration when used ranges from about 5 to about 50 ng/ml, about 10 to about 30 ng/ml, about 15 to 25 ng/ml, about 20 ng/ml. In preferred aspects bFGF is excluded from the differentiation medium in order to produce dopaminergic progenitor cells and motor neuron cells. In the case of glial cell-line derived neurotrophic factor (GDNF), the concentration when used ranges from about 5 to about 50 ng/ml, about 10 to about 35 ng/ml, about 20 to 30 ng/ml, about 25 ng/ml. While any one or more of LIF, bFGF and GDNF may be used in medium to differentiate neural progenitor cells to dopaminergic progenitor cells and/or dopaminergic neuron cells, the inclusion of LIF at about 10ng/ml and GDNF at about 25 ng/ml (in the absence of bFGF) is preferred.

In a further alternative embodiment, hNPs, preferably propagated hNPs as otherwise described herein are exposed to a differentiation medium comprising an effective amount of Sonic Hedgehog and FGFR8 and optionally, BDNF and/or TGF β 3 to produce human dopaminergic progenitor cells and/or human dopaminergic neurons as otherwise described herein. In this method, hNPs are preferably propagated for a period of about 3 days or more with an effective amount of LIF and optionally, an effective amount of bFGF as otherwise described herein or alternatively and preferably, with both LIF (e.g., about 2 ng/ml to about 25 ng/ml, about 5 to 20 ng/ml, about 5 to 15 ng/ml, about 8-12 ng/ml, preferably about 10 ng/ml) and bFGF (e.g., about 5 ng/ml to about 50 ng/ml, about 10 ng/ml to about 40 ng/ml, about 15 ng/ml to about 30 ng/ml, about 15 to about 25 ng/ml, about 20 ng/ml) for a period of between about 10 to 40 passages, preferably about 20 and 30 passages with each passage occurring on day 3-4, preferably day 4). The propagated hNPs produced as described above, are exposed to a cellular differentiation medium as otherwise described herein to produce human dopaminergic progenitors and/or human dopaminergic, wherein the cellular differentiation medium comprises an effective amount of Sonic Hedgehog (about 50-500 ng/ml, about 100-400 ng/ml, about 150-300 ng/ml, about 200 ng/ml.) and an effective amount of fibroblast growth factor 8 (FGF8) (about 25ng/ml to about 400 ng/ml, about 50 ng/ml to about 300 ng/ml, about 75 ng/ml to about 200 ng/ml, about 100 ng/ml and optionally, effective amounts of brain-derived neurotrophic factor (BDNF) (about 2 ng/ml to about 50 ng/ml, about 5 ng/ml to about 35 ng/ml, about 10 ng/ml to about 20 ng/ml) and/or transforming growth factor type β 3 (TGF β 3) (about 0.1 ng/ml to about 20 ng/ml, about 0.25 ng/ml to about 10 ng/ml, about 0.5 to about 5 ng/ml, about 1-2 ng/ml, about 1 ng/ml) for a period ranging from about 3 days to about 21 days, about 3 days to about 18 days, about 3 days to about 14 days, about 7 days to about 14 days, about 3 days to about 7 days (preferably, without passaging and with the media including differentiation factors being changed about every 1-4 days, preferably every other day) to produce human dopaminergic progenitor cells and/or human dopaminergic neuron cells, which are optionally separated and cryopreserved. In this method, the cellular differentiation medium comprising Sonic Hedgehog and FGF8 may further comprise GDNF and/or LIF in effective amounts as otherwise described herein, in addition to, or as a substitute for the BDNF and/or the

TGF β 3 to produce greater concentrations of human dopaminergic progenitor cells and/or dopaminergic neuron cells.

The following examples are provided to further illustrate the present invention. It is to be noted here that the examples presented are not to be construed as limiting the invention in any way. Appendix A also contains examples according to the present invention.

Examples- First Set

Materials and Methods hNP Cultures

Human neural progenitor (hNP) cells were derived from hESC line WA09 by our lab as previously described (Shin et al. 2006). Briefly, after one week of culture on mouse fibroblast feeder layers, WA09 hESCs were grown with derivation media containing Dubecco's modified Eagle medium (DMEM)/F12 medium (Gibco) supplemented with 2mM L-glutamine (Gibco), 2 UImL penicillin (Gibco), 2pglmL streptomycin (Gibco), N2 (Gibco), and 4 ng/ml basic fibroblast growth factor (bFGF; R&D) for 7 days. The feeder layer was removed and rosettes were allowed to develop after 3 days. These rosettes were propagated on polyornithine (Sigma) and laminin (Sigma) coated dishes in neurobasal medium (Gibco) supplemented with 2mM L-glutamine, 2 UImL penicillin, 2 pg1mL streptomycin, 827 (Gibco), 20 ng/ml bFGF, and 10nglmL leukemia inhibitory factor (LIF; Millipore). Cells were grown on polyornithinellarninin coated 100mm dishes (Falcon) in growth media consisting of neural basal media, 1X penicillin1 streptomycin, 2mM L-glutamate, B27, 10nglmL LIF, and 20nglmL bFGF. Media was changed every other day and cells were passaged every fourth day or as needed. Cells used for this experiment were passage 22-39.

Dopaminergic Neural Differentiation

hNP cells were grown on polyornithinellaminin coated 35mm plates (Falcon) for flow cytometry or on polyornithinellarninin coated 4 well slides (Falcon) for

immunocytochemistry staining. Cells were plated in growth media consisting of neural basal media, 1X penicillin/streptomycin, 2mM L-glutamate, 827, 10ng/mL LIF and 20ng/mL bFGF. After 24 hours, the media was changed to neural differentiation media, which consisted of growth media without bFGF or neural differentiation media plus 25ng/mL GDNF (Neuromics). Media was changed every three days. Cells were harvested at Day 0, 3, 7, 14 and 21 for further analysis.

Immunocytochemistry and Cell Quantification

Cells were fixed with 2% paraformaldehyde (PFA; Electron Microscopy Sciences) in PBS with calcium and magnesium (PBS+I+) (Thermo Scientific) for 20 minutes and processed for immunocytochemistry. Cells were washed in PBS+/+ 3 times followed by 3 washes for 5 minutes each of permeabilization buffer consisting of 25mL of Tween 20 (EMD Chemicals) in 50mL of high salt buffer. Cells were then blocked in 6% goat serum (Jackson Immuno) for 45 minutes. The following primary antibodies were used: mouse antiTuj1 (1 : 200, Neuromics), chicken antiTH (1 : 1 000), rat antiDAT (1 : 1000), and rabbit antiVMAT2 (1 : 500), antiPitx3 (1 : 1 000) (all from Millipore, Inc), antiNurr1 (1 : 1 000, R&D), and antiEN1 (1 : 250, SantaCruz). Reaction was revealed using AlexasFlour goat 488, 594, or 633 secondary antibodies (1 : 1 000, Molecular Probes). Cell nuclei were stained using DAPI (Invitrogen). Fluorescence was visualized using spinning disk confocal microscope (Olympus). Negative controls included human mesenchymal cells and secondary only staining. Cell counting was performed using Image Pro software Media Cybernetics. Five random visual fields were selected and counted in triplicate. Data is presented as mean $\pm$ SD. Values of $p < 0.05$ were considered significant using ANOVA and Tukey's Pair-Wise test (Statistical Analysis Software, SAS Institute).

Flow Cytometry

Cells were fixed with 4% PFA in PBS without calcium and magnesium (PBS-I-) for 10 minutes. Cells were washed in PBS+/+ 3 times followed by 3 washes for 5 minutes

each of permeabilization buffer consisting of 25mL of Tween 20 in 50mL of high salt buffer. Cells were blocked in 6% goat serum for 45 minutes. The following primary antibodies were used: mouse anti-Tuj1 (1:200, Neuromics), chicken antiTH (1:100), rat antiDAT (1 : 1 000), rabbit antiVMAT2 (1 500) rabbit antiPitx3 (1 :1 00) (all from Millipore), mouse antiNurr1 (1: 10 0, R&D), and rabbit antiEN1 (1: 250, SantaCruz). Reaction was revealed using AlexasFlour goat 488 or 633 secondary antibodies (1 : 1 000, Molecular Probes). Cells were quantified on Dako Cyan (Beckman Coulter). Negative controls were secondary only and cell only staining. Cell quantification was done using FlowJo (Treestar) software. Each experiment was run in triplicate. Data is presented as mean +SD. Values of pc.05 was considered significant using ANOVA and Tukey's Pair-Wise (Statistical Analysis Software, SAS Institute).

PCR

RNA was extracted using the Qiashtredder and RNeasy kits (Qiagen) according to manufacturer's instructions. The RNA quality and quantity was verified using a RNA 600 Nano Assay (Agilent Technologies) and the Agilent 21 00 Bioanalyzer. Total RNA (5 pg) was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems Inc.) according to manufacturer's instructions. Reactions were initially incubated at 25OC for 10 minutes and subsequently at 37OC for 120 minutes. Primers were selected using Primer Blast (National Center for Biotechnology Information) and were as follows:

DRD1sense: TCTCGAAAGGAAGCCAAGAA
antisense: TTCCCCAAATAAAGCACTG,

DRD2sense: GCTCCACTAAAGGGCAACTG
antisense: TTCTCCTTCTGCTGGGAGAG,

DRD3 sense: TACCTGGAGGTGACAGGTGG
antisense:CTATGGTGGGACTCAGGGAA,

DRD4 sense TCGTCTACTCCGAGGTCCAG

antisense: AGCACACGGACGAGTAGACC,

DRD5 sense: CCATCTCTTCCTCGCTCATC

antisense: CCCAGACAGACTCAGCAACA.

cDNA, primers, GoTaq Green (Promega) and water were added to PCR tubes and incubated at 95°C for 3 minutes and then 35 cycles at 57°C for 30 seconds and then 72°C for 30 seconds. Finally, the reactions were incubated at 72°C for 10 minutes. The PCR reactions were run on a 2% agarose gel (Bio-Rad) with ethidium bromide (Promega) for 45 minutes at 100V. Gel was visualized on Ugenius (Syngene). HPLC hNP cells, hNP cells differentiated for 21 days, and hNP cells differentiated for 21 days with GDNF were exposed to 56mM KCl (Sigma) for 30 minutes. Media was collected and acidified with HCl (Sigma) and stored at -80°C until HPLC was performed. Media was sonicated with 0.2 ml of ice-cold buffer/mobile phase (0.1 mM NaHSO₄/0.1 mM EDTA/0.2 mM octanesulfonic acid/6.5% acetonitrile, pH 3.1). The homogenate was centrifuged at 4°C at 16,100' g for 30 min, and then subjected to the same centrifugation at 16,100 ' g for 30 min in a 0.22-mm spin column. The resulting supernatant (20 ml) was injected by using a Waters 717 autoinjector (Whatman, Milford, MA) and run through a C₁₈-mm max reverse-phase column (4-mm, 80-14 silica; 150 ' 4.6 mm; Phenomenex, Torrance, CA) and an electrochemical (HPLC-EC) detector (cells maintained at 0.5 nA), where dopamine and dopac were analyzed. Samples were delivered at a constant rate of 1 ml/min (retention times: DA, 8.32 min; DOPAC, 13.05 min). The position and height of DA and DOPAC peaks were compared with reference standard solutions (Sigma-Aldrich, St. Louis, MO). Peak areas were quantified by Millennium software (Waters).

Results

hNP cells can be induced and specified towards a dopaminergic neuron

hNP cells used in this study were evaluated for the receptor involved in GDNF

activation, the RET receptor, which is expressed in the substantia nigra of mice (Airaksinen and Saarma 2002). While human mesenchymal cells (hMSCs; Figure 8 B) and hESCs did not express the RET receptor, hNP cells (Figure 8 C), differentiated neurons (Figure 8 D) and neurons differentiated with GDNF (Figure 8E) expressed RET at the cell membrane suggesting an active site for GDNF signaling.

Neither hNP cells nor hMSC (data not shown) expressed RET, EN1, TH, PITX3, DAT or VMAT2 at the protein level. However, hNP cells did express NURR1, a transcription factor necessary for regulating TH, DAT and VMAT2 expression in dopaminergic neurons, as did GDNF differentiation cultures and cultures with differentiation media only through day 21 (Figure 9A, B). While hNP cells did not express EN1 (Figure 9D), differentiation of hNP cells with GDNF induced expression of EN1 at day 3 through 21 (Figure 9E). Flow cytometry further confirmed immunocytochemistry results, the hNP cells and differentiated neurons with GDNF were NURR1+ (Figure 9G, H) and differentiated neurons with GDNF were EN1+ (Figure 9I, J). The expression of NURR1 increased significantly ($p < 0.05$) at day 7 with GDNF (68.0 ± 4.0) compared with neurons differentiated without GDNF (46.0 ± 2.0 ; Figure 9C). As previously reported, NURR1 and EN1 were localized to the perinuclear space [(Figure 9B,E) (Di Nardo et al. 2007; Smidt and Burbach 2007)]. Also at day 7, flow cytometry indicated that there was a significant ($p < 0.05$) increase in EN1 expression with GDNF exposure (36.3 ± 4.0) compared to cells differentiated without GDNF (21.4 ± 5.7 ; Figure 9F). These data indicated the presence of early induced and specified NURR1 stage cells in the hNP cell population that increased significantly ($p < 0.05$) when GDNF is present (62.9 ± 6.0 ; Figure 9C) and the EN1+ population is increased at day 21 with GDNF (74.0 ± 1.0) compared with neurons differentiated without GDNF (37.7 ± 5.7 ; Figure 9F). The hNPs also expressed the biomarker LRRK2 (figure 14).

hNP cells differentiate toward dopaminergic progenitors

hNP cells differentiated with GDNF for 21 days expressed TH (Figure 10A, B), the rate limiting enzyme for dopamine synthesis, and PITX3 (Figure 10D, E), a

transcription factor expressed only in substantia nigra dopaminergic neurons. Similar to previous studies, TH expression was in the cytoplasm (Figure 10B), while PITX3 expression was in the nucleus (Figure 10E; Messmer et al. 2007; Smidt and Burbach 2007). Flow cytometry further confirmed the immunocytochemistry results (Figure 10G-J). The percentage of cells expressing PITX3 was significantly higher (pc.05) at day 14 (28.4 ± 4.6) and 21 (65.7 ± 2.0) with GDNF when compared to the percentage of PITX3+ cells (0%) in groups without GDNF at either time point (Figure 10C). At day 21, there was a significant increase in the percentage of TH expressing cells in the GDNF cultures (51.0 ± 2.0) relative to cells differentiated without GDNF (2.9 ± 0.1 ; Figure 10F).

