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Dopaminergic Neurons Derived from Human Embryonic Stem Cell Derived Neural Progenitors: Biological Relevance and Application

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1. Introduction

Dopaminergic neurons are studied at length for their role in Parkinson's disease (PD), schizophrenia and addiction (Iversen and Iversen, 2007). While these commonly known roles for dopamine involve a similar neural subtype, the brain areas and identifying genetic markers involved in each pathway differ. These differences lead to selective involvement of each pathway (nigrostriatal, mesolimbic and mesocortical), allowing for derivation of dopaminergic neurons from human embryonic stem cells (hESCs) as well as from human neural progenitors (hNPs) that can be used for drug development or for cell therapy in PD.

Ever since their isolation in 1998, hESCs have been touted as having potential for cell therapies, drug development assays and as a source for studying human development (Thomson et al., 1998). Due to PD effecting 1% of the American population over 65 as well as the specificity of the cell type affected, PD presents as a neurodegenerative disease with potential to be helped with hESCs (Weintraub et al., 2008a). In 2004, the first report of tyrosine hydroxylase (TH) positive neurons derived from hESCs demonstrated that obtaining dopaminergic neurons would be possible in humans (Perrier et al., 2004). The stromal cell-derived inducing activity (SDIA) method enhanced dopaminergic differentiation through co-culture with mouse derived stromal cells, which secreted factors that directed differentiation towards a dopaminergic phenotype. Following the SDIA method, a 5-stage method for deriving dopaminergic neurons from hESCs that did not require co-culture with contaminating feeder layers obtained fewer dopaminergic neurons from hESCs (Schulz et al., 2004). Since this work, there has been limited success in obtaining high levels of TH+ neurons without the addition of feeder layers.

A selective dopaminergic neuron neuroprotectant was discovered in 1993, glial cell-line derived neurotrophic factor (GDNF) (Lin et al., 1993). The potential for this neurotrophic factor to protect substantia nigra dopaminergic neurons was explored, and in rat models of PD, GDNF administration was effective in protecting those cells lost in PD as well as in protecting neural cells transplanted into lesioned rat midbrains (Kearns and Gash, 1995, Hou et al., 1996). Methods for administering GDNF into human patients have been developed and clinical trials utilizing GDNF as a protectant for dopaminergic neurons have

proceeded with unfavorable results due to localization of GDNF administration as well as invasiveness of the surgery required (Kordower et al., 2000, Maswood et al., 2002, Kordower et al., 2008, Su et al., 2009). Methods for GDNF administration other than lesions have not lead to successful results. However, GDNF has potential as a dopaminergic neuroprotection agent in the differentiation of dopaminergic neurons from hESCs or hNPs.

In this chapter, we intend to cover dopaminergic development in the mouse and human brain in order to understand more fully dopaminergic derivation from hESCs and hNPs. We also intend to examine the processes of dopaminergic derivation that have been used as well as the role the GDNF plays in this process. Finally, we intend to cover the potential applications for hNP derived dopaminergic neurons.

2. Parkinson's disease

2.1 Epidemiology

PD is a progressive neurodegenerative disease that is second in prevalence only to Alzheimer's disease (Weintraub et al., 2008a). While typically thought of as a disorder only affecting the elderly population, early onset PD (appearance of symptoms between 45 and 65) currently accounts for 10% of the diagnosed cases of Parkinson's (Rao et al., 2006).

There are two main subtypes of PD, idiopathic and secondary. Idiopathic forms of PD can be either sporadic (90% of cases) or genetic. Most often seen in young onset PD, the most common genetic mutation is in the PARK8 (LRRK2) gene and the second most common is in the PARK1 gene which encodes for the alpha synuclein protein (Obeso et al., 2010). Sporadic PD has no clear etiology but may be caused by environmental factors, toxins or aging. Secondary PD is caused by medications, infection or metabolic disorders (Poewe, 2006, Elbaz and Moisan, 2008).

Diagnosis usually begins with the presentation of motor symptoms which fall into four categories: 1) resting tremor, 2) bradykinesia, 3) rigidity and 4) postural instability (Weintraub et al., 2008a). Younger patients present with tremor as their primary symptom and older patients present with bradykinesia as their primary symptom (Poewe, 2006). The resting tremor appears unilaterally and moves bilateral as the disease progresses. Most often, the tremor is seen in the distal portion of the limbs in the hands or a shaking leg. Bradykinesia, the inability to initiate movement, leads to the shuffling gait associated with PD. Most often this is noticed in the slowness and difficulty a PD patient has when walking, but it can also lead to difficulty in turning in bed or rising from a chair (Poewe, 2006). Rigidity, stiffness of the muscles in the limbs and trunk, often leads to postural instability (Poewe, 2006). Postural instability, the inability to maintain balance and coordination, occurs in the most advanced stages of PD (Poewe, 2006). This affliction often leads to the falls that can lead to rapid decline in a person's quality of life. In addition to decreasing quality of life, postural instability has very little response to the current treatments for PD (Weintraub et al., 2008a).

In addition to the motor symptoms, PD patients are affected by non-motor symptoms. This is due to the large involvement of other neurons in the limbic area of the brain, the compensation for the loss of dopaminergic neurons by other neurons and the connections between the basal ganglia and the frontal cortex. The most prevalent non-motor symptom is depression beyond which would be expected for the average population affected by a debilitating disorder with between 20 and 45% of people with PD being diagnosed with depression post diagnosis of PD (Weintraub et al., 2008b). The second most common non-motor symptom is psychosis, most often manifesting in hallucinations (Weintraub et al., 2008b). Cognitive decline is seen as the

disease progresses with memory loss, attention impairment and executive function deficits reported most often (Lim and Lang, 2010). The co-morbidity of these non-motor symptoms with the motor symptoms paints an image of PD as a whole body and mind disorder not just as a motor disorder (Weintraub et al., 2008b, Gaig and Tolosa, 2009).

The first effective treatment for PD and still the leading treatment is a dopamine precursor that crosses the blood brain barrier (BBB) and is converted into dopamine in the brain known as levo-dopa (L-dopa) (Poewe, 2006). However, L-dopa often produces side effects that are worse than the disease itself. In addition, over time, patients require higher and higher dosages to be effective, a concern with younger patients (Rao et al., 2006). The final hallmark of PD, postural instability, is resistant to L-dopa treatment. Another common treatment is dopamine agonists, which can be used in monotherapy or in combination with L-dopa. Argument for their use alone as a first treatment is to delay L-dopa treatment slowing the wearing off of L-dopa (Rao et al., 2006). However, due to the lack of robustness of dopamine agonists, almost all patients will require L-dopa at some point. In the many years since the beginning of a search for treatment, the lack of progress demonstrates the complexity of the disease (Obeso et al., 2010).

2.2 Pathophysiology

The earliest and most studied cause of PD is the degeneration of the dopaminergic neurons in the substantia nigra (SN). In the normal brain, dopaminergic neurons are found in three main areas, the olfactory bulb, the hypothalamus and the midbrain, which consists of the SN and the ventral tegmental area (VTA). From the midbrain, there are three main projections of the dopaminergic neurons. The mesolimbic pathway projects dopaminergic axons from the VTA to the nucleus accumbens, plays a role in addiction and reward and is the pathway most often affected in schizophrenia (Sillitoe and Vogel, 2008). The mesocortical pathway projects axons from the VTA to the frontal cortex and is most often associated with motivation and memory (Sillitoe and Vogel, 2008). The nigrostriatal pathway projects from the SN to the basal ganglia (BG) and is associated with motor control (Smith and Bolam, 1990). This pathway is involved in PD, and will be the focus of this review.

In the normal brain, dopaminergic projections from the substantia nigra pars compacta (SNc) synapse on the striatum, which consists of the caudate nucleus and the putaman (Figure 2.1) (Smith et al., 1998). From the striatum, a direct or an indirect pathway leads to the substantia nigra pars reticula (SNr) (Smith et al., 1998). The direct pathway sends inhibitory GABA and substance P axons to the globus pallidus internal (GPi)/SNr (Mora et al., 2008, Weintraub et al., 2008b). The indirect pathway projects inhibitory GABA and enkephalin axons to the globus pallidus external (GPe) which then sends GABAergic projections to the subthalamic nucleus (STN) which then sends glutamatergic (excitatory) outputs to the GPi/SNr (Mora et al., 2008) The projections to the SNr proceed to the thalamus. From the thalamus, glutamatergic projections head toward the cortex or GABAergic projections proceed to the brain stem and from the brain stem axons project back to the SNc completing the loop (Mora et al., 2008). Both pathways lead to activation of muscle movement and control. Through the activation the motor cortex or brain stem as well as through feedback loops within the basal ganglia, fine motor movements can be controlled (Mora et al., 2008, Gaig and Tolosa, 2009), the decision to move can be separated from the movement itself and other outside inputs can be factored into muscle movement decision.

