Characterization of the Dopaminergic Potential of the Human NTera2/D1 (NT2) Cell Line In Vitro

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Characterization of the Dopaminergic Potential of the Human NTera2/D1 (NT2) Cell Line In Vitro

By

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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DISCLOSURE

Results from some of the experiments required the use of color in the technique to analyze the data. If color cannot be seen in this version of the dissertation, the color copy can be found at the Shimberg Health Sciences Library, 12901 Bruce B. Downs Blvd., Tampa, Florida 33612-4479, (813) 974-2243.
DEDICATION

In memory of an extraordinary person, amazing scientist, and a wonderful mentor, Dr. Tanja Zigova.
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Characterization of the Dopaminergic Potential of the Human NTera2/D1 (NT2) Cell Line In Vitro

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ABSTRACT

Our laboratory is working with the human NTera2/D1 (NT2) cell line which have properties similar to progenitor cells in the CNS. These neural-like precursors cells can differentiate into all three major lineages - neurons, astrocytes, and oligodendrocytes. The pure neuronal population, called the hNT cells, possess characteristics of dopamine (DA) cells. In this dissertation, we performed various experiments to examine the neuronal and dopaminergic development of this cell line. We first cultured our hNT neurons with cells from the developing nigrostriatal (NS) pathway, the ventral mesencephalon and striatum, to determine their influence on survival, neuritic outgrowth, and DA phenotype. The survival of hNT neurons was substantially greater when they were cultured with embryonic day (E) 18 cells, compared to monocultures or cocultures with either E14 or postnatal day (P) 1 cells. The neuritic outgrowth of hNT neurons as assessed by the number of primary neurites per cell was increased when cultured with the areas of the brain from E14 and P1. The DA phenotype, as determined by the expression of the rate-limiting enzyme of DA synthesis was not increased in hNT neurons when they were cultured with primary rat cells from the NS pathway.

Next we analyzed if the retinoic acid (RA)-treated hNT neurons and the NT2 precursor cells expressed three transcription factors required for development of the DA phenotype. We report that NT2 cells endogenously expressed Engrailed-1, Ptx3, and Nurr1 while RA treatment increased Nurr1 but down-regulated Engrailed-1 and Ptx3. Finally, lithium has been shown to stimulate neurogenesis in adult hippocampal precursors as well as influence the Wnt pathway known to
be important for the induction of the DA phenotype. When we exposed the NT2 cells to lithium (1.0 mM for 4 day), there was an increase in NT2 cell survival and proliferation however the expression of essential transcription factors for the induction and maintenance of the DA phenotype were not increased in NT2 after lithium treatment.
CHAPTER 1

Introduction

Parkinson’s disease (PD) is a severe neurological disorder that affects more than 2% of the population 65 years and older. The neurodegenerative condition is caused by the loss of midbrain dopamine (DA) neurons in the substantia nigra pars compacta (SNc) which results in an extensive reduction of DA in the striatum (STR) (Hornykiewicz, 1966). This reduction in DA to the STR produces the major classical symptoms associated with PD which include resting tremor, bradykinesia, rigidity, and postural instability. Although traditional pharmacological therapies, deep brain stimulation, and stereotactic surgical procedures have reduced some of the symptoms, the illness still continues due to the progressive degeneration of the DA neurons.

Over the past two decades, scientists have explored the potential of cell replacement therapies to treat neurodegenerative disorders (Bjorklund and Lindvall, 2000a, b). The theory behind cell replacement therapy is based on the idea that lost neurological function due to injury, disease, or death can be improved by introducing new cells to replace the function of the degenerating neurons. The critical component of this line of research is to find the ideal cell source – one that is dopaminergic and available in large quantities. Human fetal tissue from the ventral mesencephalon (VM) has been shown to be at least partially successful for the treatment of PD, however ethical controversy associated with obtaining human fetal tissue as well as the limited availability of donors profoundly decreases the chances of fetal VM to be used as an effective therapy. While similar issues apply to stem cells, stem cells can differentiate into many cells types and the prospect of establishing stem cell lines makes them a potential source for cell replacement therapy. However before stem cell derived treatment can be put into practice, a better understanding of the developmental mechanisms underlying specification of the dopaminergic phenotype (Gage et
Development of the ventral mesencephalon and striatum in rat

The CNS is derived from the in folding of the ectoderm layer which creates the neural tube. From the rostral part of the neural tube, three major brain areas are formed, the prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain). The striatum (STR) develops in the forebrain, while the ventral mesencephalon (VM) develops in the midbrain. Neuronal precursors are committed to generate specific neurons according to strict neurogenetic timetables that deal with proper anatomical and functional maturation (Bayer et al., 1995). There is little variation between different animals of the same strain (Bayer et al., 1993). The development of both structure (VM and STR) occurs in parallel (Altman and Bayer, 1981; van der Kooy and Fishell, 1987; Voorn et al., 1988; Bayer et al., 1993). Clearly the developmental time period is different across each species, however significant events are conserved across species. Recently, a model system was created that related the timing of about 100 neural events during development across nine species (Bayer et al., 1993; Clancy et al., 2001). This time-line correlated the average day between the start and end dates of neurogenesis called the ‘peak’ time. For instance, the peak period of VM neurogenesis was at rat embryonic day (E) 14.6 and human E50.5, while the intended target for VM cells, the STR peaked at rat E15.8 and human E55.9.