Mature dopaminergic neurons express functional markers

hNP cells differentiated with GDNF for 21 days expressed the dopamine transporter (DAT; Figure 11A, B) and vesicular monoamine transporter (VMAT2; Figure 11D, E); similar to a previous report. DAT and VMAT2 was localized to the cytoplasm (Figure 11B,E; Smidt and Burbach 2007). Flow cytometry confirmed immunocytochemistry results (Figure 11G-J). DAT expression significantly increased (pc.05) at day 14 in the treated cells (16.5 ± 3.1) compared with neurons differentiated without GDNF (5.4 ± 1.7 ; Figure 11C). At day 14, there was a significant (pc.05) increase in VMAT2 expression at day 14 with or without GDNF exposure (58.0 ± 25.2 ; 61.8 ± 9.5 ; Figure 11F).

Differentiated hNP cells release dopamine

We examined whether the differentiated hNP cells expressed members of the dopamine receptor 1 (D1) family, which consists of D1 and dopamine receptor 5 (D5), and of the dopamine receptor 2 (D2) family, which consists of D2, dopamine receptor 3 (D3), and dopamine receptor 4 (D4). Receptors in the D1 family are associated with neural development; whereas, those receptors in the D2 family are more closely deregulated in neurodegenerative diseases and schizophrenia (Missale et al. 1998). hNP cells, differentiated neurons and neurons differentiated with GDNF expressed the D1, D4, and

D5 mRNA in all three cell types, while D2 and D3 mRNA expression was not seen in any cell type (Figure 12A).

To determine if the dopamine-like neurons were active, dopamine release was evoked with KCl and measured with HPLC. Dopamine-like neurons showed a significant ($p < 0.05$) increase in dopamine release ($0.98 \text{ ng/ml} \pm 0.05$) when differentiated with GDNF compared to hNP cells ($0.96 \text{ ng/ml} \pm 0.02$; Figure 12B). In addition, neurons differentiated with GDNF had a significant ($p < 0.05$) increase of L-dopa ($0.18 \text{ ng/ml} \pm 0.05$) compared with differentiation without GDNF ($0.04 \text{ ng/ml} \pm 0.02$; Figure 12C).

PCR for markers of other neural cell types showed no expression for G protein-coupled inwardly rectifying potassium channel (GIRK) or tryptophan hydroxylase 1 (TPH1), with expression in differentiated neurons for choline acetyltransferase (ChAT). Neurons differentiated with GDNF also expressed ChAT, glutamate dehydroxylase (GAD), phenylethanolamine-N-methyl transferase (PMNT), and dopamine-beta-hydroxylase (DBH). DBH was expressed in hNP cells, differentiated neurons, and neurons differentiated in the presence of GDNF (Figure 13). To confirm that the cells that were reactive for TH were not also reactive for DBH, immunocytochemistry was performed on hNP cells differentiated for 14 days with GDNF. Separate populations, one that expressed DBH only, one that expressed TH only, and one that expressed DBH and TH, were found within the differentiated cultures (Figure 13).

Discussion

Pluripotent hESCs differentiated towards a dopaminergic phenotype offer a potential source of cells to study PD in vitro, for developing PD specific cell based assays for drug discovery and eventually a cell source for therapy (Perrier et al. 2004; Wernig et al. 2008). The goal of this study was to examine the progressive in vitro differentiation of hESC derived propagated hNP cells to a dopaminergic fate and to determine whether hNP cells were responsive to a one-step dopaminergic differentiation process using LIF alone or in combination with GDNF. Here we demonstrated for the first time a method of

deriving dopaminergic neurons from a starting hNP cell population without the use of feeder cells. The resulting differentiated population produced up to 50.5% TH+ cells when GDNF was added to the differentiation culture containing LIF, and corresponded with an increase in PITX3 expression. This population of TH and PITX3 positive cells express the dopamine receptors D1, D4 and D5 and release dopamine as measured by HPLC in comparison with the levels seen by other groups (Anwar et al. 2008).

Previously, hESCs had been differentiated to dopaminergic neurons using the five-stage method and the SDIA method. The five-stage method had proven to be less efficient than the SDIA method (Perrier et al. 2004). However, the SDIA method involved the use of contaminating animal feeder layers, preventing their eventual movement to clinical trials. Co-culture of cells would hamper the use of these cultures in assays and potential therapeutic applications. In the present study, the hNP cells progressed through the dopaminergic specification stage marked by co-expression of NURRI and ENI. Longer differentiation culture in the presence of GDNF and LIF led to the dopamine progenitors expressing TH and PITX3. These progenitors became phenotypically mature dopaminergic neurons (DAT and VMAT2 positive). This study suggests that continually cultured populations of hNP cell can differentiate to dopaminergic like fate. These cells expressed traits similar to their *in vivo* counterparts and GDNF enhanced the *in vitro* process. We hypothesize that under these conditions, caudalization was likely induced through bFGF and LIF effects on propagated hNP cells. Then the removal of bFGF and addition of GDNF to the LIF containing differentiation media induced the hNP cells to a dopaminergic phenotype.

In this study, all hNP cells were exposed to bFGF and LIF during hNP cell propagation. Mouse ESCs (mESC) cultured in the presence of bFGF developed into hindbrain and brain stem neural cells (Chiba et al. 2005). Chick embryos cultured with bFGF demonstrated induction of caudal properties in the paraxial mesoderm of the primitive streak (Muhr et al. 1997). A study of the effect of bFGF on hESCs showed increased expression of HOX genes that are suggestive of a caudalization of the neural cells differentiated from the hESCs (Erceg et al. 2008). Our use of bFGF prior to dopaminergic cell differentiation may have had a caudalizing priming effect on hNP cells. In addition, others establish NP cells using epidermal growth factor (EGF) and

bFGF but not LIF (Carpenter et al. 2001; Elkabetz and Studer 2008). Since our hNP cells were established in an adherent monolayer continuous culture with LIF and bFGF and without EGF, these conditions potentially led to a population of hNP cells that were primed for differentiation toward a dopaminergic fate. We found that under these conditions hNP cells expressed NURRI, a marker representative of the dopaminergic induction stage and potentially eliminating the need to induce a dopaminergic specification in these NP cell cultures.

Similar to our findings with hNP cells, when LIF was used in addition to GDNF to differentiate mESCs, increased TH expression was observed, suggesting a role for LIF and GDNF in differentiation and neuroprotection of dopaminergic neurons (Kim et al. 2008). LIF, a cytokine from the interleukin 6 family, has been implicated in the differentiation of dopaminergic neurons in murine models (Carvey et al. 2001). Murine substantia nigra derived NP cells exposed to LIF were protected from 6-OHDA damage, which selectively affects the cells of the dopaminergic system. In addition, rat primary cervical ganglia exposed to LIF had decreased dopamine beta-hydroxylase (DPH) expression through LIF's suppression of the noradrenergic properties of neural cells (Dziennis and Habecker 2003; Liu and Zang 2009). LIF used in combination with GDNF in rat fetal mesencephalic neural progenitor cells (NPCs) increased differentiation towards dopaminergic neurons as shown by increased TH expression in the cultures (Carvey et al. 2001; Storch et al. 2001). GDNF increased axonal outgrowth, target innervation, dopaminergic neuron survival, RET localization and neuroprotection in the dopaminergic neurons of the striatum (Airaksinen and Saarma 2002). Therefore, LIF in combination with GDNF has been shown by others and confirmed in this study using hNP cells to have a synergistic and potentially additive effect on differentiation and survival of dopaminergic cells in vitro.

The present invention represents a one-step process for dopaminergic derivation from a primed source of proliferative hNP cells. This work differed from previous dopaminergic differentiation studies by utilizing GDNF's effect on primed hNP cells that expressed the GDNF receptor RET without the addition of dopaminergic induction

factors or feeder cells. Previous work in hESCs (Perrier et al. 2004; Roy et al. 2006) first induced early neural differentiation and then used the midbrainhindbrain organizing factors SHH and fibroblast growth factor 8 (FGF8). These two factors were involved in organizing the borders of midbrain dopaminergic development (Smidt and Burbach 2007). In contrast, the hNP cells used in this study were predetermined to a neural lineage based on previous work showing expression of neural markers (Dhara and Stice 2008; Shin et al. 2006). The hNP cells were NURR1 positive suggesting that they were primed to become dopaminergic neurons. The most effective dopaminergic differentiation occurred when LIF and GDNF were used on these LIF and bFGF primed hNP cells. These factors played important roles in directing the hNP cells toward a dopaminergic fate: LIF tended to repress the noradrenergic fate in the mouse and bFGF induced caudalization (Chiba et al. 2005; Dziennis and Habecker 2003; Hynes et al. 1995). The hNP cells expressed the RET receptor; whereas, hESCs or in the hMSCs did not. Expression of the RET receptor suggested an active site for GDNF. The use of bFGF in propagation and LIF and GDNF in differentiation leading to an increase in TH and PITX3 positive neurons suggests that the hNPs used in this study were primed for dopaminergic differentiation. This population of proliferative primed adherent hNP cells provides a novel cell source for study of dopaminergic differentiation and PD drug development as well as indicates that LIF and GDNF are effective growth factors involved in the in vitro differentiation of hNP cells towards a dopaminergic fate.

Genetic Expression Profile of GDNF Differentiated Neurons

Inhibitors to the MAPK (PD-98059) and PI3K (LY-294002) pathway showed a decrease in TH expression at day 21 post differentiation of human neural progenitor cells with the addition of glial cell-line derived neurotrophic factor (GDNF). Due to the unknown role of MAPK and PI3K pathway in GDNF effects of dopaminergic differentiation, further studies were performed to determine their mechanism of action following GDNF binding. MAP2K3 (MEK3) acts upstream to Erk1/2 and was found up-regulated with GDNF exposure to differentiated cells compared to cells differentiated without GDNF. Cdk2, another up-regulated gene, is activated by Erk1/2 and is involved

in actin regulation. This suggests that the MAPK pathway is involved in GDNF differentiation through the increase of axonal length or arborization. The significant increase of MST1 and CDC42 further confirms this conclusion. One final activated protein in the MAPK pathway is the MAPKp38 gene which is activated in response to environmental stress to help control apoptosis. A potential mechanism of action for this signaling factor is to prevent apoptosis in differentiating cells. eIF4G1, up-regulated in neurons differentiated with GDNF compared to those differentiated without GDNF, is involved in transcriptional regulation and is modulated by mTOR activity. eIF4G1 further activates other eIF proteins and turns on translation of various genes. The pathway activated by mTOR and eIF4G1 potentially may be involved in preventing apoptosis or enhancing catabolic processes during times of stress. JUN was also significantly up-regulated and it also has a role in apoptosis suggesting apoptosis as a mechanism for GDNF differentiation. Up-regulation of ILK in neurons differentiated with GDNF compared to those differentiated without leads to GSK-3 activation. GSK-3 acts upstream of mTOR to block its activity and to increase cell proliferation. A balance between the activation/deactivation of these two pathways may demonstrate a key role for them in timing of GDNF differentiation. A final part of the PI3K pathway that was up-regulated was Shc. Shc, through RAS, activates Erk just as the MAPK pathway does. This may provide the source of pathway crosstalk in GDNF differentiation. The genetic profile of neurons differentiated with GDNF compared to those differentiated without GDNF demonstrates key roles for neural arborization, increase in axonal length and regulation of apoptosis as potential mechanisms of action for GDNF control of dopaminergic differentiation in human neural progenitor cells

The work presented herein examined the effect of GDNF on the differentiation of dopaminergic neurons and their developmental progression to a mature dopaminergic neuron. Mature dopaminergic neurons require a specific progression of proteins and transcription factors which aid in the development of machinery required to produce, release and reuptake dopamine from the synapse. These factors were examined in the work proposed in this patent application. Nurr1 and EN1 are early markers which represent dopaminergic specified neurons. Following this, TH and PITX3 expression

represent dopaminergic progenitors. This expression is driven and maintained by NURR1 and EN1. In order to obtain a functional dopaminergic neuron, DAT and VMAT2 are needed. These proteins allow for the transport and reuptake of the dopamine released from the synapse. We further confirmed these differentiated neurons to be dopaminergic neurons through the presence of dopamine receptors 1, 2 and 5 as well as the presence of dopamine through HPLC analysis.

Examples- Second Set

Materials and Methods

hESC Cultures

WA09 (H9) hESCs were cultured on mouse embryonic fibroblast (Harlan) feeders inactivated by mitomycin C (Sigma-Aldrich) in 20% knockout serum replacement media consisting of Dulbecco's modified Eagle medium/F12 medium (Gibco) supplemented with 20% knockout serum replacement, 2mM L-glutamine, 0.1 mM non-essential amino acids, 50 units/ml penicillin/50 μ /ml streptomycin (Invitrogen, Carlsbad, CA), 0.1mM β -mercaptoethanol (Sigma-Aldrich) and 4ng/ml basic fibroblast growth factor (bFGF ; R&D). They were maintained in 5% CO₂ and at 37°C. Cells were passaged every 3 days by mechanical dissociation, re-plated on fresh feeders to prevent undirected differentiation with daily media changes as previously described (Milalipova, et al., 2003).

hNP Cultures

Human neural progenitor (hNP) cells were derived from hESC line H9 by our lab as previously described [9]. Briefly, after one week of culture on mouse embryonic fibroblast layers, H9 hESCs were grown with derivation medium containing Dulbecco's modified Eagle medium /F12 medium (Gibco) supplemented with 2mM L-glutamine (Gibco), 2 U/mL penicillin (Gibco), 2 μ g/mL streptomycin (Gibco), N2 (Gibco), and 4 ng/ml bFGF (R&D) for 7 days in the absence of feeder cells. Rosettes were selected with hook passaging from culture dishes and re-plated on polyornithine (Sigma-Aldrich) and

laminin (Sigma-Aldrich) coated dishes. These rosettes were propagated for 3 days on polyornithine and laminin coated dishes in neurobasal medium (Gibco) supplemented with 2mM L-glutamine, 2 U/mL penicillin, 2 µg/mL streptomycin, 1X B27 (Gibco), 20 ng/mL bFGF, and 10ng/mL leukemia inhibitory factor (Millipore). Media were changed every other day and cells were passaged every fourth day or as needed. Cells used for this experiment were passaged 22-32 times.

hNP Differentiation

hNP cells were grown on polyornithine/laminin coated 100mm plates in growth medium consisting of neural basal medium, 1X penicillin/streptomycin, 2mM L-glutamate, 1X B27, 10ng/mL LIF and 20ng/mL bFGF. After 24 hours, the media were changed to neural differentiation media, which consisted of growth medium without bFGF. Media were changed every three days. Cells were collected at 21 days post differentiation for analysis.

hNP Dopaminergic Differentiation

hNPs were differentiated towards dopaminergic-like neurons as described previously. Briefly, hNP cells were grown on polyornithine/laminin coated 35mm plates (Falcon) for flow cytometry or on polyornithine/laminin coated 4 well slides (Falcon) for immunocytochemistry staining. Cells were plated in growth medium consisting of neural basal medium, 1X penicillin/streptomycin, 2mM L-glutamate, 1X B27, 10ng/mL LIF and 20ng/mL bFGF. After 24 hours, the media were changed to neural differentiation media, which consisted of growth medium without bFGF or neural differentiation medium plus 25ng/ml GDNF (Neuromics). Media were changed every three days. Cells were harvested at Day 21 for further analysis.