In PD patients, the dopaminergic projections to the striatum deteriorate. The decline in dopaminergic modulation of the basal ganglia leads to problems in controlling muscle movements and to the symptoms seen in PD (Figure 2.1). Often the symptoms do not present until approximately 60% of the dopaminergic cells in the SNc have died suggesting a compensating mechanism for controlling movement (Gaig and Tolosa, 2009). Proposed mechanisms for this redundancy include the feedback loops located within the basal ganglia as well as movement of serotonergic neurons located nearby into the basal ganglia (Smith et al., 1998). These mechanisms from the serotonergic neurons may be responsible for some of the early non-motor symptoms (Weintraub et al., 2008b). Additionally, the relationship of the basal ganglia with the frontal cortex may be responsible for cognitive decline (Weintraub et al., 2008b). The dopaminergic neurons in the SNc deteriorate selectively in PD, leaving the dopaminergic neurons in the rest of the brain intact and not leading to symptoms typically seen in other dopaminergic disorders.

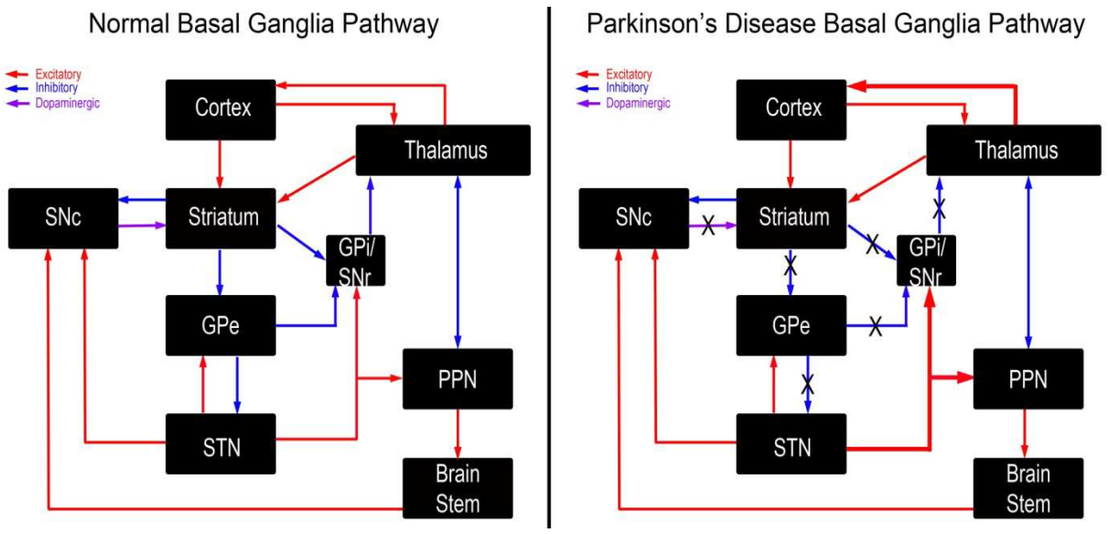

Fig. 2.1. Dopamine Signaling to the Basal Ganglia in Normal and Parkinson's Disease State. In normal state, dopaminergic neurons from the substantia nigra pars compacta (SNc) project onto the striatum. Activation of the striatum leads to motor movement modulation through the direct pathway (globus pallidus internal, GPi) or the indirect pathway (globus pallidus external, GPi). Both pathways lead to the thalamus and then to the cortex and brainstem (Smith et al., 1998). In the Parkinson's disease (PD) state, the dopaminergic neurons from the SNc are absent. This lack of input prevents the inhibitory signaling to the indirect and direct pathways, which causes a disruption in motor control (Obeso et al., 2008).

3. Human embryonic stem cells and derivatives

3.1 Human embryonic stem cells

In 1998, James Thomson and colleagues derived hESCs from the inner cell mast of discarded blastocysts (Thomson et al., 1998). From 14 inner cell masts collected, 5 embryonic stem cell lines could be created. Thomson and colleagues established early characteristics of hESCs which included high nuclear to cytoplasmic ratio, prominent nucleoli, the formation of a distinct colony, high telomerase activity, the ability to form cells from all three germ layers, and teratoma formation in addition to embryonic markers SSEA-3, SSEA-4, Tra-1-60, Tra-1-

81 and alkaline phosphytase (AP) (Thomson et al., 1998). Mouse embryonic fibroblast (MEF) feeder layers were found to support continued proliferation, and hESCs demonstrated the ability to form embryoid bodies, which contain all three germ layers (Thomson et al., 1998).
Mouse embryonic stem cells (mESCs) can be maintained in an undifferentiated state using leukemia inhibitory factor (LIF) alone without feeder layers. LIF activates the signal transducer gp130 there by activating of STAT3 and maintaining the state self-renewal in mESCs (Figure 2.3) (Humphrey et al., 2004). BMPs can be used in the place of serum in addition to LIF to maintain pluripotency through the activation of Id genes (Ying et al., 2003, Humphrey et al., 2004, Rao, 2004). This has not been the case for hESCs as LIF does not maintain the pluripotency of hESCs and is not necessary for maintenance of self-renewal (Xu et al., 2005). Initial attempts at feeder free culture of hESCs expanded upon the knowledge that hESC populations express α6 and β1 integrins leading to successful culture on laminin and Matrigel as extracellular matrices for hESCs in MEF conditioned media with differentiation results similar to what was found in previous studies (Xu et al., 2001). Basic fibroblast growth factor (bFGF) has been used to maintain clonally derived hESCs suggesting potential in a feeder free, serum free culture (Amit et al., 2004). Differentiation studies in which BMPs were blocked in hESC culture initiated neural differentiation. Taking these two together, Xu and colleagues used bFGF and BMP to maintain hESC self-renewal in the absence of MEFs or MEF conditioned media (Xu et al., 2005).
Our lab derived three lines from discarded embryos in 2001. These lines were isolated from the inner cell mass of 19 embryos and resulted in 4 cell lines. These cell lines were maintained in a pluripotent state on MEF feeder layers (Mitalipova et al., 2003). Two of these cell lines (BG01 and BG02) have the ability to form EBs and to differentiate into neural cells and cardiac cells (Mitalipova et al., 2003).

3.2 Neural progenitor cells

Directing the differentiation of hESCs towards neural cells allows for controlled culture system to develop specific neural subtypes including motor neurons, dopaminergic neurons and forebrain neurons. Several groups have attempted to establish a proliferative population of multipotent hNPs, which can be differentiated to neurons, astrocytes or oligodentrocytes.
Differentiation of hESCs toward hNPs occurs through either an embyroid body (EB) or a monolayer culture system. In EB differentiation, hESCs are grown in suspension and allowed to form masses of cells, which form a mixed population that includes hNPs (Schuldiner et al., 2001, Zhang et al., 2001). From these masses, the neural cells were selected and used in further proliferation or differentiation experiments. In monolayer differentiation, hESCs are induced with various morphogens in the tissue culture dish and neural rosette structures are allowed to form. From these structures, neural cells are selected, re-plated and allowed to proliferate or differentiate (Shin et al., 2006).
Each type of differentiation (EB or monolayer) requires various morphogens to direct the differentiation toward a neural multipotent cell. Retinoic acid (RA) plays a role in neural patterning and neural differentiation in the developing embryo (Reubinoff et al., 2001, Carpenter et al., 2003, Maden, 2007). Bone morphogenetic proteins are often inhibited by the antagonist Noggin, which leads to development of the neural phenotype in the mouse (Pera et al., 2004, Itsykson et al., 2005). bFGF signaling maintains the proliferative capacity of neural cells as well as involvement in induction and patterning (Jordan et al., 2009). bFGF was used in a neural differentiation protocol for its known caualzaling factors. Originally,

bFGF was shown to be important in the brain as a neural growth factor that maintained the pluripotency of immortalized NSCs (Figure 2.2) (Li et al., 2000). Later, bFGF has been shown to be a caudalizing factor within the neural plate and the neural floor (Jordan et al., 2009). Epidermal growth factor (EGF) is a mitogen that was used in many hESC neural differentiation protocols to maintain self-renewal potentially through crosstalk with Notch or through EGF's suppression of apoptosis (Carpenter et al., 2003, Elkabetz et al., 2008). LIF is another factor known to maintain proliferation of neural cells (Shin et al., 2006). Several reports of hNP differentiation have used varying combinations of these factors to achieve hNP differentiation from hESCs.