Factors influencing survival and neuritic outgrowth during development

During early development (before E10 in rat) the important factors in cell specification are cell-intrinsic signals which are established between the nucleus and the cytoplasmic environment. These events set the stage for cells to be influenced by nearby cells. After E12, cell-cell interactions increase the survival, maturation and migration to the proper positions. These interactions may occur through paracrine signaling or direct contact between cells. The observation that
the development of cells is influenced by the activities of other cells was first demonstrated by embryologists Hans Spemann and Hilde Mangold (Spemann and Mangold, 1924). For instance, VM cells differentiation occurs as a function of signals released by neighboring cells in the midbrain floor plate (Ye et al., 1998). Direct contact with the VM cells target, the striatum (STR) increases their survival and maturation (Prochiantz et al., 1979; Daguet et al., 1980; Hemmendinger et al., 1981; Prochiantz et al., 1981; Dal Tosè et al., 1988; Manier et al., 1997). Finally, the VM precursor cells themselves express various neurotrophic and transcription factors which enhance their development (review (Perrone-Capano and di Porzio, 1996).

Neuritic outgrowth may also be enhanced by either cell adhesion molecules (CAMs) present on the surfaces of neurons and include neural cell adhesion molecule (NCAM), N-cadherin and L1 (Letourneau et al., 1988). NCAMs, for example, were shown to increase neurite outgrowth by interacting with fibroblast growth factor receptors which leads to receptor activation by autophosphorylation (review (Hall et al., 1996). Through the activation of various second messengers, phospholipase C-γ is activated and it can phosphorylate cytoskeletal proteins such as III β-tubulin which are necessary for neurite growth.

1.3 Development of dopaminergic neurons
The induction of VM precursor cells in the rat starts at least by E7 and the first post-mitotic tyrosine hydroxylase (TH)-positive cells are observed around E12.5 (Hokfelt et al., 1976; Voorn et al., 1988). By E14, two dopaminergic cells groups are present in the VM and their fibers start innervating the STR. In the next two days, there is an increase in the size of the cells in the VM as well as the number of projections innervating the STR. On E17 the dopaminergic fibers to the STR form huge bundles closely associated with the fascicles of the internal capsule. From E17 onward, the cells in the VM become arranged into a dorsal and a ventral group which eventually become the substantia nigra pars reticulata and substantia nigra pars compacta, respectively. The first varicosities on the
dopamine (DA) axons appear after birth and their number increase rapidly during the first and second postnatal (P) weeks, reaching near adult levels on P20.

1.3.1 Molecular development of midbrain DA neurons

The distribution of DA-containing neurons in the brain is well established and restricted to relatively few areas of the brain and more than 90% of the DA cell bodies are contained in the mesencephalon (midbrain) as shown in biochemical studies of rat brains (Hokfelt et al., 1976, 1977). The DA-synthesizing cells have been grouped into areas called the A8 (retroubral nucleus), A9 (substantia nigra), and A10 (ventral tegmental area) (Kizer et al., 1976; Gerfen et al., 1987a). These DA neurons project along three different pathways with some overlap existing between the cell groups (FIG. 1.1). The DA cells in the substantia nigra send their projections along the nigrostriatal pathway and innervate the STR. The ventral tegmental area neurons project along mesolimbic pathway into the nucleus accumbens, hippocampus, and amygdala. The ventral tegmental area DA neurons also project to the prefrontal and frontal cortex along the mesocortical pathway. The DA cells in the retroubral nucleus were shown to contribute to the DA innervation in the STR, nucleus accumbens, olfactory tubercles, and amygdala (review (Deutch et al., 1988).

DA belongs to a group of neurotransmitters called catecholamines which are a group of biogenic amines having sympathetic action such as norepinephrine and epinephrine. Their distinctive structural features are the single amine group, a nucleus of catechol (a benzene ring with two adjacent hydroxyl groups) and a side chain of ethylamine or one of its derivatives. The precursor for the synthesis of DA is the aromatic amino acid, tyrosine (FIG. 1.2). Two reactions transform tyrosine to DA, the first step is catalyzed by the rate-limiting enzyme TH which converts tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA). The second step is the carboxylation of DOPA to DA catalyzed by the enzyme aromatic L-amino acid decarboxylase (AADC) (Nagatsu et al., 1964).
FIGURE 1.1 Midbrain dopamine cell groups and their areas of innervation. The A8 - A10 cell groups contain the majority of the DA in the brain. An overlap exists between the various DA-synthesizing cell groups.

FIGURE 1.2. Dopamine synthesis. Dopamine (DA) is synthesized from tyrosine by two enzymes. The first enzyme, tyrosine hydroxylase (TH) synthesizes L-3,4-dihydroxyphenylalanine (L-DOPA) from tyrosine. The second enzyme, aromatic L-amino acid decarboxylase catalyzes L-DOPA into dopamine (DA).
The development of midbrain DA neurons involves an array of complex and unique events involving both transcription factors and diffusible signals from the developing CNS. These regulators control the initiation of differentiation and maintenance of the DA phenotype. These events that drive the neural precursors down the dopaminergic pathway occur at least by E9 in rat. The current insight on the development of midbrain DA neurons involves two signaling centers (also called organizers), the mid / hindbrain boundary (also known as the isthmus organizer) and the floor plate where a group of specialized cells lie along the CNS ventral midline (Hynes et al., 1995; Ye et al., 1998; Liu and Joyner, 2001). Two important known inducing molecules are secreted during this time, fibroblast growth factor 8 (FGF8) from the isthmus organizer and sonic hedgehog (SHH) from the floor plate. In addition, other proteins which are known to influence the development of VM cells are secreted by organizing centers along the neural tube at this time such as bone morphogenetic protein (BMP) and RA (Durston et al., 1989; Lee and Jessell, 1999).

1.3.2 Phases of DA development

1.3.2.1 Induction

During the first phase, called the induction phase (E9 – E11.5), numerous critical transcription factors start being expressed by VM cells. There is an overlap of three transcription factors, Oxt2, Pax2, and Pax5 which control the general development of the forebrain, midbrain and hindbrain (Acampora et al., 1995; Ang, 1996; Schwarz et al., 1997). More specific to the midbrain VM neurons, the earliest markers expressed at this time period involve the signaling molecule Wnt1 which controls the expression of Engrailed-1 (En1) and Engrailed-2 (En2) (McMahon and Bradley, 1990; Danielian and McMahon, 1996). Another homeobox gene implicated during this early specification of VM cells is Lmbx1b (Smidt et al., 1997). En1, En2 and Lmbx1b all continue to be expressed in the adult DA midbrain neurons (Wurst et al., 1994; Joyner et al., 2000; Burbach et al., 2003). From around E10 to E12.5, the VM cells that express these essential
transcription factors are proliferating, migrating and starting to differentiate into post-mitotic neurons.