Immunocytochemistry and Cell Quantification

hNPs were differentiated towards dopaminergic-like neurons as described above. In parallel, hNPs were differentiated towards dopaminergic-like neurons as described above with inhibitors to GDNF (GDNF antibody, R&D; 1ng/ml, 10ng/ml, 100ng/ml), RET (SU-5416, Sigma-Aldrich; 1ng/ml, 10ng/ml, 100ng/ml) MAPK (PD98059, BioMol; 1ng/ml, 10ng/ml, 100ng/ml) and PI3K (LY-294002, BioMol; 1ng/ml, 10ng/ml, 100ng/ml) for 21 days. Cells were fixed with 2% paraformaldehyde (Electron Microscopy Sciences) in PBS with calcium and magnesium (PBS+/+) (Thermo Scientific) for 20 minutes and processed for immunocytochemistry. Cells were washed in PBS with calcium and magnesium (PBS+/+) 3 times followed by 3 washes for 5 minutes each of permeabilization buffer consisting of 25 μ L (0.5%) Tween 20 (EMD Chemicals) in 50mL of high salt buffer. Cells were then blocked in 6% goat serum (JacksonImmuno) for 45 minutes. The following primary antibodies were used: mouse antiTuj1 (1:200, Neuromics), chicken antiTH (1:100; all from Millipore, Inc). Reaction was revealed using AlexaFluor goat 488 or 633 secondary antibodies (1:1000, Molecular Probes). Cell nuclei were stained using DAPI (Invitrogen). Fluorescence was visualized using spinning disk confocal microscope (Olympus). Negative controls included secondary only staining. Cell counting was performed using Image Pro software (Media Cybernetics). Five random visual fields were selected and counted in triplicate. Data are presented as mean $\pm$ SD. Values of $p < .05$ was considered significant using ANOVA and Tukey's Pair-Wise test (Statistical Analysis Software, SAS Institute).

Quantitative Polymerase Chain Reaction (qPCR)

RNA was extracted using the Qiashrepper and RNeasy kits (Qiagen) according to manufacturer's instructions. The RNA quality and quantity was verified using a RNA 600 Nano Assay (Agilent Technologies) and the Agilent 2100 Bioanalyzer. Total RNA (5 μ g) was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems Inc.) according to manufacturer's instructions. Reactions were initially incubated at 25°C for 10 minutes and subsequently at 37°C for 120 minutes. RT-PCR (RT² Profiler PCR Array,

SABiosciences) assays were used for the MAPK pathway (Human MAP Kinase Signaling Pathway, SABiosciences) and PI3K pathway (Human PI3K-AKT Signaling Pathway, SABiosciences) to analyze the expression of 84 genes for each pathway. The cDNA samples were diluted in 91 μ L of ddH₂O. From the cDNA samples 120 μ L were mixed with 550 μ L of 2X RT² SYBR Green qPCR Master Mix (SABiosciences) and 448 μ L of ddH₂O, then loaded into respective channels on the microfluidic cards followed by centrifugation. The card was sealed and real-time PCR and relative quantification was carried out on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Inc). All failed (undertermined) reactions were excluded and Δ Ct values were calculated. For calculation of relative fold change values, the software provided with the assays on the SABioscience's website (RT² Profiler PCR Array Data Analysis, SABiosciences). All failed (undetermined) reactions were excluded and Δ Ct values were calculated. For calculation of relative fold change values, initial normalization was achieved against endogenous 18S ribosomal RNA using the $\Delta\Delta$ CT method of quantification. Average fold change from three independent runs were calculated as $2^{\Delta\Delta CT}$. Significance was determined by running a 2-way ANOVA and Tukey's Pair-Wise (SAS) comparisons for each gene. Treatments where there was a fold change of greater than 4-fold were considered significant following manufacturer's recommendation.

Cell Cycle Analysis

H9s, hNPs, differentiated hNPs and dopaminergic-like neurons were analyzed for cell cycle effects using propidium iodide (Invitrogen). Differentiated hNPs and dopaminergic-like neurons were differentiated for 21 days before inhibitors for insulin-like growth factor 1 (IGF1; Tyrphostin AG-1024, Enzo Life Sciences; 1ng/ml), cyclin-dependent kinase 2 (CDK2; AG-494, Enzo Life Sciences; 1ng/ml), G1 (CI898, Tocris; 10ng/ml), G1 (Daidzein, Tocris; 10ng/ml), glycogen synthase kinase 3 beta (GSK3 β ; Indirubin, Tocris; 1ng/ml), Rac1 (NSC23766, Tocris; 10ng/ml), mammalian target of rapamycin (mTOR ;Rapamycin, EMD Biosciences; .3ng/ml), MAPK (PD98059, Enzo Life Sciences; 10ng/ml), ERK (PD035901, Cayman Chemicals; 10ng/ml), p38 (SB202190, Enzo Life Sciences; 10ng/ml) and Shc (Sclerotiorin, Cayman Chemicals;

10ng/ml) were added for 24 hours. H9s and hNPs were grown as described previously and then media were changed to media containing inhibitors as listed above for 24 hours before analysis. After 24 hours, cells were harvested and washed in PBS-/- before being fixed in cold 70% ethanol at 4°C for 30 minutes. Cells were then washed 2 times in PBS-/- before adding 200µL of 50µg/ml PI. Cells were quantified on Dako Cyan (Beckman Coulter). Negative controls were secondary only and cell only staining. Cell quantification was done using FlowJo (TreeStar) software. Each experiment was run in triplicate. Data are presented as mean $\pm$ SD. Values of $p < .05$ was considered significant using ANOVA and Tukey's Pair-Wise (Statistical Analysis Software, SAS Institute). Each treatment was normalized to the non-treated control cells and background staining.

Apoptosis Assay

H9s, hNPs, differentiated hNPs and dopaminergic-like neurons were analyzed for apoptosis effects using Caspase Glo 3/7 assay (Promega). Differentiated hNPs and dopaminergic-like neurons were differentiated for 21 days before transfer to a 96 well plate and inhibitors for IGF1 (Tyrphostin AG-1024, Enzo Life Sciences; 1ng/ml), CDK2 (AG-494, Enzo Life Sciences; 1ng/ml), G1 (CI898, Tocris; 10ng/ml), G1 (Daidzein, Tocris; 10ng/ml), GSK3 β (Indirubin, Tocris; 1ng/ml), Rac1 (NSC23766, Tocris; 10ng/ml), mTOR (Rapamycin, EMD Biosciences; .3ng/ml), MAPK (PD98059, Enzo Life Sciences; 10ng/ml), ERK (PD035901, Cayman Chemicals; 10ng/ml), p38 (SB202190, Enzo Life Sciences; 10ng/ml) and Shc (Sclerotiorin, Cayman Chemicals; 10ng/ml) were added for 24 hours. H9s and hNPs were grown as described previously and then transferred to a 96 well plate and media were changed to media containing inhibitors as listed above for 24 hours before analysis. Caspase Glo 3/7 assay (Promega) was used following manufacturer's directions to analyze apoptosis. The buffer was added to the substrate and the substrate dissolved. 100µL of this mix was added to each well of the 96 well plate and incubated for 1 hour at RT protected from light. The plate was then analyzed on the Flexstation 3 (Molecular Devices). Data are presented as mean $\pm$ SD. Values of $p < .05$ was considered significant using ANOVA and Tukey's Pair-Wise (Statistical Analysis Software, SAS Institute). Each treatment was normalized to the non-treated control cells and background staining.

Proliferation Assay

H9s, hNPs, differentiated hNPs and dopaminergic-like neurons were analyzed for proliferation effects using Click-iT EdU High Content Screen kit (Invitrogen). Differentiated hNPs and dopaminergic-like neurons were differentiated for 21 days before transfer to a 96 well plate and inhibitors for IGF1 (Tyrphostin AG-1024, Enzo Life Sciences; 1ng/ml), CDK2 (AG-494, Enzo Life Sciences; 1ng/ml), G1 (CI898, Tocris; 10ng/ml), G1 (Daidzein, Tocris; 10ng/ml), GSK3 β (Indirubin, Tocris; 1ng/ml), Rac1 (NSC23766, Tocris; 10ng/ml), mTOR (Rapamycin, EMD Biosciences; .3ng/ml), MAPK (PD98059, Enzo Life Sciences; 10ng/ml), ERK (PD035901, Cayman Chemicals; 10ng/ml), p38 (SB202190, Enzo Life Sciences; 10ng/ml) and Shc (Sclerotiorin, Cayman Chemicals; 10ng/ml) were added for 24 hours. H9s and hNPs were grown as described previously and then transferred to a 96 well plate and media were changed to media containing inhibitors as listed above for 24 hours before analysis. Click-iT EdU High Content Screen kit (Invitrogen) was used following manufacturer's instructions to analyze proliferation. EdU expression level was measured on the Flexstation 3 (Molecular Devices). Cell nuclei were stained using DAPI (Invitrogen). Fluorescence was visualized using spinning disk confocal microscope (Olympus). Negative controls included secondary only staining. Data are presented as mean $\pm$ SD. Values of $p < .05$ was considered significant using ANOVA and Tukey's Pair-Wise (Statistical Analysis Software, SAS Institute). Each treatment was normalized to the non-treated control cells and background staining.

Results

Blocking GDNF and its Downstream Pathways Inhibits Dopaminergic Enhancement

Differentiating hNPs in normal differentiation media produces 11% TH⁺ neurons. This process is enhanced by the addition of 25ng/ml GDNF to the differentiation media significantly ($p < .05$) increasing the number of TH⁺ neurons to 52%. An antibody to

GDNF which has been shown to neutralize GDNF activity and prevent it from binding to the RET receptor decreased TH expression significantly ($p < 0.05$) to 19% at 1ng/ml, 3% at 10ng/ml (Figure 15A) and removed all expression at 100ng/ml after 21 days in culture (Figure 15A, B, C). SU-5416, a competitive inhibitor of the RET receptor, decreased TH expression significantly ($p < 0.05$) to 8% at 1ng/ml and killed all cells at dosages higher than that (Figure 1D, E, F). LY294002, a reversible inhibitor of PI3K, only slightly decreased the TH expression to 41% with 1ng/ml of the compound added to the differentiation media; cells cultured with greater than that concentration did not survive (Figure 15G, H, I). PD-98059, an inhibitor which prevents mitogen-activated protein kinase kinase I (MEK1) from activating downstream pathways by binding to the receptor and preventing MEK1 activation, reduced TH expression significantly ($p < 0.05$) to 8% with 1ng/ml of inhibitor in the differentiation media. 10ng/ml of inhibitor reduced expression of TH completely while higher dosages killed the cells (Figure 15J, K, L). Following evidence supporting the inhibition of GDNF and its downstream signaling pathways inhibited dopaminergic enhancement, genes involved in the MAPK and PI3K pathway were analyzed by RT-PCR.

Changes in MAPK Pathway Affect Dopaminergic Differentiation

The MAPK pathway expression changes with the differentiation of differentiated hNPs to dopaminergic-like neurons. Specific to the changes in GDNF enhancement of dopaminergic neurons, dopaminergic-like neurons relative to differentiated hNPs up regulate significantly ($p > 0.05$) *Creb1* (360 fold $\pm$ 3), *Mapk8* (5 fold $\pm$ 4), *Mapk13* (5 fold $\pm$ 2) and *Mef2c* (54 fold $\pm$ 3; Figure 16A).

Examination of the functional changes that occur because of inhibition of these pathways occurred through measurement of apoptosis and proliferation. Inhibition of MEK with PD98059 decreased apoptosis significantly ($p < 0.05$) in dopamine-like neurons 1.1 fold relative to differentiated hNPs with inhibitor and decreased apoptosis 24 fold relative to dopamine-like neurons cultured without inhibitor (Figure 16B). Differentiated hNPs cultured with inhibitor decrease significantly ($p > 0.05$) 9.1 fold relative to

differentiated hNPs cultured without inhibitor in caspase levels (Figure 16B). Dopamine-like neurons decreased in proliferation 104.7 fold relative to differentiated hNPs and 2.7 fold to dopamine-like neurons without inhibitors when cultured with PD98059 (Figure 16B). PD035901 inhibits ERK in the MAPK pathway. Addition of PD035901 to culture of dopamine-like neurons decreased apoptosis significantly ($p < 0.05$) 1.2 fold relative to differentiated hNPs with inhibitor and decreases apoptosis level significantly ($p > 0.05$) 8.1 fold relative to dopamine-like neurons cultured without inhibitor (Figure 15C). Differentiated hNPs cultured with inhibitor decreased in apoptosis significantly ($p > 0.05$) 8.8 fold relative to differentiated hNPs without inhibitor. Proliferation of dopamine-like neurons with inhibitor increased 16 fold relative to differentiated hNPs with inhibitor (Figure 15C).

Inhibiting the p38 pathway with SB202190 significantly ($p < 0.05$) decreased apoptosis of dopamine-like neurons 17 fold compared to differentiated hNPs cultured with inhibitor while significantly ($p < 0.05$) decreasing proliferation in dopamine-like neurons 3.6 fold relative to differentiated hNPs cultured with inhibitor (Figure 15D). Dopamine-like neuron apoptosis is decreased significantly ($p > 0.05$) 6.1 fold when cultured with inhibitor relative to culture without inhibitor and proliferation is significantly ($p > 0.05$) decreased 1.1 fold when cultured with inhibitor relative to culture without inhibitor (Figure 15D). The mechanism through which the MEK and ERK pathways are activated by GDNF is outlined in Figure 15E and the mechanism through which p38 is activated by GDNF is shown in Figure 15F.