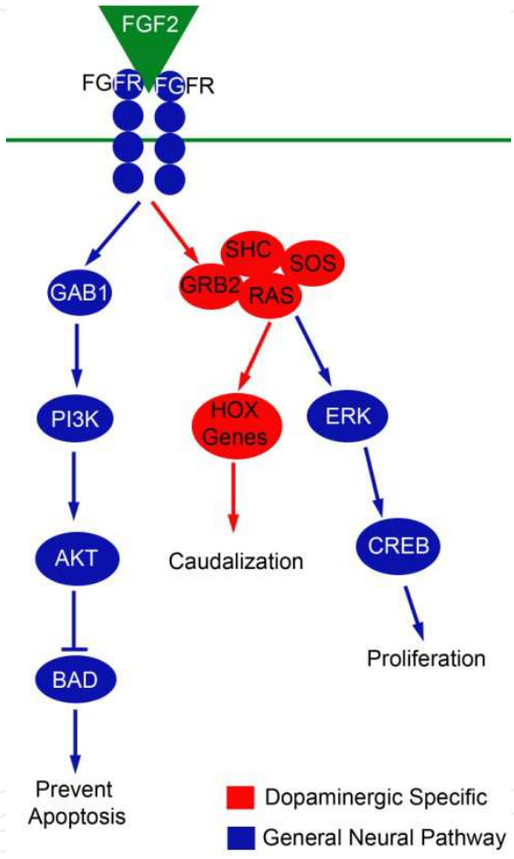

Fig. 2.2. Fibroblast Growth Factor 2 Induces Caudalization and Prevents Apoptosis
Fibroblast growth factor 2 (FGF2) binds to the fibroblast growth factor receptor in developing neurons to activate the PI3K pathway or the MAPK pathway. In neural progenitors (hNPs) and neural stem cells (NSCs), FGF2 activates AKT, which blocks BAD signaling to prevent apoptosis (Sato et al., 2010). MAPK activation of the transcription factor CREB increases proliferation in hNPs and NSCs (Sato et al., 2010). These pathways are general neural pathways seen in all neurons prior to specification and represented by the general neural pathway. Additionally, FGF2 activates HOX genes in a gradient within the developing brain to caudalize neurons towards an anterior cell fate, which is the action of FGF2 in the dopaminergic neurons differentiated in this chapter and represented by the dopaminergic specific pathway (Chiba et al., 2005).

Differentiation of these hNPs into specific neural subtypes creates neural cells that are better models for transplanation specific developmental patterns, disease progression or transplantation. Differentiation of motor neurons has required the addition of RA, sonic hedgehog (SHH) and bFGF (Li et al., 2005, Shin et al., 2005). Forebrain differentiation has required *Otx1*, *Otx2* and *Bf1* expression and is thought to involved Wnt signaling (Elkabetz et al., 2008). Serotonergic neuron differentiation requires SHH and fibroblast growth factor 4 (FGF4) (Barberi et al., 2003). Differentiation toward a dopaminergic fate begins with SHH and fibroblast growth factor 8 (FGF8) and will be further explored in this review (Perrier et al., 2004).

In 2006, our lab derived hNPs from hESCs using a monolayer culture system. Neural derivation media was used to induce neural rosette structures from which neural cells were selected and transferred to a monolayer culture (Shin et al., 2006). The combination of bFGF and LIF added to the culture media allowed for the maintenance of neural progenitor cells in a monolayer that could be continually cultured for several (>40 passages) while maintaining a stable karyotype (Shin et al., 2006). We have demonstrated the ability to differentiate these hNPs to motor neurons with the addition of RA (Shin et al., 2005) and to dopaminergic neurons with the addition of GDNF (Young et al., 2010).

4. Factors involved in dopaminergic differentiation

4.1 Sonic hedgehog

In the developing embryo, signaling factors in the developing nervous system control the movement of the different types of neurons in the brain and spinal cord to their correct position. In dopaminergic neuron development, sonic hedgehog (SHH) modulates the dorsal/ventral placement of the midbrain dopaminergic neurons (Hynes et al., 1995). SHH is secreted from the notochord to induce floor plate cells through a decreasing gradient and to signal for the ventral forebrain and midbrain development of serotonergic and dopaminergic neurons (Hynes et al., 1995, Smidt and Burbach, 2007). SHH signaling is closely regulated to ensure proper enlargement of the midbrain area and is turned off to allow for post-mitotic differentiation. Dopaminergic neurons will arise from the pool of neuroepithelial progenitors found in the ventricular floor plate (Smidt and Burbach, 2007). Wnt causes a down regulation of SHH signaling allowing for the end of neural proliferation and the beginning of neurogenesis (Joksimovic et al., 2009). In 1995, SHH was discovered to be important for dopaminergic neural development through its activation of cAMP and PKA (Hynes et al., 1995). Transplantation of floor plate tissue to other areas or induced expression of SHH in other brain areas will cause ventralization of those areas. Over expression of the SHH target Gli1 causes the same effects as SHH itself, further confirming SHH's role in dopaminergic neuron development (Gulino et al., 2007). The ability for floor plate tissue combined with FGF8 beads to induce the formation of midbrain dopaminergic neurons further added increased evidence for SHH in the midbrain/hindbrain organization. SHH activates Patched (Ptc), releasing its negative control on Smoothened (Smo) and activating downstream transcription factors Gli1, Gli2 and Gli3 (Gulino et al., 2007). Each Gli has distinct actions; Gli1 acts to increase SHH activation. Gli2 acts to modulate Wnt, Brachyury, Xhox3 and Bcl-2 genes. Gli3 activates Ptc as a negative control of SHH signaling (Gulino et al., 2007). The decreasing gradient outward from the ventral midbrain signals the induction of neural precursor cells, which is suppressed by Wnt signaling the beginning neurogenesis of the floor plate derived dopaminergic and serotonergic neurons. SHH interacts with FGF8 to induce the correct size pool of dopaminergic neurons (Joksimovic et al., 2009).

4.2 Fibroblast Growth Factor 8

In combination with SHH, FGF8 controls the boundaries of the midbrain-hindbrain organizer (MHO) which direct the area in which dopaminergic neurons will be expressed. FGF8 expression originates at the isthmus and radiates anterior/posterior (Ye et al., 1998). The size of the MHO is determined by outside induction factors including the Hox genes at the anterior edge and FGF4 which signals with SHH for serotonergic neuron development. FGF8 interacts with other early regulatory genes involved in dopaminergic neuron development (Otx2, Gbx2, EN1, EN2, Pax2 and Pax5) to maintain and regulate the dopaminergic field of development (Smits et al., 2006). If ectopically applied, FGF8 and SHH induce a two-dimensional system of midbrain neural precursor cells (Smidt and Burbach, 2007).

4.3 Leukemia Inhibitory Factor

Leukemia inhibitory factor (LIF) is a member of the interleukin 6 family of cytokines and supports cell growth and development. LIF has known function in maintaining the pluripotency of mESCs (Pease et al., 1990). This function does not carry over into hESCs, as the addition of LIF to the culture media for hESCs does not maintain the pluripotency (Xu et al., 2005). In the non-dividing cells of the neural crest, LIF induces sensory neuron development (Murphy et al., 1991). Later it was discovered that LIF also promotes proliferation of the progenitor pool found in the olfactory bulb and in fetal neural stem cells (Satoh and Yoshida, 1997, Galli et al., 2000). This is thought to occur through the gp130 receptor regulation Notch signaling which controls neural stem cell proliferation. LIF has been used to maintain of pluripotency in hNPs derived from hESCs as well as in NSC cultures (Chojnacki et al., 2003).