1.3.2.2 Differentiation
At the start of the second phase, called differentiation (E11 – E13), two essential transcription factors are expressed by VM cells, Nurr1 (NR4A3) and Ptx3 (Pitx3). Nurr1 is an orphan steroid receptor shown to be critical for the development, differentiation, maintenance and survival of midbrain DA neurons (Zetterstrom et al., 1997; Castillo et al., 1998; Saucedo-Cardenas et al., 1998; Wallen et al., 1999). Ptx3 (also known as paired-like homeodomain transcription factor 3 or pituitary homeobox 3) is almost exclusively expressed in VM cells in the CNS (Smidt et al., 1997). During differentiation from E11-E13, various VM specific markers start being expressed including TH, aldehyde dehydrogenase (AHD-2), D2 receptor, and vesicular monoamine transporter 2 (VMAT2). AHD-2 is an enzyme found in VM cells that oxidizes retinaldehyde to RA, while D2 receptor is found in the presynaptic terminals of midbrain DA neurons and modulate DA neurotransmission to the STR (Sokoloff et al., 1980; McCaffery and Drager, 1994). VMAT2 belongs to the vesicular neurotransmitter transporter family and allows transport and storage of monoamines into dense core vesicles (Amara, 1992, 1996). Maturation of midbrain DA neurons occurs around the time DA fibers arrive in the STR. DA is detected in the rat STR by E15 – E16 (Fishell and van der Kooy, 1987, 1989, 1991) which occurs at around the same time dopamine transporter (DAT) is expressed (Perrone-Capano and di Porzio, 1996). DAT mediates the uptake of DA into neurons and is a major target for various pharmacologically active drugs and environmental toxins.

1.3.2.2.1 Essential transcription factors in DA differentiation
1.3.2.2.1.1 Nurr1
The nuclear orphan receptor Nurr1 (NR4A3) was shown to be essential for the development, differentiation, maintenance, and survival of midbrain neurons. In
Nurr1 null mice, midbrain DA neurons failed to mature (Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998; Le et al., 1999b; Wallen et al., 1999). Furthermore, Nurr1 was shown to regulate key genes involved in the DA phenotype including TH, VMAT2, DAT, and Ret which is a co-receptor with high affinity glycoprophosphatidylinositol (GPI)-anchored receptors, collectively called GDNF family receptors \( \alpha \) (Sakurada et al., 1999; Wallen et al., 1999; Iwawaki et al., 2000; Sacchetti et al., 2001; Airaksinen and Saarma, 2002). Nurr1 was also shown to regulate DA synthesis and storage in MN9D cells (Hermanson et al., 2003). The differentiation of embryonic cells into DA neurons is accompanied by the activation of the Nurr1 gene (Carvey et al., 2001; Haas and Wree, 2002). The transfection of Nurr1 into stem cells increases the differentiation, the maturation and the expression of TH (Wagner et al., 1999; Chung et al., 2002; Kim et al., 2002; Kim et al., 2003).

Nurr1 expression continues into adulthood suggesting its importance in the maintenance of the DA phenotype (Saucedo-Cardenas and Conneely, 1996; Zetterstrom et al., 1996). The DA neurons in mice lacking an allele for Nurr1 have been shown to have reduced levels of DA and increased vulnerability to neurotoxins (Le et al., 1999a; Eells et al., 2002). Mice over-expressing Nurr1 have decreased susceptibility to neurotoxins (Lee et al., 2002). Nurr1 mutations have been implicated in diseases such as PD, schizophrenia, and manic-depressive disorders as well as cocaine abusers (Buervenich et al., 2000; Chen et al., 2001; Bannon et al., 2002; Xu et al., 2002; Zheng et al., 2003).

1.3.2.2.2 Ptx3

The expression of the bicoid-related homeodomain-containing transcription factor Ptx3, which is also known as Ptx3, is highly restricted to VM neurons (Semina et al., 1997; Smidt et al., 1997). Ptx3 is known to be important in the proper development of midbrain DA neurons since Ptx3-deficient aphakia mice failed to develop DA neurons of the substantia nigra pars compacta. Furthermore, DA in the dorsal STR was severely reduced in these mice to 10% of that of the wild-
type mice (Burbach et al., 2003; Hwang et al., 2003; Nunes et al., 2003; van den Munckhof et al., 2003; Smidt et al., 2004b; Smidt et al., 2004a). Ptx3 was shown to increase expression of AHD2 and TH by binding to the promoter region (Cazorla et al., 2000; Lebel et al., 2001; Chung et al., 2004). Furthermore, Ptx3 expression was shown to be decreased in cocaine users (Bannon et al., 2004).

1.3.2.2.3 Engrailed
During early embryogenesis, the Engrailed (En) genes are involved in the regionalization of the neural tube. Each region has a specific group of cells located in the organizers with each organizer expressing specific differentiating factors for that region (Joyner, 1996; Lundell et al., 1996; Simon et al., 2001). By E7, two homologues, Engrailed-1 (En1) and Engrailed-2 (En2), are expressed in cells in the mid / hindbrain boundary which will eventually become VM cells as well as the cerebellar cells (CER) (Simon et al., 2001). Mice null for En1 and En2 have a reduction in the size of their midbrain and hindbrain due to a decrease in the number of proliferating En1-positive/En2-positive precursor cells (Wurst et al., 1994; Liu and Joyner, 2001; Simon et al., 2001). TH expression is induced in En1/En2 null mice but there is a reduction in the number of TH-positive neurons and by birth there are no TH-positive neurons found in the VM. This suggests TH induction can be initiated without En1/En2 expression, however the DA phenotype and cell survival cannot be maintained in these null mice (Simon et al., 2001; Alberi et al., 2004; Simon et al., 2004). Recently, it was shown that En is required to prevent apoptosis in midbrain DA neurons (Alberi et al., 2004).