Dopaminergic-like neuron e expression of *Pak1* (71 fold) and *Rac1* (33 fold) expression is significantly (>4 fold) up regulated relative to differentiated hNPs while *Cdc42* expression is unchanged (Figure 17A). NSC23766, a RAC1 inhibitor, decreased apoptosis on dopaminergic-like neurons cultured with inhibitor 1.2 fold relative to differentiated hNPs cultured with inhibitor (Figure 17B). Apoptosis significantly ($p > 0.05$) increased in dopaminergic-like neurons cultured with inhibitor 1.5 fold relative to dopaminergic-like neurons cultured without inhibitor and proliferation significantly ($p > 0.05$) decreased 1.2 fold in differentiated hNPs cultured with inhibitor relative to

differentiated hNPs cultured without inhibitor (Figure 17B). Proliferation of dopaminergic-like neurons increased 1.75 fold relative to differentiated hNPs when cultured with inhibitor (Figure 17B). The mechanism through which the RAC1 pathway is activated by GDNF is outlined in Figure 17C.

Downstream Targets of PI3K Pathways Unchanged in GDNF Cultured hNPs

Dopaminergic-like neurons show no change in expression of GSK3 β pathway genes APC and GSK3 β (Figure 4A). Indirubin, an inhibitor of GSK3 β , decreased apoptosis 1.2 fold in dopamine-like neurons relative to differentiated hNPs while proliferation increased 2880 fold in dopamine-like neurons relative to differentiation hNPs (Figure 4B). The mechanism through which GDNF activates the GSK3 β pathway is outlined in the pathway in Figure 18C.

Relative to differentiated hNPs, *Pten* (60 fold) is significantly (>4 fold) up regulated while *Tsc1* (55 fold) and *Tsc2* (34 fold) are down regulated (Figure 18D). Inhibition of the mTOR pathway with rapamycin decreased dopamine-like neuron apoptosis 3.7 fold relative to differentiated hNPs cultured with rapamycin (Figure 18E). Proliferation decreased significantly ($p < 0.05$) 5.6 fold in dopaminergic-like neurons cultured with rapamycin relative to differentiated hNPs (Figure 18E). The mechanism through which GDNF activates the mTOR pathway is outlined in the pathway in Figure 18F.

Cell Cycle in Dopaminergic-like Neurons

IGF1, a cell growth factor, is significantly ($p > 0.05$) down regulated 11 fold in dopaminergic-like neurons relative to differentiated hNPs. *Shc1*, responsible for modulating IGF1 insertion into the membrane, is significantly (>4 fold) down regulated 13 fold in dopaminergic-like neurons relative to differentiated hNPs (Figure 5A). The mechanism through which SHC modulated IGF1 insertion into the membrane is outlined in Figure 19B.

hNPs were significantly (> 4 fold) up regulated in *Ccnb1* (6 fold), *Ccnd1* (42 fold), *Ccnd3* (6 fold), *Cdkn1a* (51 fold), *Cdkn1b* (16 fold), *Cdkn1c* (9 fold; Figure 19C). Differentiated hNPs were significantly (>4 fold) up regulated in *Ccnb1* (9 fold), *Ccnd1* (19 fold), *Ccnd3* (20 fold), *Cdkn1a* (65 fold), *Cdkn1b* (10 fold), *Cdkn1c* (7 fold) relative to hNPs (Figure 19C). Dopaminergic-like neurons were significantly (>4 fold) down regulated in *Ccnb1* (12 fold), *Ccnd1* (19 fold), *Ccnd3* (20 fold), *Cdkn1a* (55 fold), *Cdkn1b* (19 fold), *Cdkn1c* (25 fold) relative to hNPs (Figure 19C). CI-898, an inhibitor of cell cycle that causes cell cycle arrest at late G1 phase or early S phase, was cultured in H9s, hNPs, differentiated hNPs and dopamine-like neurons at 10ng/ml. In H9s, CI-898 arrested the cells in late G1, increasing the number of cells in G1 when measured with propidium iodide (PI; Figure 19D). hNPs cultured with CI-898 were also arrested at late G1 increasing the G1 phase and number of cells in the phase (Figure 19E). There was no effect on the cell cycle of differentiated hNPs and dopaminergic-like neurons (Figure 19F, G).

Discussion

The goal of this study was to examine the pathway through which GDNF enhances dopaminergic differentiation from hNPs derived from hESCs. Inhibition of GDNF and its receptor RET led to complete abolishment of TH expression when cultured for 21 days. Additionally, inhibitors to the MAPK (MEK inhibitor) and PI3K (AKT inhibitor) pathway caused complete abolishment of TH expression. This led us to examine further the potential signaling pathways that could be causing enhancement of dopaminergic differentiation through culture with GDNF.

The MAPK pathway can be divided into three sub-pathways, the ERK pathway, the JNK pathway and the p38 pathway. The JNK pathway responds to growth factors, cellular stress, cytokines, oxidative stress and G protein coupled receptors [19]. The JNK pathway sends stress signals through the MAP3K1-4 to MAP2K4/7 to activate cellular growth, differentiation, survival or apoptosis through regulating transcription factors [20]. The p38 MAPK pathway is activated by G protein coupled receptors, DNA damage,

oxidative stress, cytokines and transforming growth factor beta TGF- β [21]. MAP3K1-4 activates MAP2K3/6 or MAP2K4, which leads to activation of transcription factors within the nucleus that increase cytokine production and apoptosis [21]. Regulation of the p38MAPK pathway is important for the control of apoptosis and response to cellular stresses [22]. The ERK pathway can be activated by G protein coupled receptors, integrins, and ion channels leading to activation of MAP2K1/2 and ERK1/2 [23]. ERK1/2 activates transcription factors, which modulate growth and development within the cell [24].

When GDNF is cultured with the hNPs in this study for 21 days, genes involved in the ERK and p38 aspects of the MAPK pathway were up regulated relative to differentiated hNPs cultured without GDNF including *Creb1*, *Mapk8*, *Mapk13* and *Mef2c*. The MEK activation of ERK leads to transcription of *Creb1*. A suggested mechanism for this activation is through promoting survival of dopaminergic neurons through ERK's known role in promoting TH protein expression [25]. The decreased level of apoptosis in hNPs differentiated with GDNF compared with the level seen when hNPs are differentiated without GDNF when inhibited with a MEK and an ERK inhibitor suggests that GDNF does act through the MEK and ERK pathways to promote dopaminergic survival in this mechanism (Figure 20B). The p38 pathway activates *Mef2c* to promote dopamine neuron survival through selective synaptic pruning [26, 27]. In those neurons which are primed to become dopaminergic, GDNF enhances the synaptic connections between neurons improve their survival. The p38 pathway increases apoptosis of the non-primed dopaminergic neurons as is suggested by the decrease in apoptosis in the hNPs differentiated with GDNF when cultured with a p38 inhibitor. An increase in non-dopaminergic neuron apoptosis would be seen during the enhancement with GDNF through the p38 pathway; however, with that pathway blocked, that selective enhancement would not occur (Figure 20A).

RAC1 regulates actin within the neuron as well as the morphology of the neuron [28]. The increase in Rac1 and Pak1 expression with differentiation of hNPs with GDNF suggests activation of this pathway in dopaminergic-like neurons used in this study. The

lack of significant change in apoptosis and proliferation when this pathway is inhibited further suggests the role of the Rac1 pathway in GDNF enhancement of dopaminergic-like neurons lies outside of apoptosis and proliferation and might lay in the known roles of RAC1 in actin regulation and morphology modulation [29].

The mammalian target of rapamycin (mTOR) acts downstream of AKT in the PI3K pathway can be activated by RAS homolog enriched in brain and blocked by TSC1/TSC2 [30]. While there are two mTOR complexes, mTORC2 activation is not understood. MTORC1, which can be activated by growth actors, acts to activate eukaryotic translation initiation factor 4EBP1, which leads to mRNA translation that increases cell growth. mTORC1 also suppresses autophagy and signals for ribosome biogenesis [31]. mTORC2 acts to control actin organization and cellular survival [32, 33]. GDNF activation of RET also leads to PI3K activation of the mTOR pathway activates eukaryotic initiation factors that regulate synaptic plasticity and dopamine neural survival (Figure 20C). The inhibition of TSC1/TSC2 on mTOR was lifted with the decrease in genetic expression of TSC1/TSC2 in differentiated hNPs with GDNF increasing the expression of *EIF4B* and *EIF4G*. Dopaminergic-like neurons relative to differentiated hNPs up regulate PTEN, which has been shown to regulate neural growth [34, 35], potentially slowing neural growth as the hNPs differentiate. TSC1 and TSC2 complex regulate neural growth and are down regulated in dopamine-like neurons relative differentiated hNPs [36]. Rapamycin, an inhibitor of the mTOR pathway has been shown to prevent PD cell death [37]. The dopaminergic-like neurons used in this study decreased in apoptosis when inhibited with rapamycin confirming these results (Figure 6C).

GSK3 β signaling, activated by growth factors or WNT signaling through the Frizzled receptor, modulated glucose metabolism and glycogen synthesis through activation of the enzyme necessary for glycogen, glycogen synthesis [38]. Additionally, GSK3 β modulates cell cycle through blocking Cyclin D1 and p21Cip, which lead to increase in proliferation and growth. Activation of β -Catenin leads to activation of transcription factors [39, 40]. GSK3 β activation has less of a role in GDNF differentiation and more of a role in neural progenitor cell growth and development [41]. Blocking GSK3 β increases

proliferation in dopaminergic-like neurons suggesting that with the lack of glucose synthesis, the dopaminergic-like neurons revert to an immature fate. The enhancement of GDNF on dopaminergic-like neuron differentiation likely does not occur through the GSK3 β pathway (Figure 6C).

SHC is a protein involved in receptor trafficking within the cell. SHC binds to the IGFR and controls its insertion into the membrane [42, 43]. IGF1 plays a role in neural growth and development early in embryogenesis [44, 45]. The IGF pathway and its downstream activation of GSK3 α has less involvement in the GDNF activation of RET and dopaminergic differentiation but more to do with the growth and proliferation of the neural progenitor cells. *Igfl* and *Shc* is down regulated in dopaminergic-like neurons relative to differentiated neurons suggesting a post-mitotic neuron. Supporting the movement of differentiated hNPs with and without GDNF towards a post-mitotic neuron is the decrease in cell cycle genes as the hNPs differentiate. Additionally, the cell cycle in differentiated hNPs and dopaminergic-like neurons resemble that of a post-mitotic neuron. The increased decrease in *Igfl* and *Shc* in dopaminergic-like neurons relative to differentiated hNPs suggests that GDNF acts to establish a more mature neural phenotype.

Conclusion

GDNF has been shown to enhance dopamine differentiation of hNPs [10]; this differentiation is completely blocked when GDNF and its receptor RET are blocked. The MEK and ERK pathways support dopaminergic neural survival while the p38MAPK pathway modulates dopamine enhancement through pruning of non-primed dopaminergic neurons. RAC1 is involved in neurite outgrowth. While inhibiting the mTOR pathway helped to support the maintenance of the dopaminergic-like neurons through up regulating eukaryotic initiation factors that improve synaptic plasticity and dopamine neural survival, GSK3 α has no role in GDNF enhancement of dopaminergic-like neurons. This study has further elucidated the mechanisms through which GDNF helps to

support dopaminergic neurons and to enhance the differentiation of dopaminergic-like neurons from hNPs allowing for potential future targets for PD therapies.

The work presented in this application represents developmentally mature dopaminergic neurons expressing the necessary transcription factors, proteins for dopamine production, proteins for dopamine release and proteins for dopamine reuptake.

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Claims:

1. A method of producing a population of isolated dopaminergic progenitor cells from neural progenitor cells comprising exposing said neural progenitor cells to a differentiation medium comprising an effective amount at least one growth factor selected from the group leukaemia inhibition factor (LIF), glial cell-line derived neurotrophic factor (GDNF) and mixtures thereof for a period ranging from about 3 days to 21 days, isolating said population of progenitor cells from said differentiation medium and optionally cryopreserving said progenitor cells for storage.
2. The method according to claim 1 wherein said growth factor is a mixture of LIF and GDNF.
3. The method according to claim 1 wherein said growth factor is GDNF.
4. The method according to claim 1 wherein said growth factor is LIF.
5. The method according to any of claims 1-4 wherein said dopaminergic progenitor cells express the markers Nurr1 and EN1, but not TH and Pitx3.
6. The method according to any of claims claim 1-3 wherein said dopaminergic progenitor cells express the markers Nurr 1, EN1 and PITX3, but not TH.
7. The method according to any of claims 1-3 wherein said dopaminergic progenitor cells express Nurr 1, EN1, PITX3 and TH, but not VMAT2 or DAT.
8. The method according to any of claims 1-6 wherein said medium is changed every three days and said cells are not passaged after plating at day 0.
9. The method according to any of claims 1-8 wherein said period is about 7-14 days.
10. The method according to any of claims 1-8 wherein said period is about 14-21 days.
11. The method according to any of claims 1-10 wherein said hNPs are feeder cell free.
12. The method according to any of claims 1-11 wherein said dopaminergic progenitor cells are feeder cell free.
13. The method according to any of claims 1-12 wherein said isolated cells are cryopreserved.

14. A method of producing a population of isolated feeder cell free dopaminergic progenitor cells from feeder free neural progenitor cells comprising providing feeder free neural progenitor cells; exposing said neural progenitor cells to a differentiation medium comprising an effective amount of leukaemia inhibition factor (LIF) and basic fibroblast growth factor (bFGF) for a period of about 1-3 days, followed by exposing said cells to at least one growth factor selected from the group consisting of leukaemia inhibition factor (LIF) and glial cell-line derived neurotrophic factor (GDNF) in the absence of bFGF for a period ranging from about 3 days to about 21 days and isolating said population of progenitor cells from said differentiation medium.

15. The method according to claim 14 wherein said growth factor is a mixture of LIF and GDNF.

16. The method according to claim 14 wherein said growth factor is GDNF.

17. The method according to claim 14 wherein said growth factor is LIF.

18. The method according to any of claims 14-17 wherein said dopaminergic progenitor cells express the markers Nurr1 and EN1, but not TH and Pitx3.

19. The method according to any of claims claim 14-17 wherein said dopaminergic progenitor cells express the markers Nurr 1, EN1 and PITX3, but not TH.

20. The method according to any of claims 14-17 wherein said dopaminergic progenitor cells express Nurr 1, EN1, PITX3 and TH, but not VMAT2 or DAT.