While it was known that LIF supported glial cell differentiation, in 2003 it was discovered that LIF acts through the ERK pathway to decrease the expression of dopamine beta hydroxylase (DβH) (Figure 2.3) (Dziennis and Habecker, 2003). Mouse and rat mesencephalic derived progenitors were differentiated into dopaminergic neurons using both LIF and GDNF (Storch et al., 2001). These differentiated dopaminergic neurons were maintained in culture for extended periods as well as used for deriving a clonal line (Storch et al., 2001). LIF has also been used in a rat model of PD to increase the number of mesencephalic dopaminergic neurons. Support for the use of LIF as a factor to enhance dopaminergic differentiation from hNPs in this chapter comes from the suppression of DβH by LIF (Figure 2.3) in addition to the known success in a rat and mouse model of dopaminergic differentiation with GDNF and LIF (Ling et al., 1998, Liu and Zang, 2009).

4.4 Glial cell-line Derived Neurotrophic Factor

GDNF was discovered as a neurotrophic factor for dopaminergic neurons in 1993 in rat glial cell cultures (Lin et al., 1993). Since this time, its use as a potential treatment for PD has been explored in several animal models (rat, mouse, non-human primate), cell culture models (rat, mouse, non-human primate, mESCs, hESCs, human fetal tissue) and in human drug trials. GDNF was first tested as a recovery factor in animal models of PD (Bowenkamp et al., 1995, Shults et al., 1996). Rats lesioned with 6-OHDA and then injected with GDNF showed increase in TH expression and a reduction in apomorphine induced turning (Bowenkamp et al., 1995, Shults et al., 1996). Retrograde tracing studies show that GDNF injected into the midbrain was transported back to the SN (Tomac et al., 1995). In C57/B1 mice, injection of

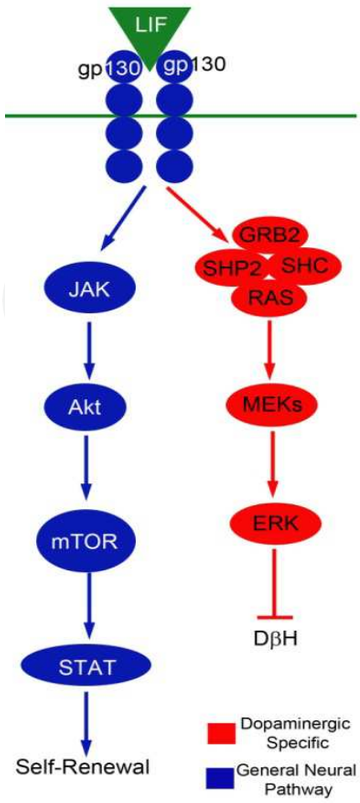

Fig. 2.3. Leukemia Inhibitor Factor action in dopaminergic and non-dopaminergic neurons
Leukemia inhibitory factor (LIF) binds to the gp130 receptor on the cell surface causing activation of the JAK/STAT pathway and the MAPK pathway (Matsuda et al., 1999). Activation of the JAK/STAT pathway modulates self-renewal in neurons other than dopaminergic (Matsuda et al., 1999). The MAPK pathway is activated by LIF in dopaminergic neurons to suppress dopamine beta hydroxylase production, which would lead to production of norepinephrine instead of dopamine (Dziennis and Habecker, 2003). General neural pathway refers to any neural subtype other than dopaminergic while dopaminergic specific refers to dopaminergic neurons similar to those derived in this chapter.

GDNF in the SN protects from degeneration and aids in the recovery of dopaminergic neurons in an MPTP model (Hou et al., 1996). The first study of GDNF in a non-human primate, a rhesus monkey, showed recovery in bradykinesia, rigidity and postural instability in an MPTP lesioned striatum that was maintained with injections of GDNF every 4 weeks (Gash et al., 1996). As injections into the brain are not desired for a potential treatment option for PD, fetal mesencephalic neurons from rat brains that excrete GDNF were injected into 6-OHDA lesioned rats and an increase in TH expression and expansion of neurite tracts were seen postmortem (Rosenblad et al., 1996, Wang et al., 1996, Winkler et al., 1996). Adenovirus' created to promote GDNF expression were tested by several groups for their ability to protect the SN from 6-OHDA neurotoxicity with limited results and lack of long-term effectiveness (Choi-Lundberg et al., 1997, Lapchak et al., 1997). Most of the data has shown limited results with long-term data unavailable; however, due to the known protective role for GDNF in DA neurons, enthusiasm is still high.

A 12-week study of GDNF injection into the ventricle of a 6-OHDA lesioned rat showed an increase in response to amphetamine to those seen in normal animals (Sullivan et al., 1998). Injections into the SN are found to be protective where as injections into the putamen are not (Gerhardt et al., 1999). Long-term adenovirus vector administration in the SN led to re-innervation of the striatum after 6 months of administration (Kirik et al., 2000). Lentiviral administration of GDNF into the striatum of both mice and non-human primates that were lesioned 2 weeks later protected TH neurons in both young and old mice (Kozlowski et al., 2000, Georgievska et al., 2002). GDNF administration in a mouse α-synuclein model did not protect the SN from neurodegeneration presenting a complication that remains for GDNF as a therapy for PD (Lo Bianco et al., 2004).

GDNF's role in protecting and recovering neurons in the SN that degenerate in PD led to research into its use as a growth molecule in hESCs. hESCs differentiated towards dopaminergic neurons have been touted as a potential cell therapy in PD. The neurotrophic factor has been used in several differentiation protocols (Buytaert-Hoefen et al., 2004, Perrier et al., 2004, Schulz et al., 2004). The use of GDNF along with co-culture with PA6 cells increased the number of TH positive cells produced over PA6 co-culture alone (Buytaert-Hoefen et al., 2004). Using hESC derived dopaminergic neurons as a model of PD, GDNF provided protection against MPTP toxicity (Zeng et al., 2006).

Another method of improving delivery systems involves genetically altering neural stem cells or astrocytes to release GDNF and injecting these into the brain which protected from parkinsonian motor responses in mouse models of PD (Engele and Franke, 1996, Elsworth et al., 2008). Injection of hNPs modified to secrete GDNF into MPTP monkeys increased axon fibers that express both TH and VMAT2; however, these cells remained at the area of injection and did not travel to the area of need (Emborg et al., 2008). The neuroprotection of GDNF in rat, non-human primate and hESC models of PD demonstrates its robustness as a useful tool for developing future therapies.

Further expanding on the rodent research, intracerebral injections of GDNF into MPTP treated rhesus monkeys induced a 20% increase in dopamine levels and functional recovery with GDNF injections every four weeks (Gash et al., 1996). When GDNF was administered along with the most commonly prescribed L-dopa drug in parkinsonian rhesus monkeys, a significant functional improvement in PD symptoms was seen as well a decrease in the side effects typically accompanying L-dopa drugs (Elsworth et al., 2008). Further study between the relationship of GDNF administration and functional recovery indicates a role for GDNF in modulating dopamine plasticity in the striatum. Safety and efficacy studies in non-human primates demonstrated that the injections do not cause any negative histological effects in the injected brain and that the most notable side effect of GDNF delivery was weight loss (Su et al., 2009). These studies advanced the field towards using GDNF in human clinical trials.

Following determination that human PD patient brains maintained expression of the RET receptor for GDNF, a male patient received intracerebroventricular injections of GDNF which resulted in severe side effects and no functional recovery. Several years later, a randomized double-blind study of intracerebroventricular monthly injections of various dosages of GDNF lead to no parkinsonian symptom improvement with GDNF but an increase in adverse effects including significant weight loss potentially because the GDNF did not reach the target tissues (Kordower et al., 2000). Targeted injections of GDNF into the putamen resulted in significant improvements in PD quality of life scores, dopamine uptake and dyskinesia (Kordower, 2003). In a two-year follow up study, the patients continued to

improve with no added side effects. Withdrawal of GDNF injections caused a complete reversal to pre-injection levels of quality of life and symptomatic scores (Kordower et al., 2008). At this stage, GDNF is still being evaluated as a potential treatment for PD but the route of administration and side effect profile are holding back major advances in the field.