1.3.2.3 Maturation
During the final maturation phase (E16 – P21), the intended target for midbrain DA neurons, the STR, starts to influence DA neuron survival, neuritic outgrowth and innervation. In vitro investigations demonstrated the survival of DA cells increased in the presence of the STR cells and extracts (Prochiantz et al., 1979; Daguet et al., 1980; Dal Toso et al., 1988). While some reports stated there was
no increase in the DA cell number (di Porzio et al., 1980a; Tomozawa and Appel, 1986), the maturation and the capacity of the cultured VM neurons to take up DA increased in the presence of STR cells compared to the VM monocultures (Prochiantz et al., 1979; Hemmendinger et al., 1981; Prochiantz et al., 1981; Manier et al., 1997).

1.3.2.3.1 Influence of striatal cells on ventral mesencephalon neurons

The STR is part of basal ganglia includes the STR, external segment of the globus pallidus, internal segment of the globus pallidus and subthalamic nucleus. Some of these areas are further subdivided such as the STR which consists of two parts called the caudate and putamen which are separated by the internal capsule whose myelinated fibers give the complex a striated appearance. The principal neurons of the STR, the medium spiny neurons (MSN), make up about 90% of the total STR neuronal population and are inhibitory, using \( \gamma \)-aminobutyric acid (GABA) as their primary neurotransmitter (Gabrion et al., 1995). The MSN have neurotransmitter inputs from the cortex and DA neurons of the substantia nigra and outputs to the globus pallidus.

The STR develops from the ganglionic eminences located on the floor of the telencephalic vesicle which bulges into the medial and lateral ventricular eminence (MVE and LVE) (Smets et al., 1979). The neuroepithelium of both ventricular eminences contains two distinct proliferative zones that contain neural precursors, the ventricular zone (VZ) and the subventricular zone (SVZ) where mitotic activity is occurring. After the cells exit the cell cycle in the VZ or SVZ, the neuroblasts migrate via radial glia laterally into the mantle zone where they undergo terminal differentiation. Cell-to-cell contact with cell adhesion molecules such as NCAM and/or extrinsic signals such as RA are required from the local environment for full maturation (McCaffery and Drager, 1994; Hamasaki et al., 2001; Noctor et al., 2001).
Neurogenesis in the STR of the rat begins at E12 and continues throughout embryonic life (Phelps et al., 1989; Bhide et al., 1996; Sheth and Bhide, 1997). The STR is segregated into the striosome and matrix compartments which are generated during two separate waves of neurogenesis. The medium spiny as wells as the aspiny interneurons originate in the LVE and cells born up to about E17 form the striosome, while cells born on E18 until the early postnatal period become part of the matrix compartment (Olsen et al., 1986; van der Kooy and Fishell, 1987). Cholinergic interneurons are born between E12 and E17 and are generated in the MGE with those born before E14 ending up in the striosome and those after E15 becoming part of the matrix (Brady et al., 1989; van Vulpen and van der Kooy, 1998).

The genesis of the STR striosome neurons coincides with the arrival of the first DA projections from the substantia nigra at E14. By E19 these DA fibers have clustered into islands that express dopamine- and cAMP-regulated phosphoprotein, of apparent $M_r 32000$ (DARPP-32) and substance P (Ouhibi et al., 1990). The striosome neurons undergo prolonged postnatal maturation which closely parallels the maturation and innervation of the midbrain DA neurons which suggests a preference for VM projections to cluster in the DARPP-32-positive islands (Gerfen et al., 1987a; Voorn et al., 1988; Tepper et al., 1998; Antonopoulos et al., 2002). The proportion of DA varicosities forming synapses increased gradually to reach a peak at the end of the first postnatal week and by the third week of life, the compartmentalization disappears and the DA innervation becomes uniform throughout the STR (Voorn et al., 1988).

1.4 Expression of neurotrophic factors during development

During the development of the CNS, precursor cells secrete proteins called neurotrophic factors (NFs) to promote their own survival, growth and differentiation and/or attract innervating cells (Reimann et al., 1986; Lindsay et al., 1993; Lindsay, 1994; Snaidr et al., 2002; Bai et al., 2003). Certain NFs are
produced by different areas of the brain at specific time periods, although their precise expression during development is not yet completely understood. There are two families of NFs currently known. The neurotrophin (NT) family includes brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) and NT-4/5. The NTs accomplish their functions by activating specific cell surface receptors, the tyrosine kinases (Trks). The Trk receptors have either distinct or overlapping specificities. For instance, TrkA is specific for NGF, TrkB is a receptor for BDNF and NT-4/5, while NT-3 binds to TrkC (Gaddy et al., 1979; Glass and Yancopoulos, 1993; Ip et al., 1993). All neurotrophins can also bind to the non-tyrosine kinase receptor low affinity p75NTR which is homologous to the tumor necrosis factor receptor.

The second group called the glial cell-lined derived neurotrophic factor (GDNF) family consists of GDNF, neurturin (NTN), persephin (PSP) and artemin (ART). The GDNF proteins signal through multiple receptor complexes such as GFRα receptors and receptor tyrosine kinase, ret (Knight et al., 1989; Lin et al., 1993; Jing et al., 1996; Treanor et al., 1996; Klein et al., 1997; Trupp et al., 1997; Baloh et al., 1998a; Baloh et al., 1998b; Milbrandt et al., 1998). Both GFRα and ret are expressed in the embryonic and adult CNS suggesting their importance during development in proliferation, survival, and differentiation (Pachnis et al., 1993; Avantaggiato et al., 1994; Schuchardt et al., 1994; Tsuzuki et al., 1995; Trupp et al., 1997; Bennett et al., 1998; Airaksinen et al., 1999; Schiltz et al., 1999). In addition, ret knockout mice showed similar abnormalities during development as observed in GDNF knockout mice (Schuchardt et al., 1995). Recently, Baldassare and colleagues demonstrated that the activation of ret by GDNF inhibited proliferation of NT2 cells (Baldassarre et al., 2002).