21. The method according to any of claims 14-20 wherein said medium is changed every three days, said cells are not passaged after plating at day 0 and said cells also express the biomarker LRRK2.

22. The method according to any of claims 14-21 wherein said period is about 7-14 days.

23. The method according to any of claims 14-21 wherein said period is about 7-21 days.

24. The method according to any of claims 14-21 wherein said period is about 14-21 days.

25. The method according to any of claims 14-24 wherein said hNPs are feeder cell free.

26. The method according to any of claims 14-25 wherein said dopaminergic progenitor cells are feeder cell free.

27. A method of producing dopaminergic neuron cells from neural progenitor cells in high purity comprising exposing said neural progenitor cells to a differentiation medium comprising an effective amount of glial cell-line derived neurotrophic factor (GDNF) or a mixture of GDNF and leukaemia inhibition factor (LIF) for a period ranging from about 14 days to 21 days, isolating said population of dopaminergic neuron cells and optionally cryopreserving said isolated dopaminergic neuron cells.

28. The method according to claim 27 wherein said neuron cells express the biomarkers VMAT2, DAT, TH, PITX3, EN1 and Nurr1.

29. The method according to claim 27 or 28 wherein said neuron cells express the biomarkers VMAT2, DAT, TH, PITX3, EN1, Nurr1, RET, D1, D4 and D5.

30. The method according to any of claims 27-29 wherein said medium is changed every three days and said cells are not passaged after plating at day 0.

31. The method according to any of claims 27-30 wherein said isolated cells are cryopreserved.

32. A method of producing dopaminergic neuron cells from dopaminergic progenitor cells in high purity comprising exposing said dopaminergic progenitor cells to a differentiation medium comprising an effective amount of glial cell-line derived neurotrophic factor (GDNF) or a mixture of GDNF and leukaemia inhibition factor (LIF) for a period ranging from about 3 days to about 18 days, isolating said population of neuron cells from said differentiation medium and optionally, cryopreserving said isolated dopaminergic neuron cells.

33. The method according to claim 32 wherein said period ranges from about 7 days to about 14 days.

34. The method according to claim 32 wherein said period ranges from about 3 days to about 7 days.

35. The method according to claim 32 wherein said period ranges from about 7 days to about 18 days.

36. The method according to any of claims 30-35 wherein said neuron cells express the biomarkers VMAT2, DAT, TH, PITX3, EN1 and Nurr1.

37. The method according to claim 30-36 wherein said neuron cells express the biomarkers VMAT2, DAT, TH, PITX3, EN1, Nurr1, RET, D1, D4 and D5.

38. The method according to any of claims 30-37 wherein said medium is changed every three days and said cells are not passaged after plating at day 0.

39. A population of isolated dopaminergic progenitor cells which express the biomarkers Nurr1 and EN1.

40. A population of isolated dopaminergic progenitor cells which express the biomarkers Nurr1, En1 and PITX3.

41. A population of isolated dopaminergic progenitor cells which express the biomarkers Nurr1, En1, PITX3 and TH.

42. The population of isolated dopaminergic progenitor cells according to any of claims 39-42 which express the biomarker LRRK2.

43. The dopaminergic progenitor cells according to any of claims 39-42 which are cryopreserved.

44. The dopaminergic progenitor cells according to claim 43 which have been thawed after cryopreservation.

45. The progenitor cells according to any of claims 39-44 which are feeder cell free.

46. A population of isolated dopaminergic neuron cells produced according to the method of any of claims 27-38, 67, 76 or 77 which express the biomarkers VMAT2, DAT, TH, PITX3, EN1 and Nurr1.

47. A population of isolated dopaminergic neurons cells produced according to the method of any of claims 27-38, 67, 76 or 77 which express the biomarkers VMAT2, DAT, TH, PITX3, EN1, Nurr1, RET, D1, D4 and D5.

48. A population of isolated dopaminergic progenitor cells which are produced according to the method of any of claims 1-26 or 65-75.

49. The population of cells according to claim 48 which express the biomarkers Nurr1 and EN1.

50. The population of cells according to claim 48 which express the biomarkers Nurr1, En1 and PITX3.

51. The population of cells according to claim 48 which express the biomarkers Nurr1, En1, PITX3 and TH.

52. The population of cells according to any of claims 48-51 which express the biomarker LRRK2.

53. The population of cells according to any of claims 48-52 which are cryopreserved.

54. The population of cells according to claim 53 which have been thawed after cryopreservation.

55. The population of cells according to any of claims 48-54 which are feeder cell free.

56. A method of treating a neurodegenerative disease in a patient in need thereof, said method comprising administering to said patient an effective number of dopaminergic progenitor cells according to any of claims 39-45, 48-55 or 67-74 or an effective number of dopaminergic neuron cells according to 46, 47, 67, 76 or 77 or a mixture of said dopaminergic progenitor cells and said dopaminergic neuron cells, optionally in combination with GDNF and further optionally LIF.

57. The method according to claim 56 wherein said neurodegenerative disease is Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis (Lou Gehrig's disease), Tay Sachs disease, multiple sclerosis or lysosomal storage disease.

58. The method according to claim 56 or 57 wherein said cells are administered in a cell medium, further in combination with GDNF and optionally, LIF.

59. The method according to any of claims 56-58 wherein said cells are administered by injection directly into the affected tissue of the brain or spinal column of said patient.

60. The method according to any of claims 56-58 wherein said cells are administered by injection into the brain or spinal column and allowed to migrate to the affected tissue.

61. The method according to any of claims 56-60 wherein said neurodegenerative disease is Alzheimer's disease, Huntington's disease or Parkinson's disease.

62. A method of treating a patient in need to repair damage to a patient's central nervous system comprising administering to said patient an effective number of dopaminergic progenitor cells according to any of claims 39-45, 48-55, 67-75 or an effective number of dopaminergic neuron cells according to 46, 47, 67, 76 or 77 or a mixture of dopaminergic progenitor cells and dopaminergic neuron cells, optionally in combination with GDNF and further optionally LIF.

63. The method according to claim 62 wherein said damage occurred secondary to stroke, cardiovascular disease, a heart attack, physical injury or trauma, genetic damage or an environmental insult to the brain and/or spinal cord of said patient.

64. The method according to claim 62 or 63 wherein said cells are administered in a cell medium, further in combination with GDNF and optionally, LIF.

65. The method according to any of claims 62-64 wherein said cells are administered by injection directly into the affected tissue of the brain or spinal column of said patient.

66. The method according to any of claims 62-64 wherein said cells are administered by injection into the brain or spinal column and allowed to migrate to the site of damage.

67. A method of producing a population of isolated dopaminergic progenitor cells and/or dopaminergic neuronal cells from neural progenitor cells comprising exposing said neural progenitor cells to a differentiation medium comprising an effective amount of Sonic Hedgehog and fibroblast growth factor 8 (FGF8) as differentiation agents and optionally, an effective amount of at least one additional differentiation agent selected from the group consisting of BDNF, TGF β 3, LIF, GDNF and mixtures thereof for a period ranging from about 3 days to 21 days, isolating said population of progenitor cells from said differentiation medium and optionally cryopreserving said progenitor cells for storage.

68. The method according to claim 67 wherein said differentiation agent comprises a mixture of Sonic Hedgehog, FGF8, and optionally, at least one of BDNF, TGF β 3 or mixtures thereof.

69. The method according to claim 67 wherein said differentiation agent comprises a mixture of Sonic Hedgehog, FGF8, and optionally, at least one of LIF, GDNF and mixtures thereof.

70. The method according to claim 67 wherein said differentiation agent comprises a mixture of Sonic Hedgehog, FGF8 and a mixture of BDNF, TGF β 3 and LIF.

71. The method according to any of claims 67-70 wherein said neural progenitor cells are propagated in a nutrient medium comprising LIF and bFGF for a period of at least three days before exposing said propagated cells to said differentiation medium.

72. The method according to any of claims 67-70 wherein said neural progenitor cells are propagated in nutrient medium comprising LIF and bFGF for between 10 and 40 passages before exposing said propagated cells to said differentiation medium.

73. The method according to any of claims 67-72 wherein said dopaminergic progenitor cells express the markers Nurr1 and EN1, but not TH and Pitx3.

74. The method according to any of claims claim 67-72 wherein said dopaminergic progenitor cells express the markers Nurr 1, EN1 and PITX3, but not TH.

75. The method according to any of claims 67-72 wherein said dopaminergic progenitor cells express Nurr 1, EN1, PITX3 and TH, but not VMAT2 or DAT.

76. The method according to any of claims 67-72 wherein said neuron cells express the biomarkers VMAT2, DAT, TH, PITX3, EN1 and Nurr1.

77. The method according to claim 30-36 wherein said neuron cells express the biomarkers VMAT2, DAT, TH, PITX3, EN1, Nurr1, RET, D1, D4 and D5.

78. Use of a population of dopaminergic progenitor cells according to any of claims 39-45, 48-55 or 67-75, a population of dopaminergic neuron cells according to claims 46, 47, 67, 76 or 77 or mixtures thereof in the manufacture of a medicament for the treatment of neurodegenerative disease in a patient in need thereof, said medicament optionally including GDNF and further optionally LIF.

79. Use according to claim 78 wherein said neurodegenerative disease is Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis (Lou Gehrig's disease), Tay Sachs disease, multiple sclerosis or lysosomal storage disease.

80. Use according to claim 78 or 79 wherein said medicament includes GDNF and optionally, LIF.

81. Use according to any of claims 78-80 wherein said medicament is administered by injection directly into the affected tissue of the brain or spinal column of said patient.

82. Use according to any of claims 78-80 wherein said medicament is administered by injection into the brain or spinal column and the cells contained therein are allowed to migrate to the affected tissue.

83. Use according to any of claims 78-82 wherein said neurodegenerative disease is Alzheimer's disease, Huntington's disease or Parkinson's disease.

84. Use of a population of dopaminergic progenitor cells according to any of claims 39-45, 48-55 or 67-75, a population of dopaminergic neuron cells according to claims 46, 47, 67, 76 or 77 or mixtures thereof in the manufacture of a medicament for treating a patient in need of repair of damage to said patient's central nervous system, optionally in combination with GDNF and further optionally LIF.

85. Use according to claim 84 wherein said damage occurred secondary to stroke, cardiovascular disease, a heart attack, physical injury or trauma, genetic damage or an environmental insult to the brain and/or spinal cord of said patient.

86. Use according to claim 84 or 85 wherein said cells are administered in a cell medium, further in combination with GDNF and optionally, LIF.

87. Use according to any one of claims 84-86 wherein said cells are administered by injection directly into the affected tissue of the brain or spinal column of said patient.

88. Use according to any one of claims 84-86 wherein said cells are administered by injection into the brain or spinal column and allowed to migrate to the site of damage.

89. Use of a population of dopaminergic progenitor cells according to any of claims 39-45, 48-55 or 67-75, a population of dopaminergic neuron cells according to claims 46, 47, 67, 76 or 77 or mixtures thereof in the manufacture of a medicament.