5. Genes involved in dopamine development

Protein	Expression (mouse)	Role in Dopaminergic Neurons	References
NURR1	E10.5	• Drive expression of TH, AADC, RET, VMAT2, DAT • Support development of DA neurons • Maintain post-mitotic DA neurons	(Zetterstrom et al., 1997, Saucedo-Cardenas et al., 1998, Wallen et al., 2001)
EN1	E7.5	• Expressed in neuroepithelium • Secreted to maintain mid-/hindbrain boundary • Induced by FGF8 • Maintain post-mitotic DA neurons	(Liu and Joyner, 2001, Sgado et al., 2006)
TH	E11.5	• Driven by NURR1 • Rate limiting enzyme in DA synthesis	(Lehnert and Wurtman, 1993, Maxwell et al., 2005)
PITX3	E11.5	• Drive expression of VMAT2, DAT and RA • Maintain SN neurons	(Lebel et al., 2001, Hwang et al., 2003, Smidt et al., 2004, Jacobs et al., 2007)
DAT	E13.5	• Gives MPTP access to DA neurons • Denser in SN neurons • Removes DA from synapse	(Storch et al., 2004, Schiff et al., 2009)
VMAT2	E18	• Package MPTP to prevent it from damaging cell • Less VMAT2 expression in PD brains	(Harrington et al., 1996, Hansson et al., 1998, Speciale et al., 1998)

Table 2.1 Proteins Expressed in Dopamine Neurons_AADC - Aromatic L-Amino Acid Decarboxylase, DA - Dopamine, DAT – Dopamine Transporter, EN1 – Engrailed 1, FGF8 – Fibroblast Growth Factor 8, MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, NURR1 - Nuclear Receptor Related 1, PD – Parkinson's Disease, PITX3 - Paired-like Homeodomain Transcription Factor 3 , RA – Retinoic Acid, RET – Rearranged in Transfection, SN – Substantia Nigra, TH – Tyrosine Hydroxylase, VMAT2 - Vesicular Monoamine Transporter 2

5.1 Nuclear receptor related 1

Nuclear receptor related 1 (Nurr1) is a member of the Nur family of proteins which are involved in cell growth and apoptosis. Beginning expression at E10.5 in the mouse, Nurr1 is required for normal dopaminergic development (Table 2.1) (Zetterstrom et al., 1997). Nurr1 was discovered to be similar to Nur77 already found in the olfactory bulb, cortex, hippocampus, SN and VTA of the mouse (Law et al., 1992, Zetterstrom et al., 1996). Mice administered 6-OHDA not only show a loss in dopaminergic neurons but also in Nurr1 expression. Nurr1 knockout (Nurr1-/-) mice do not express TH in brain areas A8 (retrorubral nucleus), A9 (SN) and A10 (VTA) (Zetterstrom et al., 1997). Discovery of the need for Nurr1 to regulate TH expression led to further examination of the role that Nurr1 plays in maintenance of other proteins important for a properly functional dopaminergic neuron (Table 2.1). In Nurr1-/- mice, aromatic L-amino acid decarboxylase (AADC), the enzyme responsible for converting L-dopa to dopamine or 5-hydroxytryptophan to serotonin, was found to be absent in dopaminergic neurons only; however, paired-like homeodomain transcription factor 3 (PITX3), a gene found only in SN dopaminergic neurons, expression was unaffected in Nurr-/- mice (Table 2.1) (Saucedo-Cardenas et al., 1998). As discussed earlier, GDNF signals through co-receptor GFRα1 binding to RET (Table 2.1). Nurr1 knockouts are deficient in RET but not in GFRα1 suggesting the importance of Nurr1 not only in pathways involved in dopamine production but in neuron maintenance and support (Wallen et al., 2001).

Nurr1 is not only important for embryonic development of dopaminergic neurons but also for the maintenance of these neurons in the postnatal and adult brain. Conditional knockouts induced by *Cre* ablation of Nurr1 at E13.5 to E15.5 show a loss of TH and the dopamine transporter (DAT) expression in postnatal rats while adult ablation leads to reduction in TH expression in the SN preferentially over the VTA (Table 2.1) (Kadkhodaei et al., 2009). Overall, Nurr1 plays a role in activating and maintaining the expression of AADC, TH, RET and DAT. With such an importance in dopaminergic neurons, finding a decrease in Nurr1 in PD patients as well as a base pair insertion mutation is not surprising. Further expansion on the role of Nurr1 in PD patients may lead to future treatment options.

5.2 Engrailed 1

Engrailed 1 (EN1) is part of a family of homeobox genes consisting of EN1 which is expressed in the VTA and SN and EN2 which is only expressed in a subset of dopaminergic neurons and begins to be expressed later in development than EN1 (Danielian and McMahon, 1996). EN1 is a developmental regulation protein that is expressed in mouse around day E7.5 and plays a role in the development of dopaminergic neurons and the maintenance of those neurons (Table 2.1) (Danielian and McMahon, 1996). EN1 is expressed in the neuroepithelium of the ventral midbrain around the isthmus, which is responsible for controlling the midbrain/hindbrain boundary (Liu and Joyner, 2001). Induction of EN1 by FGF8 maintains the area of the brain that will consist of the dopaminergic neurons. EN1 knockout mice lose the expression of all dopaminergic neurons by birth (Ye et al., 2001). A gain of function study in mice demonstrated that EN1 would induce the midbrain/hindbrain expression in any area in which it was expressed (Table 2.1) (Alberi et al., 2004). Both EN1 and EN2 are necessary for proper induction of midbrain dopaminergic neurons and they can partially compensate for each other (Alberi et al., 2004). The other role of EN1 is in maintaining dopaminergic neurons post-mitotically in the midbrain (Table 2.1) (Sgado et al., 2006). EN1 conditional knockout mice lose their dopaminergic neurons in the

midbrain due to caspase 3 induction and apoptosis (Sgado et al., 2006, Sonnier et al., 2007). Heterogeneous EN+/- mice will progressively lose their dopaminergic neurons in a pattern that is similar to that seen in PD patients (Sgado et al., 2006, Sonnier et al., 2007).

5.3 Tyrosine hydroxylase

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in dopamine synthesis, making it the main marker for dopaminergic neurons. In the production of catecholamines, L-tyrosine is converted to L-dopamine by TH. Aromatic L-amino acid decarboxylase (AADC) then converts the L-dopamine into dopamine. In dopaminergic neurons, the process stops there. In noradrenergic neurons, dopamine is converted into norepinephrine by dopamine β hydroxylase (DBH). If the neuron releases epinephrine, then norepinephrine is converted to epinephrine by phenylethanolamine N-methyltransferase (PMNT) (Table 2.1) (Lehnert and Wurtman, 1993). As TH has an important role in several neural subtypes, it cannot be the sole marker for a dopaminergic neuron even if it is an important one. In the midbrain, TH expression occurs at E11.5 in the mouse immediately prior to PITX3 expression (Table 2.1). TH expression is driven by Nurr1, which began its expression at E10.5 (Maxwell et al., 2005).

5.4 Paired-like homeodomain transcription factor 3

Paired-like homeodomain transcription factor 3 (PITX3) is a homeodomain protein which is found only in dopaminergic neurons in the midbrain of the central nervous system. Expression of PITX3 is found outside of the CNS transiently in the eye lens. Expression of PITX3 begins at E11.5 in the mouse, immediately following expression of TH (Table 2.1) (Lebel et al., 2001). PITX3 expression completely overlaps the areas of TH expression in the SN and only a subset of the TH+ neurons in the VTA; additionally, the TH promoter has a PITX3 binding site. Aphakia mutant mice (PITX3-/-) lack SN neurons exclusively starting around E12.5, but not VTA neurons suggesting the molecular mechanism of PITX3 in the VTA differs from that in the SN and this mechanism may provide insight into the selective degeneration of dopaminergic neurons in the SN in PD (Hwang et al., 2003, Nunes et al., 2003, van den Munckhof et al., 2003). Retrograde tracing studies confirm that the absence of dopaminergic neurons in the SN in apkakia mice leads to a lack of the normal connections to the caudate putamen as is seen in PD (Hwang et al., 2003, Nunes et al., 2003, van den Munckhof et al., 2003). The absence of PITX3 in aphakia mice does not lead to an absence in many of the other genes involved in dopaminergic neuron development and maintenance (NURR1, LMX1b, EN1, EN2 and RET) (Smidt et al., 2004). AdH2 expression is affected causing a decrease in retinoic acid (RA; Table 2.1) (Jacobs et al., 2007). Restoring the levels of RA can counteract the developmental deficits seen in these mice suggesting that PITX3's role in dopaminergic neural maintenance is through regulation of RA expression. However, this is only required during early development and does not account for the continued deficits seen in aphakia mice (Jacobs et al., 2007). A possible role for PITX3 in continued deficits is in its control of VMAT2 and DAT expression (Hwang et al., 2009). Aphakia mice lack both VMAT2 and DAT as seen by both in situ hybridization and PCR (Table 2.1) (Smits et al., 2005).