Since the discovery of the first NF in the CNS (Levi-Montalcini, 1964) researchers have been actively studying NFs and their expression in developing and adult brain. The NFs are expressed in specific areas of the brain at precise time periods during development suggesting their importance to the proper
survival, differentiation and/or neuritic maturation of these cells within these brain regions. For instance, NT-3 is highly expressed in most areas of the prenatal brain where proliferation, migration, and differentiation are occurring and then decreases substantially at birth (Maisonpierre et al., 1990a). There is also an overlap of expression of various NTs and it may be that the combination of NTs is important for the specification of that brain region. For example, VM neurons respond to BDNF but not NGF, while both BDNF and NGF influence primary cholinergic neurons in the forebrain (Hartikka and Hefti, 1988; Hyman et al., 1991; Stromberg et al., 1993; Nosrat et al., 1996) Both GDNF and NT-4/5 are highly expressed in the STR and CER up to E21. After birth, NT-4/5 expression decreased substantially in all areas of the brain. On the other hand, GDNF expression decreases in the CER but remains constant in the STR up to 3 weeks postnatally (Stromberg et al., 1993; Nosrat et al., 1996). Prenatal VM cells express low levels of GDNF but high levels of PSP (Jaspers and Cools, 1988; Choi-Lundberg et al., 1998; Schafer et al., 2002). Moreover, in P1 rat, both NTN and PSP are expressed in the STR and VM, while PSP is only expressed in the CER (Widenfalk et al., 1997; Cho et al., 2004a; Cho et al., 2004b).

Recently, a connection was found between the expression of neurturin (NTN) and GDNF during the development of the nigrostriatal system (Stromberg et al., 1993; Perez-Navarro et al., 2000; Koeberle and Ball, 2002). NTN mRNA peaked at P0 in the VM and progressively decreased thereafter while in the STR NTN started out low and did not peak until P15. The increase of NTN in the STR coincided with a decrease in GDNF mRNA expression. This switch in the expression of NTN, from the soma to the target area of DA neurons, correlated with the DA innervation of the STR (Lopez-Martin et al., 1999). In addition, NTN increased VM cell survival in vitro, while GDNF induced sprouting and hypertrophy and an increase in TH expression (Beck et al., 1995; Tomac et al., 1995).
1.5 NT2 cells and hNT neurons as a unique model for dopaminergic development

Previously, our laboratory showed that hNT cells possess immunocytochemically detectable markers typical of midbrain dopaminergic neurons, the VM cells, including TH, DAT, D2 receptors, and AHD-2 (Zigova et al., 1999; Zigova et al., 2000a). Functional evidence of their neurochemical phenotype was confirmed by DA release via KCl stimulation (Iacovitti et al., 2001). Since NT2 cells have properties similar to human neural progenitor cells and can be induced to differentiate into neurons that express the DA phenotype, we examined the potential of the NT2 cells to be used as a model for DA development. As we stated earlier, one of the main concerns in cell replacement therapy for the treatment of PD is associated with finding the ideal cell source. Human fetal VM has been shown to be at least partially successful for the treatment of PD, however, ethical controversies exist in obtaining ample and suitable fetal tissue (Lindvall and Hagell, 2001). The human hNT neurons are an excellent cell source since they are available in unlimited quantities and are a neuronal population that contain numerous DA properties. The safety and beneficial effects of these neurons were observed in rat models of ischemia, Huntington’s and PD (Borlongan et al., 1998; Hurlbert et al., 1999; Philips et al., 1999; Saporta et al., 1999; Willing et al., 1999; Baker et al., 2000; Zigova et al., 2000a). Furthermore, the hNT neurons are the only cell line approved by the Federal Drug Administration for neural transplants into humans and have been used as a potential treatment in patients with cerebral infarctions (Kondziolka et al., 2002).

1.5.1 Using hNT neurons a model to examine survival and neuritic outgrowth during development

The hNT neurons survival and neuritic outgrowth could be increased by cell contact and/or exposure to factors either released by primary cells such NFs or CAMs. As previously stated, hNT neurons have numerous properties typical of primary neurons such as expression of various neuronal markers, neurotransmitters and formation of synapse. In addition, hNT neurons express
numerous receptors which can be activated by NFs and CAMs that effects their survival and neuritic outgrowth. As previously stated, Baldassare and colleagues illustrated that NT2 and hNT cells express the ret subunit and therefore are responsive to factors in the GDNF family (Baldassarre et al., 2002). In addition, RA induces the expression of NT receptors (TrkA, TrkB, TrkC, and p75NTR) in hNT neurons and permits the hNT cells to become responsive to certain NTs (Cheung et al., 1997; Piontek et al., 1999; Satoh and Kuroda, 2000). Finally, RA treatment increased the expression of fibroblast growth factor (FGF) receptors in hNT neurons and thereby may stimulate neuritic outgrowth by the interaction between CAMs (Campbell et al., 1992). It has been suggested that neurite outgrowth can be stimulated by the activation of FGF receptors by CAMs (Doherty and Walsh, 1996; Hall et al., 1996; Viollet and Doherty, 1997). Therefore, by exposure to RA, the hNT neurons have some of the necessary receptors to interact with factors released from the developing CNS. We consequently created a coculture system model with hNT neurons and primary rat cells from timed-pregnant rat at E14, E18 and P1 with three specific areas from the rat brain, the STR, VM and CER.