FIGURE 1

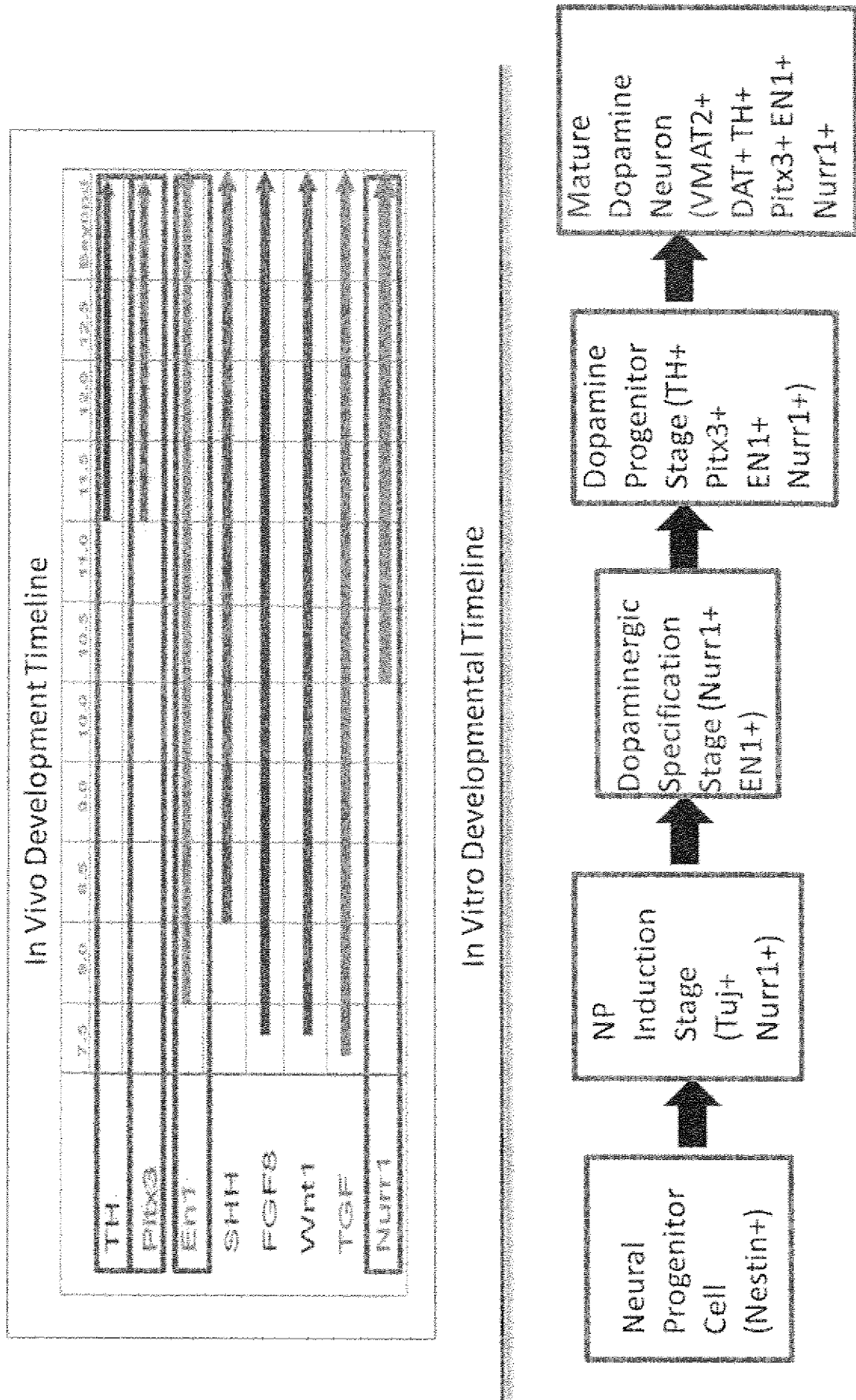


FIGURE 2

- GDNF plays a role in
 - neural survival
 - proliferation
 - differentiation

-
- Does GDNF directs dopaminergic differentiation of neural progenitor cells

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FIGURE 3

FIGURE 4

NP cells can be induced and specified

Nurr1 Expression EN1 Expression

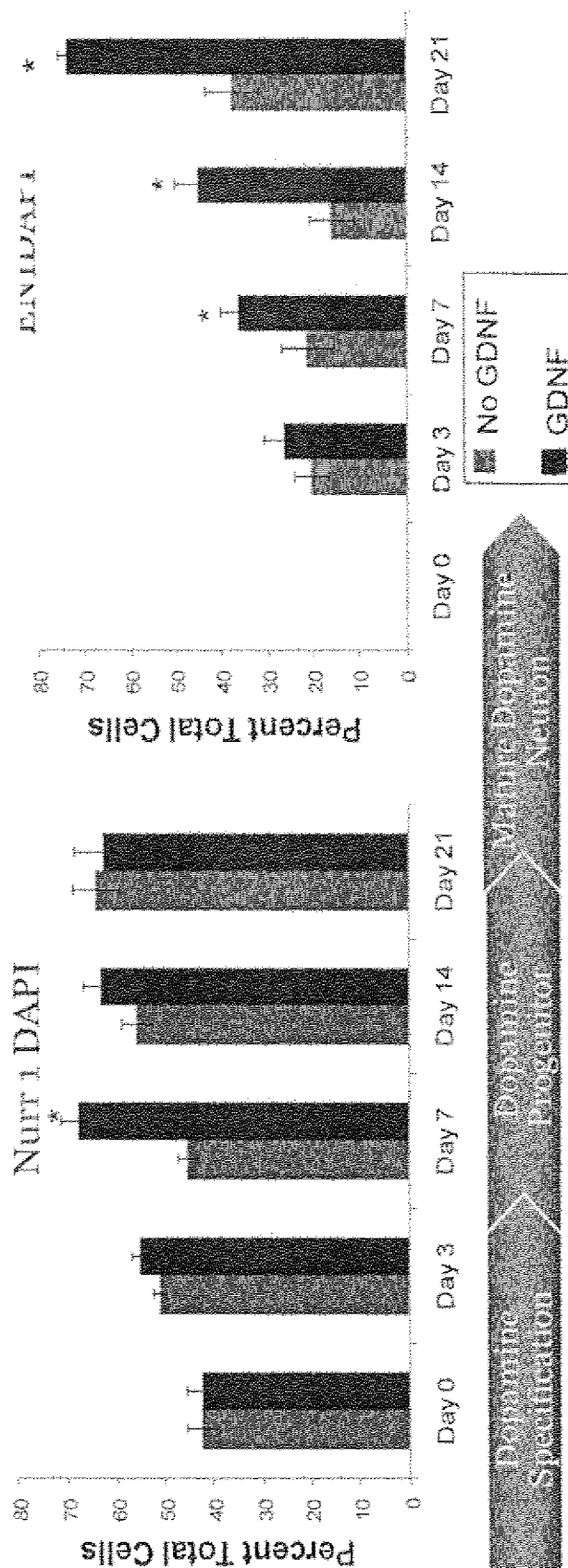


FIGURE 5

NP cells differentiate to dopaminergic progenitors

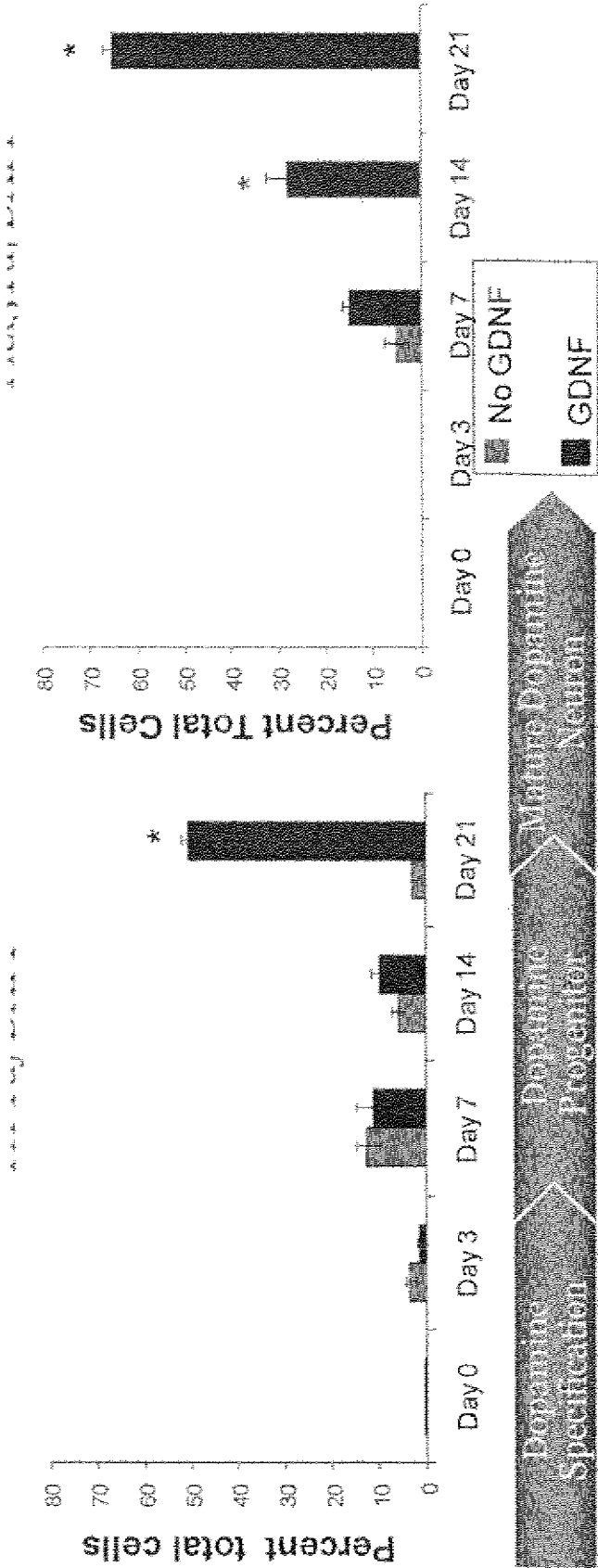


FIGURE 6

Mature dopaminergic neurons express functional markers

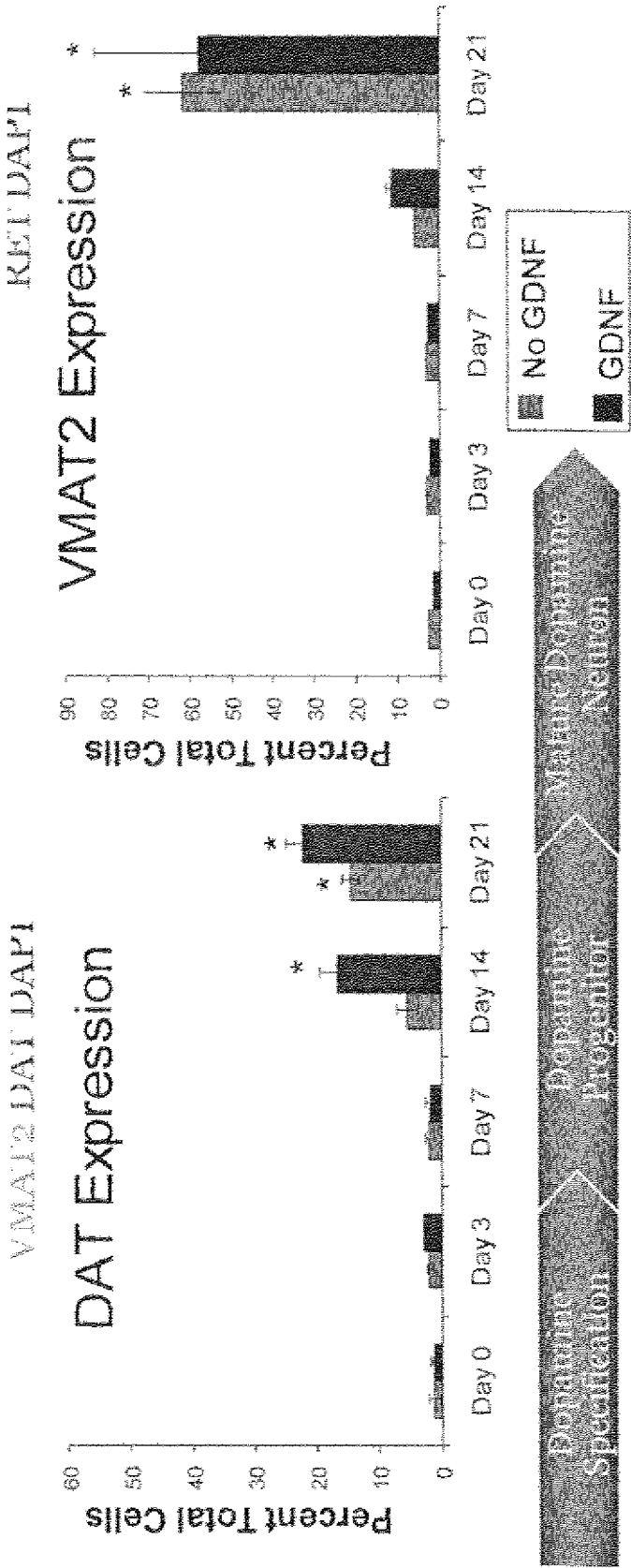


FIGURE 7

Implications

- GDNF is sufficient to enhance the differentiation of neural progenitors to a dopaminergic phenotype
- GDNF can activate many pathways, which pathway plays a role in development of dopaminergic neurons is unknown

FIGURE 8

A In Vitro Developmental Timeline

Stage	Markers	Reference
Neural Progenitor Cell	Nestin	Geisinger, et al., 2007
Neural Progenitor Induction Stage	Tuj Nutt1	Schik et al., 2003; Prasad et al., 2006; van Raden and Hirsinger, 2007
Dopamine Specification Stage	Nutt1 EN1	Alonso, et al., 2003; Smith et al., 2003
Dopamine Progenitor Stage	TH Pk3 EN1 Nutt1	Smith et al., 2003; Prasad et al., 2006
Mature Dopamine Neuron	VIMAT2 DAT TH Pk3 EN1 Nutt1	Meng, et al., 1999; Hansen, et al., 1998