5.5 Dopamine transporter

The dopamine transporter (DAT) is the protein responsible for removing dopamine from the synapse post release and taking it back into the neuron. This allows for recycling of

the neurotransmitter as well as halting the activation of the post-synaptic neuron. DAT activity depends on sodium moving down its concentration gradient, dopamine and chloride ions being recognized outside the transporter, dopamine and chloride ion translocation into the cell and unloading, and the transporter returning to its original state (Volz and Schenk, 2005). DAT mRNA is denser in the SN relative to the VTA suggesting a possible role for DAT in the pathology of PD. Over expression of DAT led to excitotoxicity and loss of dopaminergic neurons (Storch et al., 2004). Due to MPP+ entering the dopaminergic neuron through the DAT transporter, DAT knockout mice are insensitive to MPTP toxicity (Table 2.1) (Storch et al., 2004). Variable number tandem repeats (VNTRs) found in DAT occur in patients with various neurological disorders including PD (Haddley et al., 2008). These VNTRs seem to occur prior to symptomology of the disease suggesting that these VNTRs pre-dispose the dopaminergic neurons to susceptibility of the disease (Haddley et al., 2008).

5.6 Vesicular monoamine transporter 2

Vesicular monoamine transporter 2 (VMAT2) is a protein which is responsible for packaging monoamines (dopamine, serotonin, norepinephrine) into vesicles in the cytosol for transmission out of the cell (Harrington et al., 1996). VMAT2 also packages several neurotoxins such as MPTP to prevent them from causing harm to the neuron (Table 2.1) (Harrington et al., 1996). VMAT2 expression starts at E11 in the telencephalon and is seen in the caudate putamen and nucleus accumbens at P1 (Hansson et al., 1998). At E18 expression is found in the SN and VTA (Table 2.1). VMAT2 -/- mice die shortly after birth; however, VMAT2 +/- mice or blockage of the transporter yield results on the transporter function (Hansson et al., 1998). VMAT2+/- mice have a drastic decrease in dopamine despite compensation inside the neuron by more than doubling synthesis (Stankovski et al., 2007). MPTP destruction is more than twice that in normal mice through greater accumulation of the toxin to remain in the cytosol where it can cause damage to the neuron (Harrington et al., 1996). Animals that lack VMAT2 do not lack the neurons themselves, just the monoamines; cells eventually die through lack of use via the caspase 3 and caspase 9 pathways (Stankovski et al., 2007). Brains of PD patients examined postmortem express 88% less VMAT2 in the putamen, 83% less in the caudate and 70% less in the nucleus accumbens compared to brains of people who were not diagnosed with PD (Table 2.1) (Speciale et al., 1998).

6. Dopaminergic differentiation

Due to the lack of success in developing a new therapeutic for PD over the last 30 years combined with the specificity of the cells that deteriorate in PD, differentiating dopaminergic neurons from hESCs for use in cell therapy or drug discovery for PD has been a research focus for many years with the first successful attempt by Perrier and colleagues in 2004. Discovered in 2000 for its ability to induce midbrain dopaminergic neurons from mESCs, stromal cell-derived inducing activity (SDIA) refers to the factors secreted from or imbedded in the cell membrane of PA6 cells or other bone marrow cells which have been shown to promote dopaminergic differentiation (Kawasaki et al., 2000). Studies on fixed PA6 cells and on mitomycin c treated and irradiated cells show a reduction in ability to differentiate to dopaminergic neurons (Vazin et al., 2008). Microarray studies examining the factors secreted from these cells have suggested 8 possible categories (IGF, FGF, Notch,

PDGF, SHH, TGFβ, VEGF, Wnt) for potential secreted factors (Swistowska et al., 2010). Utilizing SDIA, hESCs were co-cultured with stromal cells, SHH and FGF8 to to differentiate them towards a neural fate (Perrier et al., 2004). Removal of SHH and FGF8 and replacement with brain derived neurotrophic factor (BDNF) and ascorbic acid (AA) induced 60-70% TH positive/Tuj positive cells (Perrier et al., 2004). The dopaminergic phenotype of these cells was further confirmed by VMAT2 and EN1 staining (Perrier et al., 2004). GDNF, used in co-culture with SDIA, doubled the number of TH positive cells seen with SDIA activity alone (Zeng et al., 2004). Another hESC line, SA002.5 was differentiated on PA6 cells resulting in up to 37% TH positive/Tuj1 positive neurons. These neurons were transplanted into the nigral-stratial pathway with negative consequences including proliferation following transplantation and terotoma formation (Brederlau et al., 2006). Differentiation of H9 hESCs on a SHH secreting M5S stromal feeder layer with bFGF lead to no teratoma formation when transplanted into the SN, but few TH+ cells survived (Ko et al., 2007). Attempts at differentiation with a bone marrow stromal cell feeder layer and FGF8/SHH lead to 40% TH+ cells but no cells survived the graft. In an effort to differentiate a line that would be post mitotic after injection, H9 and H1 cells were co-cultured with rat astrocytes; however, transplanted cells that survived were still undifferentiated mitotic cells (Roy et al., 2006).

Following induction using the SDIA method, focus on a method using only growth factors and no co-culture methods began and was reported in 2005 by Yan and colleagues. hESCs were differentiated to neural progenitors through an embyroid body (EB) stage. Dopaminergic induction began with 7 days of FGF8 culture followed by 7 days of FGF8 and SHH culture (Yan et al., 2005). Progression to biologically functional dopaminergic neurons required 14 days of culture with dopamine survival factors (GDNF, BDNF), dopamine inducing factor ascorbic acid (AA), neural specification factor cyclic AMP (cAMP) in addition to the FGF8 and SHH. The dopaminergic neurons expressed 31% TH positive neurons after 5 weeks of differentiation (Yan et al., 2005). Another report using all of the above factors plus a dopamine induction factor, TGFβ lead to 43% TH positive cells; however, transplantation lead to few surviving TH+ post mitotic cells and primarily neural precursors that continued to proliferate (Yang et al., 2008). The first report of of hESCs differentiated towards a dopaminergic phenotype being transplanted that resulted in significant improvements in rotational and forepaw stepping also resulted in the formation of tumors (Yang et al., 2008).

The field progressed to promoter systems that express genes known to be involved in dopaminergic development. Lmx1a is induced at E7.5 in mouse by Otx2 (Friling et al., 2009). Lmx1a helps to induce a midbrain dopaminergic neuron identity through controlling NURR1 and PITX3 expression (Chung et al., 2009). An Lmx1a promoter was used in hESCs to promote differentiation of 10 to 20% TH+ neurons (Friling et al., 2009). Efforts to improve the derivation of dopaminergic neurons have included formation of spherical neural masses (SNMs) instead of EBs prior to differentiation (Cho et al., 2008, Vazin et al., 2009). Elucidating the factors expressed in and secreted by stromal cells used to differentiate dopaminergic neurons included microarray studies. One study found that the cell membrane of stromal cells expressed FGF7, hepatocyte growth factor and vascular endothelial growth factor, which were sufficient to induce dopaminergic differentiation (Vazin et al., 2008). A microarray examining the mRNA expression of PA6 cells found IGF2 and several IGF binding proteins, FGF10, DLK1 NGF, SHH, TGF3β, VEGF and Wnt RNAs to be highly expressed in PA6 cells (Swistowska et al., 2010). Additionally, receptors for these genes were more highly expressed in NSCs compared to hESCs. A study using various

combinations of factors which activated these receptors or replicated the factors expressed by PA6 cells determined that the combination of factors termed SPIE (SDF-1, PTN, IGF2, and EFNB1) was most effective at differentiating hESCs towards dopaminergic neurons (Cho et al., 2008, Vazin et al., 2009). However, a highly efficient and effective method of dopaminergic differentiation has not been obtained.