1.5.2 Similarities between the NT2 precursors and CNS progenitor cells
The field of stem cell research would have not be as advanced as it is today if it were not for the investigations performed by cell and developmental biologist in the late 70’s and early 80’s on embryonic carcinomas (EC). EC cells lines possess strikingly similar properties to embryonic stem cells. The EC cells can self-replicate, express embryonic stem cell markers and differentiate into various cells types (Friedman and Moore, 1946; Dixon and Moore, 1953; Pierce et al., 1957; Damjanov et al., 1979). Human EC cell lines have long been used to study the mechanisms governing lineage commitment in the CNS. One of these cell lines, the NTera2/cl.D1 or NT2 cells, established by Andrews and colleagues were originally derived from a human testicular teratocarcinoma (Andrews, 1984). Teratocarcinomas are malignant tumors that contain a mixture of undifferentiated and differentiate cells from all three germ layers, the ectoderm,
endoderm, and mesoderm (Sperger et al., 2003). Xenografts of teratocarcinomas confirmed the ability of these cells to self-replicate and differentiate into various cell type lineages (Kleinsmith and Pierce, 1964; Stevens, 1967a, b; Martin and Evans, 1975).

The Tera2 cells, a clone derived from a teratocarcinoma, were capable of differentiating into various cell types including primitive gut-, smooth muscle- and neural-like cells (Andrews, 1984; Andrews et al., 1984; Miyazono et al., 1995; Miyazono et al., 1996). The Tera2 neural-like cells were re-established in culture and termed NTera2. A subclone of the NTera2 cell line, the NTera2/D1 (NT2) cells, resembled neural progenitors since they expressed neural specific markers, self-replicated and were capable of differentiating into all three neural lineages, neurons, astrocytes, and oligodendrocytes, in vitro and in vivo (Andrews, 1984; Andrews et al., 1984; Andrews et al., 1986; Lee and Andrews, 1986; Andrews, 1987; Fenderson et al., 1987; Rendt et al., 1989; Pleasure et al., 1992; Trojanowski et al., 1993; Miyazono et al., 1995; Langlois and Duval, 1997; Bani-Yaghoub et al., 1999; Sandhu et al., 2002).

Morphologically, NT2 precursors have flat, large (40 - 50 µm), irregular cell bodies with prominent single or multiple nuclei (FIG. 1.1A). The mitotically active NT2 cells express several markers (TABLE 1.1) typical for neural progenitors such as nestin, vimentin, cytokeratin, transforming growth factor α, and neurofilament, medium and light chain (Lee and Andrews, 1986; Dmitrovsky et al., 1990; Pleasure and Lee, 1993; Bani-Yaghoub et al., 1999). In addition, NT2 precursors express markers for primitive neuroectoderm including N-cadherin, neuronal cell-adhesion molecule (NCAM) as well as embryonic markers such as liver alkaline phosphate and stage-specific embryonic antigen-3 and -4 (Andrews, 1984).

When NT2 cells were transplanted into the caudateputamen of nude mice or exposed to various pharmacological agents such as hexamethylene
bisacetamide and osteogenic protein, the NT2 cells underwent morphological, genotypic and phenotypic changes (Andrews, 1984; Andrews et al., 1984; Miyazono et al., 1996). The effect of one agent in particular, retinoic acid (RA), on the genes, enzymes and receptor expression has been extensively studied both in vitro and in vivo and has been used as a model for neural development (Damjanov and Andrews, 1983; Pleasure et al., 1992; Piontek et al., 1999; Przyborski et al., 2000). Most recently, cDNA arrays have been used to detect many up- and down-regulated genes in the NT2 cells during differentiation. These studies have identified similar changes to those that occur during the differentiation of neural stem cells, such as the down-regulation of proliferative genes and the up-regulation for factors important for neuronal growth and differentiation (Cheung et al., 1997; Satoh and Kuroda, 2000; Bani-Yaghoub et al., 2001; Leypoldt et al., 2001; Freemantle et al., 2002; Przyborski et al., 2003).

For instance, the cDNA expression array analysis (588 genes) between untreated NT2 cells and their daughter cells after RA exposure resulted in elevations of 76 genes including those encoding transcription factors, intracellular signal-transducing proteins, cell death-regulatory proteins, and growth factor/cytokines/neurotransmitters and their receptors. Furthermore, 11 genes that were down-regulated after differentiation were genes of proteins involved in cellular proliferation (Satoh and Kuroda, 2000). Freemantle and colleague used microarrays to examine the gene expression after 8, 24 and 48 h of RA exposure (Freemantle et al., 2002). Within 8 h, a total of 27 genes were induced and 5 repressed, with that number increasing to about 57 genes induced and 37 repressed after 48 h of RA-treatment. The genes that were initially altered may be associated with interplay between growth suppression and differentiation. The genes that were induced after 48 hours were associated with developmental pathways such as Wnt, cell adhesion, cytoskeletal and matrix remodeling, and intracellular signaling cascades. The genes that down-regulated after 48 h of RA-treatment were associated with protein/RNA processing and turnover metabolism.
1.5.3 Retinoic acid and neural development

RA is synthesized by dehydrogenases which oxidize retinol to retinaldehyde to either all-trans or cis RA by dehydrogenases. RA acts within the cell nucleus to establish or change the pattern of gene activity in cells. RA is involved in processes as diverse as the specification of spatial patterning during morphogenesis of the CNS and limbs, cellular differentiation and control of cell proliferation (Durston et al., 1989; De Luca, 1991; Leid et al., 1992; Nagao et al., 1995; Maden, 1998a, b; Morriss-Kay and Ward, 1999). The two forms of RA, all-trans RA and cis-RA exert different effects depending on the cell type. The cis RA inhibits epithelial carcinogenesis and suppresses malignant transformation in vitro while all-trans RA influences embryonic patterning, axis formation, differentiation and proliferation (Strickland and Mahdavi, 1978; Andrews, 1984; Moasser et al., 1995; Saitoh and Katoh, 2001). The location of RA within the brain during development was analyzed using the expression of RA synthesizing enzymes as a marker for the presence of RA (aldehyde dehydrogenase and aldehyde oxidases) (review (McCaffery and Drager, 2000). The first enzyme, AHD2 (also known as ALDH1, RALDH1) is present in four regions during development – the dorsal retina (McCaffery et al., 1992), otic vesicle (unpublished data), the VM and STR (McCaffery and Drager, 1994). The second enzyme, RALDH2, accounts for the high RA levels in the embryonic trunk region and is involved in the development of the limbs, heart, kidneys, reproductive organs and spinal cord (Niederreither et al., 1999). Recently, a third enzyme RALDH3 was found to be expressed in the ventral retina (La Mantia et al., 1991).