FIGURE 9

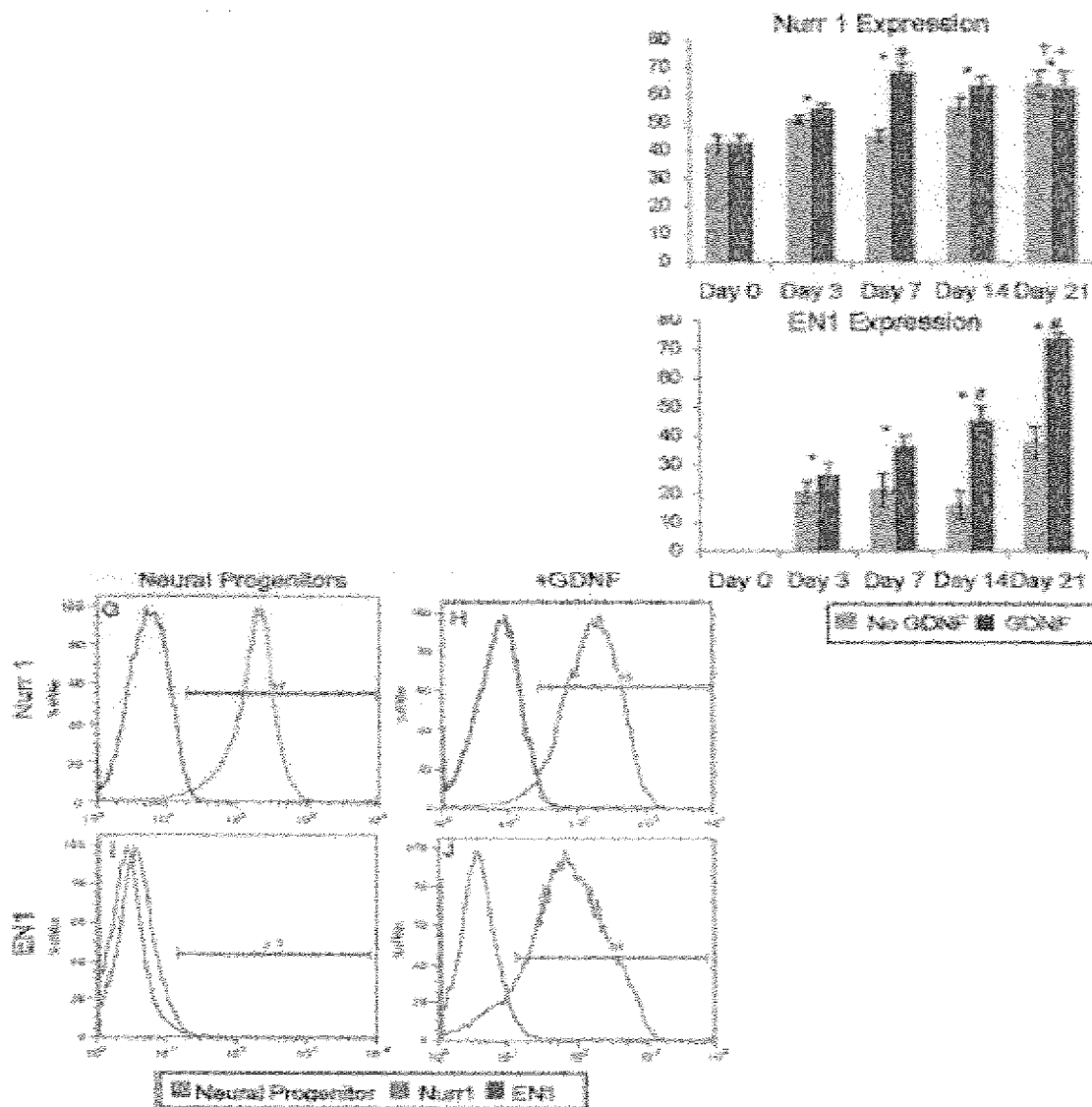


FIGURE 10

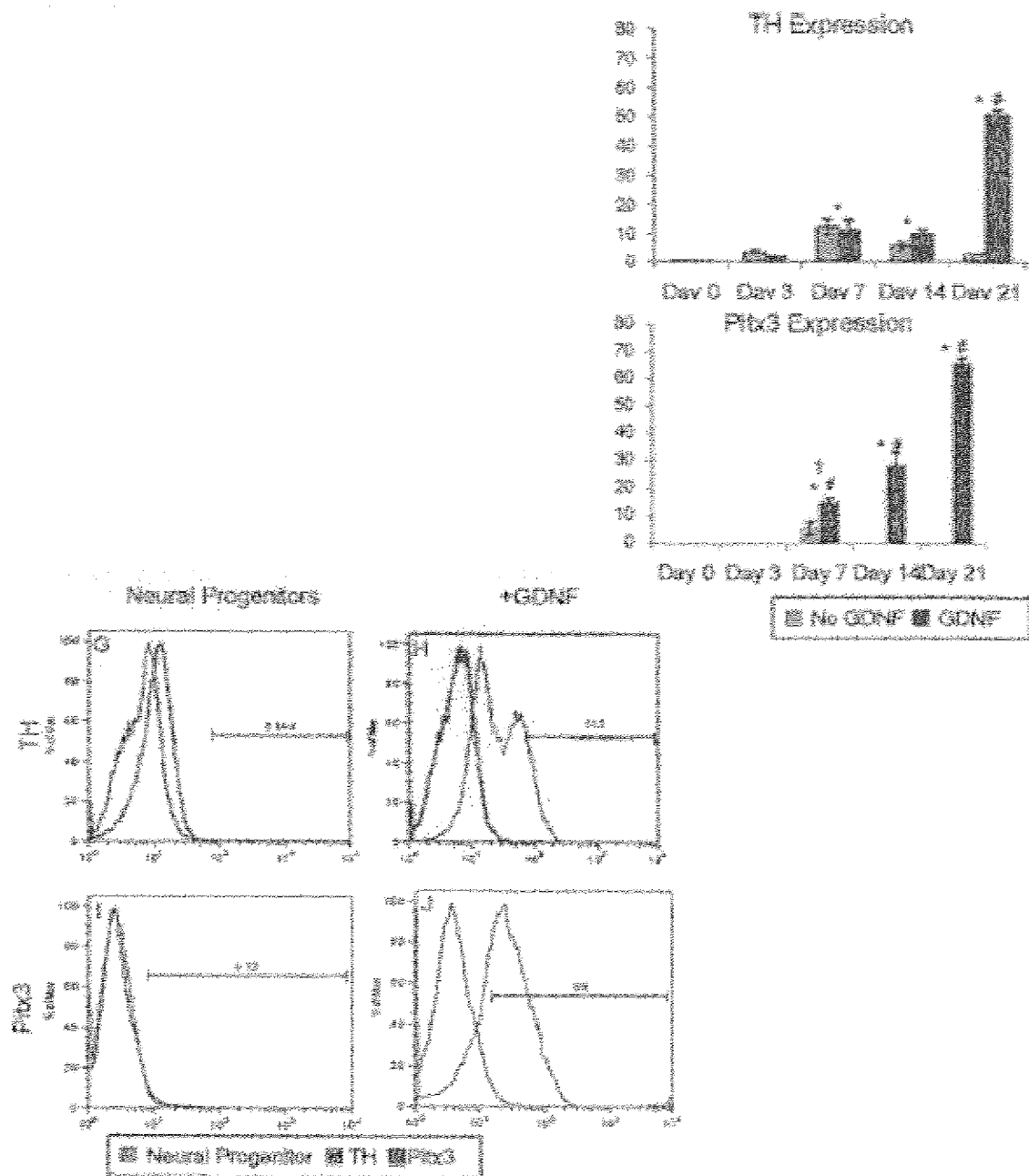


FIGURE 11

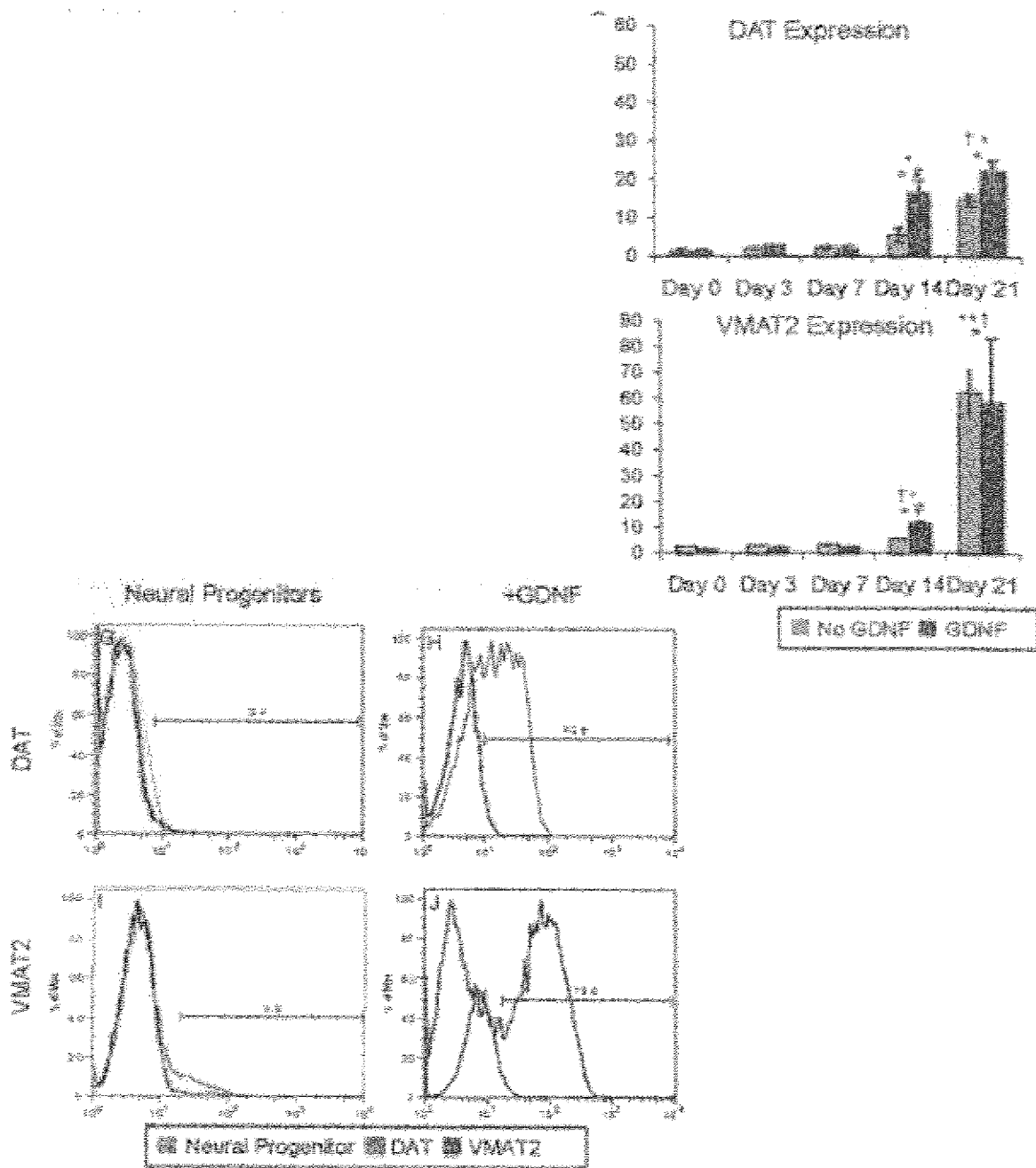


FIGURE 12

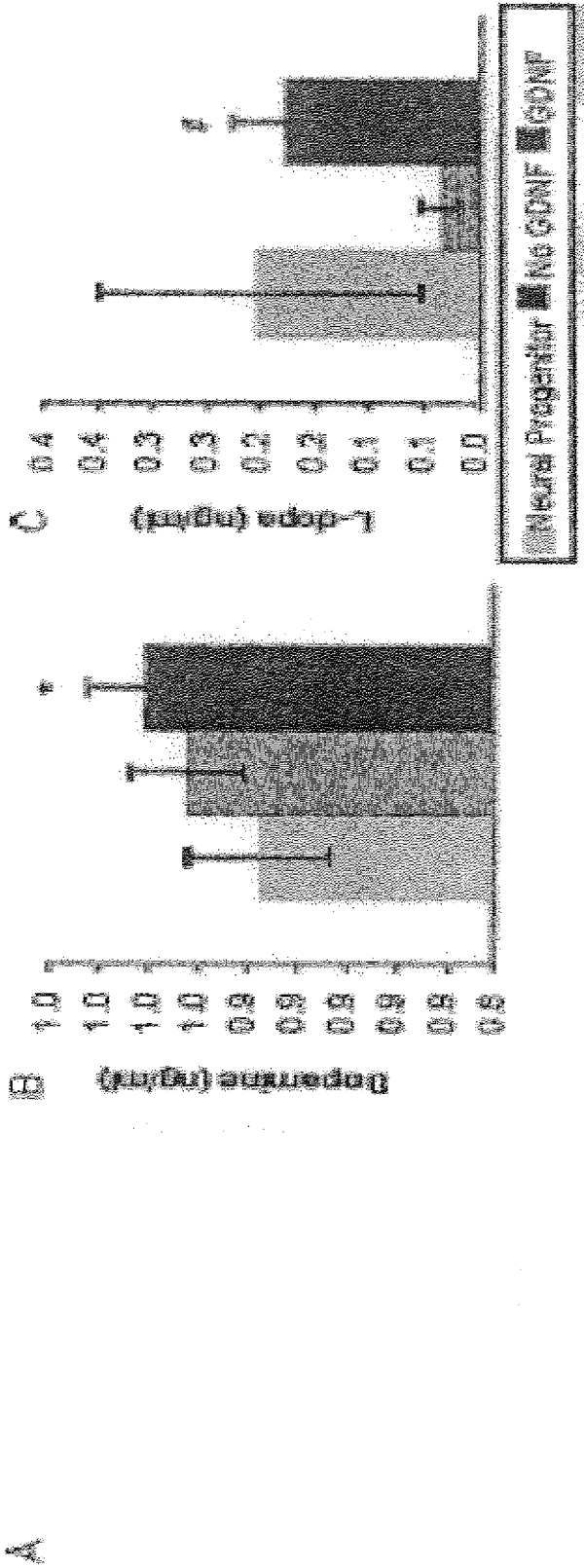


FIGURE 13

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A

FIGURE 14

MJFF-1 (c5-8) MJFF-2 (c41-2) MJFF-3 (c69-6)

MJFF-4 (c81-8) MJFF-5 (c68-7)