In an attempt to improve on the 5 stage method and to remove the feeders from the co-culture system, En-Stem A cells were differentiated with PA6 conditioned media for 4 weeks resulting in 18% TH+ cells compared with 26%TH+ cells derived from H9 derived hNPs cultured in PA6 conditioned media (Swistowska et al., 2010). The time of exposure to PA6 conditioned media was important. Cells exposed to PA6 conditioned media at the neural stem cell stage produced more TH+ neurons than did cells exposed as hESCs or cells exposed later in neural differentiation. Differentiation with FGF-20, a novel neurotrophic factor found to be expressed in the SN of rat brains, on PA6 feeder cells lead to a 5-fold increase (3% to 15%) in TH+ cells and reduced overall cell death via the caspase 8 and BAX pathways (Correia et al., 2007). Foxa2 ventralizes neural progenitors in the developing brain and leads to cell cycle arrest of ventral midbrain cells to promote differentiation over proliferation. Additionally, Foxa2 acts in an auto regulatory loop with SHH to promote dopaminergic neurons and to inhibit GABAergic differentiation (Lin et al., 2009). In order to promote Foxa2+ progenitor cells that mark ventral mesencephalic dopaminergic neurons, a high activity form of SHH and the FGF8a isoform induced dopaminergic neurons (Cooper et al., 2010) Currently research remains ongoing working to improve upon the differentiation protocol used to derive dopaminergic neurons.

7. GDNF and its mechanism of action within the neuron

GDNF belongs to the transforming growth factor-β (TGF-β) superfamily. Within the superfamily is the GDNF family of ligands, which include neurturin (NRTN), artemin (ARTN), persephin (PSPN) and GDNF (Airaksinen and Saarma, 2002). Each of these ligands bind preferentially to GDNF-family receptor-α (GFR-α) co-receptors (GDNF to GFR-α1; NRTN to GFR-α2; ARTN to GFR-α3; PSPN to GFR-α4) prior to binding to receptor tyrosine kinase (RET) protein which is attached to the plasma membrane with a glycosyl phophatidylinositol (GPI) anchor (Airaksinen and Saarma, 2002). In order to activate downstream pathways, the RET-GFRα complex must become associated with a lipid raft, recruited by Src and FRS2 (Airaksinen and Saarma, 2002). This binding activates the PI3K and MAPK pathway involved in neuron survival and neurite outgrowth (Figure 2.4) (Airaksinen and Saarma, 2002). The GDNF interaction with GFR1α1-RET promotes dopamine neuron survival, axon growth and hypertrophy (Figure 2.4) (Airaksinen and Saarma, 2002).

7.1 Mitogen activated protein kinase pathway

The mitogen activated protein kinase (MAPK) pathway consists of a network of kinases that are involved in cell survival, differentiation, proliferation, apoptosis, growth and involved in GDNF signaling (Figure 2.4) (Pimienta and Pascual, 2007), . There are currently three well known MAPK pathways: the c-JUN N-terminal kinase (JNK)/stress activated protein kinase (SAPK), the extracellular signal-regulated kinase (ERK1/2 and ERK5), and the p38 MAPK pathway (Figure2.4) (Roux and Blenis, 2004). MAPKKK1-4 will activate MAPKK 4 and 7, which in turn activates JNK 1, 2 and 3. The JNK pathway is involved in retinoic acid

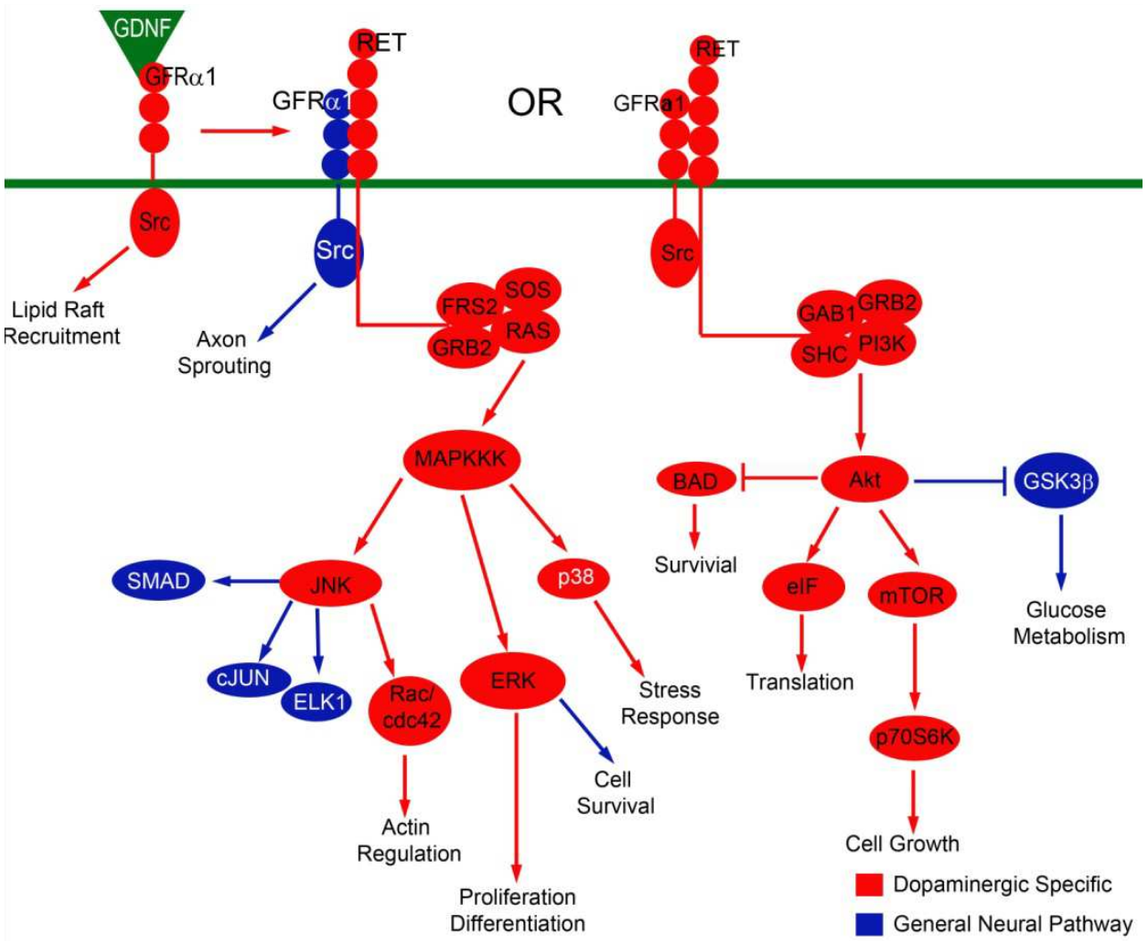

Fig. 2.4. Glial Cell-line Derived Neurotrophic Factor Supports Dopaminergic Differentiation through Activation of Several Pathways Glial cell-line derived neurotrophic factor (GDNF) binds to its co-receptor GFRα1 which then binds to the RET receptor to activate the MAPK, PI3K, JNK and Src pathways. Src recruits RET to the lipid raft for binding (Airaksinen and Saarma, 2002, Sariola and Saarma, 2003). MAPK activation of the JNK pathway regulates actin through RAC/CDC42 activation in its enhancement of the dopaminergic differentiation in this chapter. Other parts of the JNK pathway activated by GDNF not involved in dopaminergic differentiation include SMAD, cJUN and ELK1 (Airaksinen and Saarma, 2002, Sariola and Saarma, 2003). MAPK activation of the ERK pathway leads to enhancement of dopaminergic differentiation and cell survival in the cells in this chapter. The AKT pathway activated by GDNF in the dopaminergic neurons in this pathway leads to cell growth through the mTOR pathway, translation factor activation through the eIF pathway and cell survival through inhibition of BAD. Not involved in the dopaminergic differentiation of the cells in this chapter is activation of GSK3β (Airaksinen and Saarma, 2002, Sariola and Saarma, 2003).

neurogenesis in jnk knockout mESCs (Hayashi et al., 2000). The JNK pathway regulates cellular survival and neuronal migration (Figure2.4) (Garcia-Martinez et al., 2006). The ERK pathway divides into the ERK1/2 pathway and the ERK5 pathway (Nishimoto and Nishida, 2006). The ERK1/2 pathway is activated by MAPKK1/2, which is turned on by aRaf, bRaf or cRaf (Hayashi et al., 2000). The ERK pathway regulates cellular survival (Garcia-Martinez et al., 2006). ERK stimulates transcription factors such as Elk and c-myc and protein kinases such as ribosomal S6 kinase (RSK; Figure 2.4) (Roux and Blenis, 2004). ERK5 is involved in cell survival and proliferation through activation of MAPKKK 1-4 which triggers MAPKK5 (Nishimoto and Nishida, 2006). In vivo mouse models have demonstrated that ERK5 signaling is involved in both cardiovascular and neural development (Roux and Blenis, 2004). The final MAPK pathway, p38 MAPK pathway, is stimulated by MAPKKK1-4 activation of MAPKK3/6 (Figure 2.4) (Roux and Blenis, 2004). In embryonic development, there are two peaks of p38 activity (Roux and Blenis, 2004). The first acts as a switch between cardiovascular and neural development. The later peak modulates neurite formation and neural survival.