The effects of the different isoforms of RA are thought to be mediated through two families of nuclear receptors, which are members of the steroid/thyroid hormone receptor superfamily (review (Mangelsdorf, 1994; Chambon, 1996). The retinoic acid receptors (RARs) and the retinoic X receptors (RXRs) each contain α, β,γ subtypes which can produce their pleiotrophic effects by homo- or heterodimerization with one another (Mangelsdorf et al., 1991; Kliewer et al., 1992; Nagpal et al., 1992; Kastner et al., 1997a). The cis-RA binds to both RARs
and RXRs while all-\textit{trans} RA binds to RARs (Heyman et al., 1992). All three RAR and RXR subtypes are endogenously expressed at various levels in untreated NT2 cells and their expression is altered by the length of RA treatment (Moasser et al., 1995; Cheung et al., 1996a; Cheung et al., 1996b; Cheung et al., 1997; Spinella et al., 1998; Cheung et al., 2000; Borghi et al., 2003). When Moasser and colleagues exposed NT2 cells to $10^{-5}$ M of RA treatment for 4 days, there was an increase in RAR\textsubscript{α}, RAR\textsubscript{β}, RAR\textsubscript{γ}, and no marked change in RXR\textsubscript{α} (Moasser et al., 1996). Cheung and colleagues illustrated that after 3 weeks of RA treatment ($10^{-6}$M), there was an up-regulation of RXR\textsubscript{α}, RAR\textsubscript{α}, no change in RAR\textsubscript{β}, and a down-regulation of RAR\textsubscript{γ}. Clearly, these investigations demonstrate that the length and duration of RA exposure alters the expression of RARs and RXRs in NT2 cells. The expression of the combination of the RA receptors produces different effects by activating different pathways. For example, over-expression of RXR\textsubscript{β}, but not RXR\textsubscript{α} or RXR\textsubscript{γ}, leads to growth suppression and induction of non-neuronal phenotypes. Co-transfection of RAR\textsubscript{γ} and RXR\textsubscript{β} results in growth suppression and the expression of neuronal markers (Spinella et al., 1998).

In addition, when RA was added to embryonal carcinoma (EC) cells, the type of differentiated cell induced depended on the concentration of RA applied. At low doses ($10^{-9}$ M) the EC developed into cardiac cells and at intermediate doses ($10^{-8}$ M) skeletal cells appeared, while high doses ($10^{-7}$ M to $10^{-5}$ M) of RA produced neurons and astroglia (Edwards and McBurney, 1983). During embryogenesis, the differentiation of particular cells is determined by the various concentrations of inducing factors (review (Gurdon et al., 1996; Neumann and Cohen, 1997). When using the NT2 cell line to study neural development, the common recommended dose is $10^{-5}$ M to $10^{-6}$ M of RA for at least one week (Andrews, 1984).
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<th>Marker</th>
<th>Cell Type</th>
<th>Significance</th>
<th>Reference</th>
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<tr>
<td>Stage-specific embryonic antigen-3 (SSEA-3)</td>
<td>embryonic carcinomas and stem cells</td>
<td>glycoprotein at specific stages in early embryonic development; plays diverse roles in the development of the embryo, including regulation of cell growth, recognition, and differentiation</td>
<td>(Andrews, 1984)</td>
</tr>
<tr>
<td>Stage-specific embryonic antigen-4 (SSEA-4)</td>
<td>embryonic carcinomas and stem cells</td>
<td>glycoprotein at specific stages in early embryonic development; plays diverse roles in the development of the embryo, including regulation of cell growth, recognition, and differentiation</td>
<td>(Andrews, 1984)</td>
</tr>
<tr>
<td>Liver alkaline phosphate</td>
<td>embryonic carcinomas and stem cells</td>
<td>cell-surface antigen expressed by human EC cells and undifferentiated pluripotent stem cell</td>
<td>(Andrews, 1984)</td>
</tr>
<tr>
<td>TRA-1-60</td>
<td>embryonic carcinomas and stem cells</td>
<td>epitope associated with keratin sulfate; useful serum marker in germ cell tumors</td>
<td>(Andrews, 1984)</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>ectoderm</td>
<td>cell-surface molecule that promotes cell-cell interaction; indicates primitive neuroectoderm formation</td>
<td>(Chuong and Edelman, 1984)</td>
</tr>
<tr>
<td>Neuronal cell-adhesion molecule (NCAM)</td>
<td>ectoderm</td>
<td>cell-surface molecule that promotes cell-cell interaction; indicates primitive neuroectoderm formation</td>
<td>(Chuong and Edelman, 1984)</td>
</tr>
<tr>
<td>Cytokeratin, nestin, vimentin</td>
<td>neural progenitor</td>
<td>intermediate filament structural proteins characteristic of primitive neuroectoderm formation</td>
<td>(Lee and Andrews, 1986; Pleasure and Lee, 1993; Bani-Yaghoub et al., 1999)</td>
</tr>
<tr>
<td>Transforming growth factor-α</td>
<td>neural progenitor</td>
<td>important protein that binds to epidermal growth factor receptors and effects proliferation</td>
<td>(Dmitrovsky et al., 1990)</td>
</tr>
<tr>
<td>Microtubule-associated protein-1b (MAP-1β)</td>
<td>neural progenitor</td>
<td>early cytoskeletal protein present in committed neuroepithelial cells of neuronal lineage</td>
<td>(Pleasure and Lee, 1993)</td>
</tr>
<tr>
<td>Neurofilament (NF) - medium and light</td>
<td>neural progenitor</td>
<td>important structural protein expressed in primitive neural tissue</td>
<td>(Pleasure and Lee, 1993)</td>
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**TABLE 1.1** Embryonic and neural markers present in NT2 cells.
FIGURE 1.3 Photomicrographs showing the morphology of NT2 cells before and after retinoic acid treatment. To evaluate the morphology of NT2 cells and the different grades of hNT neurons, the slides were exposed to Blue counterstain. (A) NT2 cells were flat, irregular shaped with some cells seen at different stages of mitosis (arrow). (B-D) The different types of hNT neurons are generated according to the number of weeks of RA exposure (10 µm, supplement 3 times a week). Morphologically, the various types of hNT neurons were indistinguishable. The hNT cells formed small clumps (B) with extensive neuritic networks (C, arrows) between the individual neurons and clumps. The hNT neurons show typical neuronal morphology with either bi or multipolar processes (D, asterisks).
Przyborski and colleagues investigated the effects of RA on the neuronal development in NT2 cells and suggested that neuronal differentiation occurs in three phases (Przyborski et al., 2000). During Phase I, there is a peak in nestin expression, a marker expressed by proliferating neural cells. Immediately after induction with RA there is a sharp decline in Nestin expression. The second phase is characterized by an increase in basic/helix-loop-helix protein (bHLH), known to control cell proliferation and determine cell lineage, and neuroD1, an important regulator of neurogenesis. During this time period the neural precursors exit the cell cycle and start expressing A2B5, a marker expressed by differentiated neurons (Fenderson et al., 1987; Moasser et al., 1996; Spinella et al., 1999; Przyborski et al., 2000). Spinella and colleagues also reported a cell cycle arrest at the G1 phase in NT2 cells after treatment with RA (Spinella et al., 1999). During the transition from the second phase into the third, when the cell becomes post-mitotic, they start expressing neuronal properties such as neuronal tubulin proteins including III-tubulin (TuJ1) and microtubule associated protein (MAP) which will allow for outgrowth of axon and dendrites as well as synaptophysin proteins (Sandhu et al., 2002). During the final differentiation phase, the cells start expressing transcription factors and neurotransmitters that will eventually be necessary for their final phenotype. In the case of the VM precursor cells, as they start to develop and mature, they express Engraileds, Ptx3 and Nurr1, as well as TH, AHD2, DAT, D2 receptors, and VMAT2 (review (Simon et al., 2003; Thuret et al., 2004). This dissertation examined if the DA phenotype can be induced through RA treatment by studying the expression of these transcription factors during RA treatment.