FIGURE 15

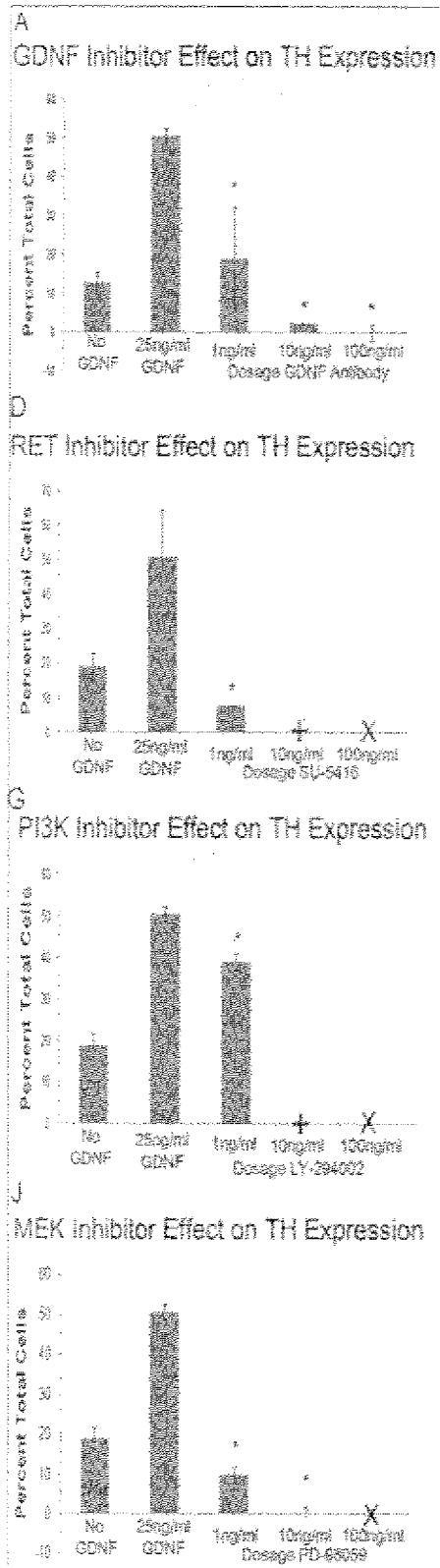


FIGURE 16

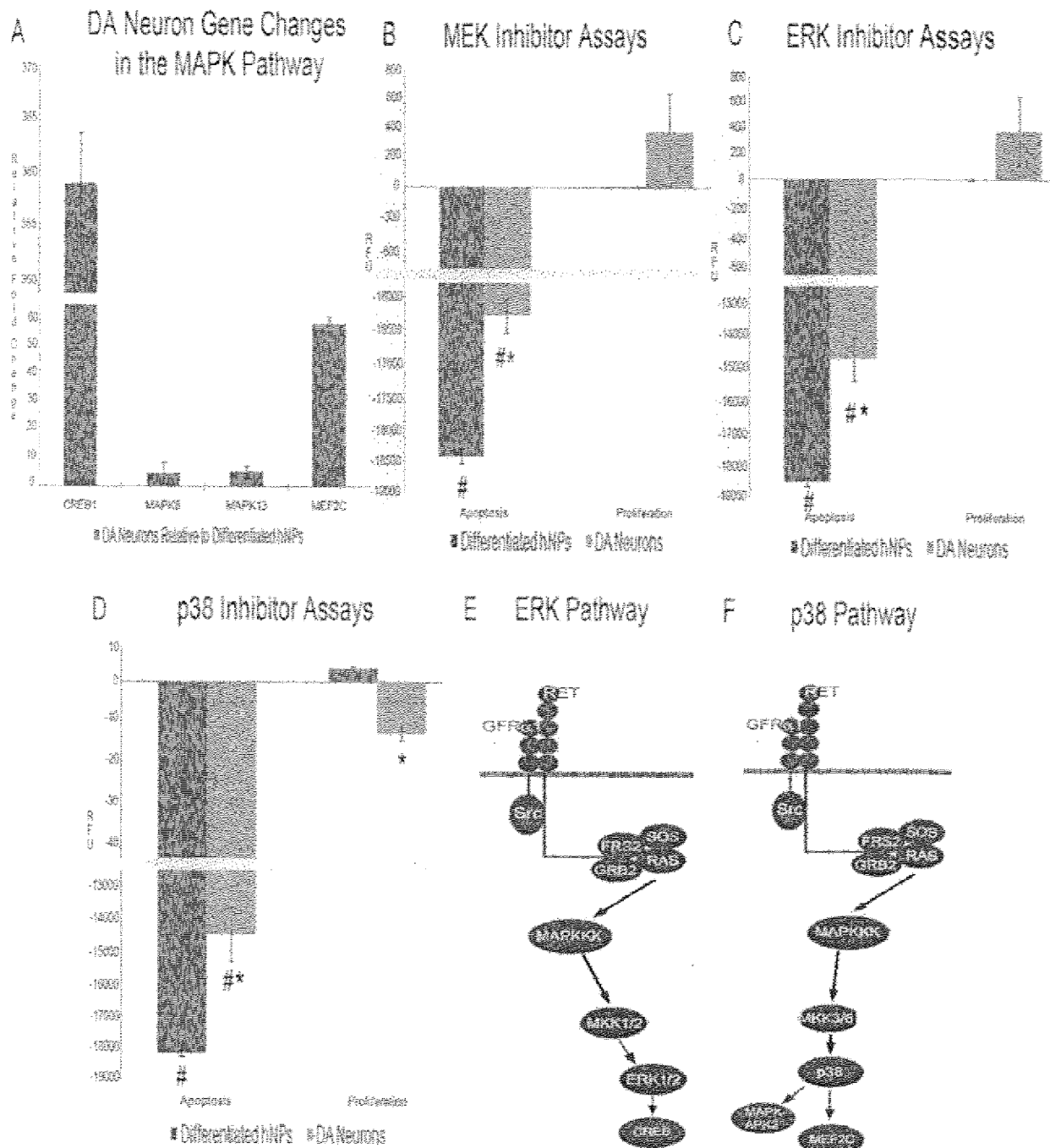
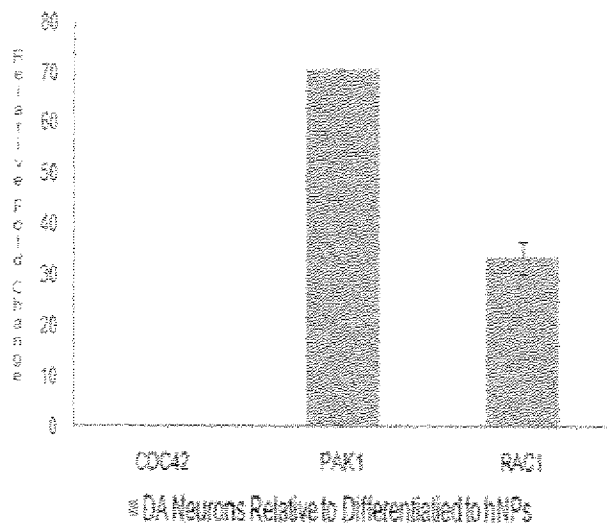
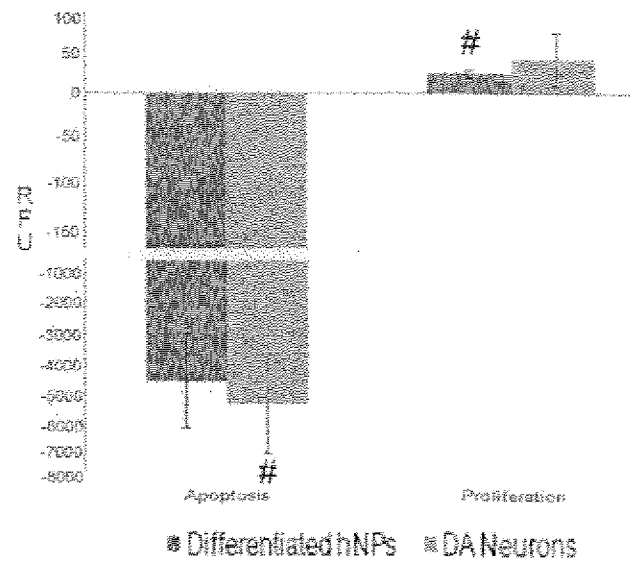


FIGURE 17

A DA Neuron Gene Expression Changes in the Rac1 Pathway



B Rac1 Inhibitor Assay



C Rac1 Pathway

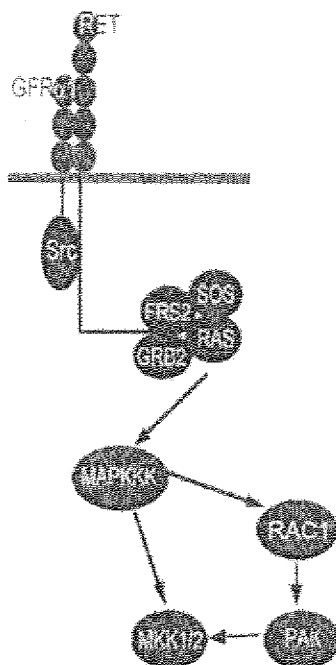
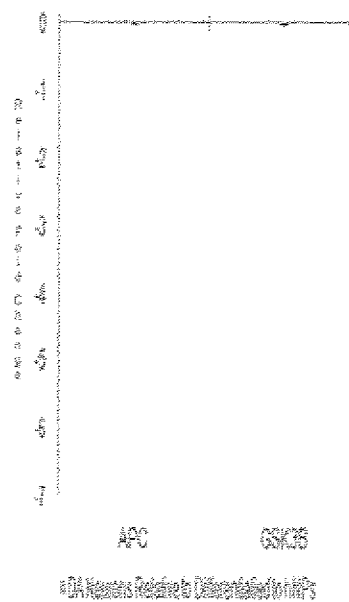
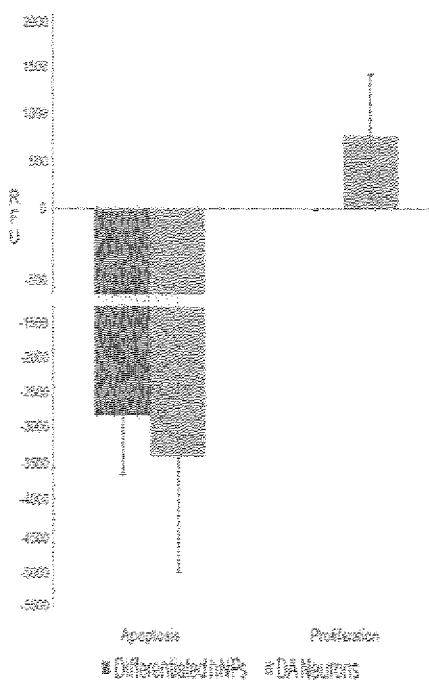
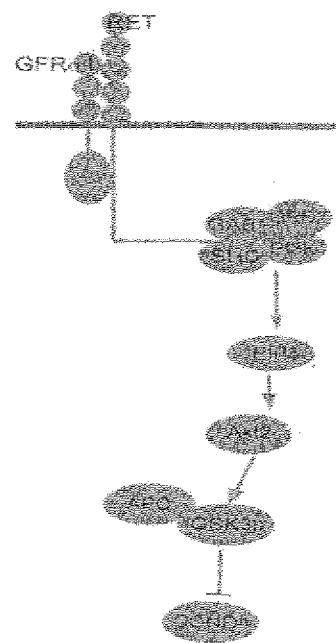
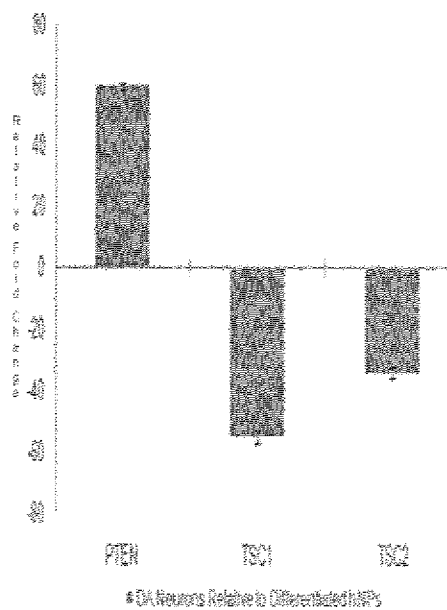
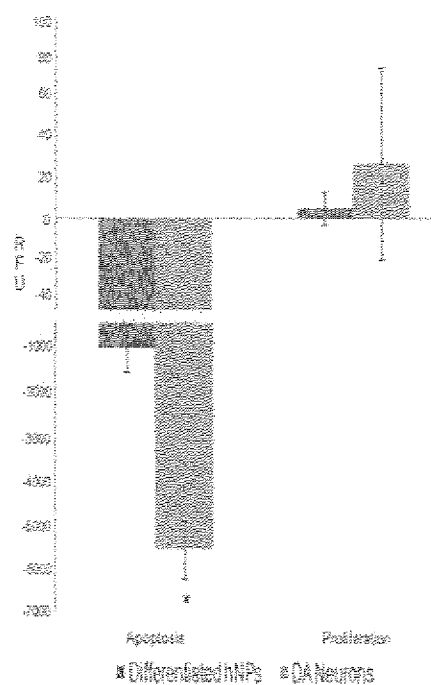


FIGURE 18

A DA Neuron Gene Expression
Changes in the GSK3 β PathwayB GSK3 β Inhibitor AssaysC GSK3 β PathwayD DA Neuron Gene Expression
Changes in mTOR Pathway

E mTOR Inhibitor Assay



F mTOR Pathway

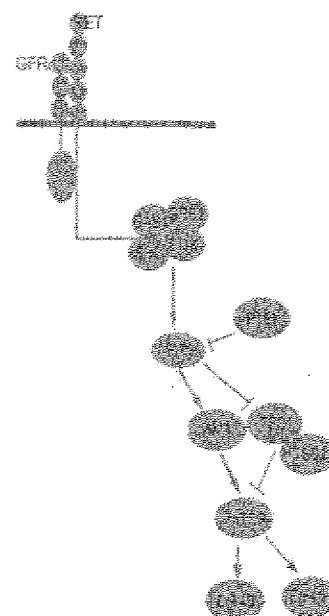


FIGURE 19

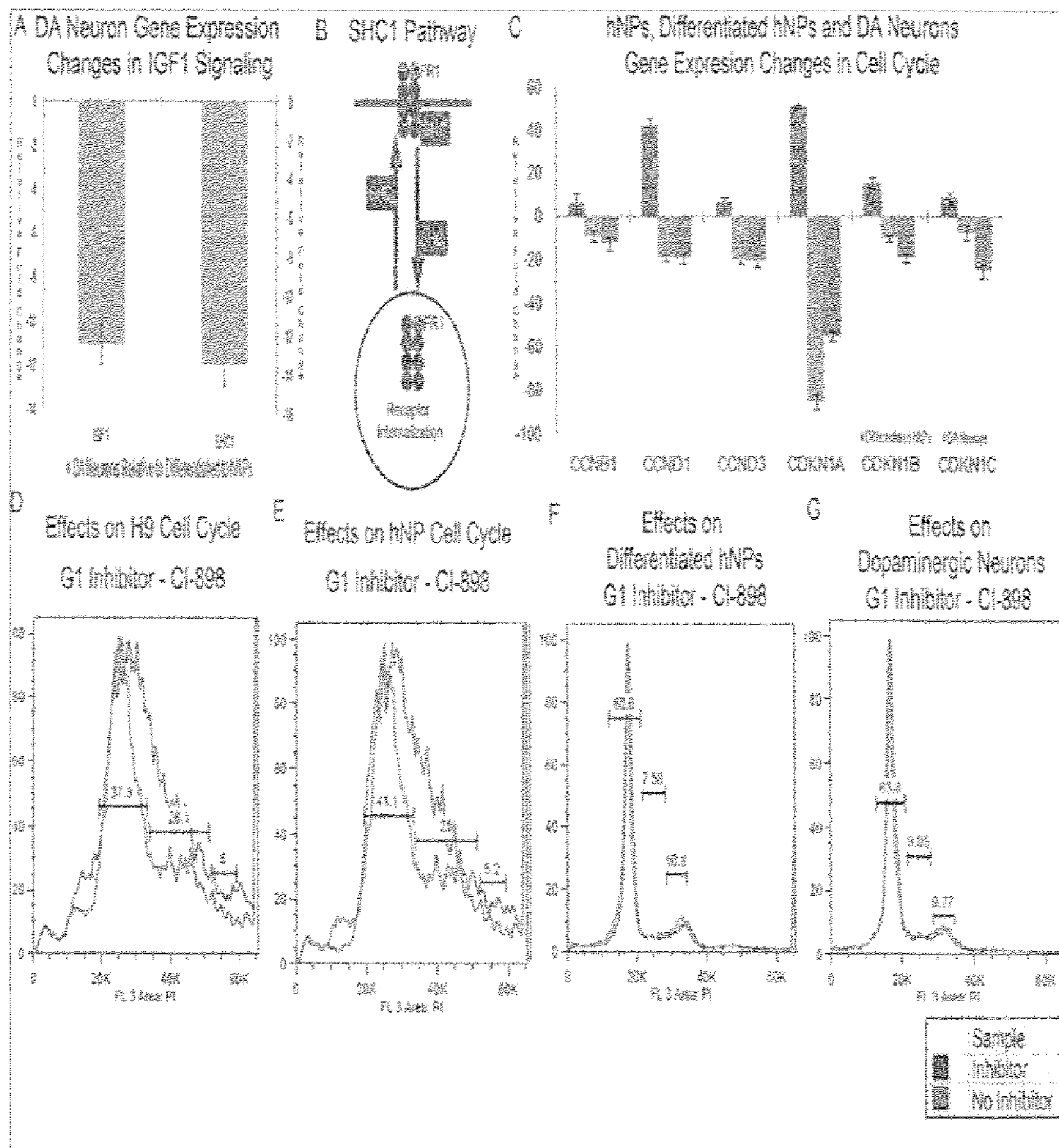


FIGURE 20