The mechanism through which GDNF acts to promote dopaminergic neural survival and differentiation is not entirely known, but it is thought that the MAPK pathway may play a role in promoting neural survival, differentiation or neurite outgrowth (Ohiwa et al., 1997, Nicole et al., 2001). Cultured embryonic rat cortical cells exposed to GDNF increased arborization and neurite outgrowth through activation of the p42/p44 MAPK pathway (Figure 2.4) (Ohiwa et al., 1997, Nicole et al., 2001). RET coupling with the Shc/Grb2 domains leads to downstream activation of the MAPK pathway (Figure 2.4) (Ohiwa et al., 1997, Nicole et al., 2001). Further research needs to be done to determine the involvement of the MAPK pathway in dopaminergic differentiation after RET activation.

7.2 Phosphoinositide 3-kinase pathway

The phosphoinositide 3-kinase (PI3K) pathway is activated by cytokines, growth factors and hormones and is involved in downstream regulation of cell survival, proliferation, apoptosis and regulation of transcription factors. PI3K exerts action on Akt, which acts in cellular functions such as survival, protein synthesis, proliferation, glucose metabolism, and neural signaling through its triggering of several other factors (Duronio, 2008). Akt inhibits pro-apoptotic signals Bad and the Forkhead family thus increasing cell survival (Figure2.4) (Manning and Cantley, 2007). Regulation of glucose metabolism occurs through glycogen synthase kinase 3 (GSK3) activation (Figure 2.4) (Manning and Cantley, 2007). Finally, Akt neural involvement occurs through regulation of the GABA receptor, ataxin-1 and huntingtin in addition to interaction with TGF-β signaling (Figure 2.4) (Manning and Cantly, 2007). Rubinsky and Meyuhas, 2006). mTOR is found in two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTOR1 integrates signals to encourage cell growth or catabolic processes depending on which condition is more favoured.

Acting along with Akt signaling are pathways involved in translation control (eIF4E and p70 S6K), cell growth and survival (mTOR) and cell cycle regulation [phosphatase and tensin homolog (PTEN) (Figure 2.4)] (Ruvinsky and Meyuhas, 2006). mTORC2 promotes cellular survival and cytoskeletal maintenance (Ruvinsky and Meyuhas, 2006). Mutations in this pathway or deregulation caused by stress leads to complications in protein translation and many wide ranging problems in cellular function (Carnero et al., 2008). The p70 S6K pathway controls phosphorylation of ribosomal protein S6 that is important for cell size and glucose homeostasis (Figure 2.4) (Ruvinsky and Meyuhas, 2006). mTORC2 promotes cellular

survival and cytoskeletal maintenance (Wullschleger et al., 2006). Mutations in mTOR signaling are involved in cancer, cardiovascular disease and metabolic disorders (Yap et al., 2008). PTEN is a tumor suppressor through its regulation of cell cycle, cell division and negative regulation of the PI3K/Akt pathway (Carnero et al., 2008).
In the mouse dopaminergic cell line, MN9D, the PI3K inhibitor LY294002 was administered prior to GDNF addition. In these studies, GDNF failed to protect the viability of the neurons exposed to 6-OHDA. In rat primary cultures, GDNF administration phosphorylates Akt (Ugarte et al., 2003). This phosphorylation was completely blocked by pre-incubating the cells with Wortmannin, a PI3K inhibitor (Figure 2.4) (Ugarte et al., 2003). When RET complexes with GAB1 and stimulates CREB, GDNF activates the PI3K pathway preferentially to the MAPK pathway (Figure 2.4) (Maeda et al., 2004).

7.3 Src

Src was the first discovered tyrosine kinase located in the cytoplasm. The family of Src tyrosine kinases (SFK) consists of Fyn, Lyn, Hck, c-Yes, Blk, Fgr, and Lck. SFKs play roles in cell growth, differentiation and survival, as well as cellular adhesion and synaptic transmission (Figure 2.4) (Encinas et al., 2001). When GDNF binds to its co-receptor GFRα1, the glycosyl phosphatidylinositol (GPI) that anchors the GFRα to the membrane recruits RET to the lipid raft and allows for activation of cellular signaling pathways that increase neural survival and differentiation (Figure 2.4) (Tansey et al., 2000). RET activation can occur in *cis* or *trans*. *Cis* activation occurs when a GPI anchored GFRα1 co-localizes on the same cell as the RET and allows for recruitment of a lipid raft in that cell (Tansey et al., 2000). When the GPI anchored GFRα1 is on an adjacent cells (such as a glial cell), the lipid raft is recruited in *trans* (Figure 2.4) (Encinas et al., 2001). *Trans* activation of RET is not sufficient to activated downstream pathways such as MAPK and PI3K. It is not known the reason for the availability of *trans* activation as it leads to decreased differentiation and decreases neural survival (Encinas et al., 2001). RET activation of Src has been shown to increase axon sprouting of dopamine (Akerud et al., 1999).

7.4 c-Jun N-terminal Kinase (JNK) pathway

The c-Jun N-terminal kinase (JNK) pathway is a subfamily of the MAPK pathway. This pathway plays a role in stress response in the cell and is activated by cytokines and environmental stresses (Figure 2.4) (Weston and Davis, 2007). MAPK phosphatases (MKP) negatively regulates the JNK pathway and these MKPs can be inhibited by reactive oxygen species, which causes increased activation of the JNK pathway and can lead to cellular death (Weston and Davis, 2007). There are 3 JNK genes (JNK1-3), but only JNK3 activates neuronal cell death (Sun et al., 2007, Weston and Davis, 2007). JNKs also include a group of scaffold proteins (JIP1-4) which interact with the mechanisms for vesicular transport, axon growth and axon repair after damage (Weston and Davis, 2007). Both Rac1 and Cdc42 activate the JNK pathway (Figure 2.4). Through this activation, they modulate cytoskeletal organization within the neuron as well as aid in neural migration (Sun et al., 2007).
GDNF also activates the JNK pathway. Through GDNF and its co-receptor GFRα1 activating RET, JNK has been shown to modulate neurite outgrowth and extension in dopaminergic neurons (Figure 2.4) (Chiariello et al., 1998). Additionally, this JNK activation causes a cell cycle delay at G2/M to allow actin reorganization to improve cell viability (Figure 2.4) (Fukuda et al., 2005).

8. Conclusion

The high prevalence of PD in the American population combined with the increasing percentage of aging population presents a need for improving upon the treatments currently available for the disease. Currently, the treatments available have not changed from the first largely available compound and the side effects obtained from this drug combined with the lack of long-term response suggest a need for a better treatment option. The models that have been used thus far have been animal models that do not offer a direct comparison to human physiology. hESC derived hNPs that are differentiated to dopaminergic neurons provide an optimal tool for studying the basic biology of dopaminergic neurons as well as for researching new drug options. The methods for deriving these neurons needs to be improved upon in order to provide better treatment options for PD.

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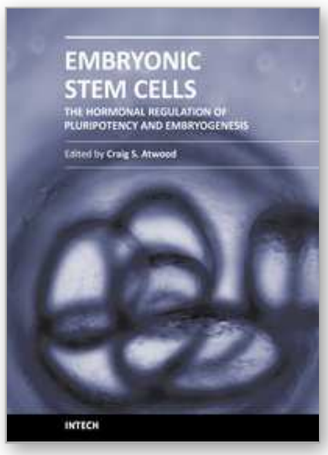

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