1.5.4 hNT neurons

When NT2 cells are exposed to 10^{-6} of RA for several weeks they differentiate into post-mitotic, neuron-like cells called hNT or NT2-N cells. The hNT neurons used in this experiment will be exposed to either 3, 4 or 5 weeks of all-trans RA-treatment (3w, 4w, 5w). Morphologically, hNT neurons resemble primary neurons with elongated perikarya with bi-or multipolar processes (FIG. 1.3D,
asterisk) that form extensive neuritic branches interconnecting with other clusters of neurons (FIG. 1.3C, arrows). The soma of these developing neuron-like cells was approximately four times smaller in comparison to their NT2 precursors.

In addition to their neuronal morphology, the hNT neurons express numerous neuronal markers such as dendritic-specific microtubule-associated protein-2β (MAP-2β) or axonal marker Tau, III β-tubulin (TuJ1), growth associated protein (GAP-43), and neurofilament - heavy (Lee and Andrews, 1986; Goslin and Banker, 1990; Pleasure et al., 1992; Pleasure and Lee, 1993; Bani-Yaghoub et al., 1999; Guillemaud et al., 2000a; Guillemaud et al., 2000b; Zigova et al., 2000a). The hNT neurons also have two distinctive characteristics found in neurons - their ability to synthesize neurotransmitters and form synapses (TABLE 1.2). Neurotransmitters expressed in these cells included cholinergic, catecholaminergic, GABAergic, and serotonergic phenotypes, along with appropriate synthetic enzymes, such as acetylcholine, glutamic acid decarboxylase (GAD) and TH (Zeller and Strauss, 1995; Iacovitti and Stull, 1997; Zigova et al., 1999; Guillemaud et al., 2000a; Guillemaud et al., 2000b; Zigova et al., 2000a). Moreover, hNT neurons formed both glutamatergic and GABAergic functional synapses when plated on primary astrocytes (Hartley et al., 1999). Further indications of functional synapses include the expression of synaptic proteins such as synaptophysin and synapsin I, II, III (Pleasure et al., 1992; Leypoldt et al., 2002). Synapsins and synaptophysins are proteins found in the presynaptic areas that regulate the release of neurotransmitters at the synapse (Lu et al., 1992).
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<th>Significance</th>
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<tr>
<td>Neurofilament-H</td>
<td>protein expressed in terminally differentiated neurons</td>
<td>(Lee and Andrews, 1986; Pleasure et al., 1992)</td>
</tr>
<tr>
<td>Growth associated protein (GAP-43)</td>
<td>protein expressed in developing or regenerating neurons</td>
<td>(Goslin and Banker, 1990)</td>
</tr>
<tr>
<td>III β-tubulin</td>
<td>tubulin protein found in immature and mature neurons</td>
<td>(Guillemain et al., 2000a; Zigova et al., 2000a)</td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>neuronal protein located in synapses</td>
<td>(Pleasure et al., 1992)</td>
</tr>
<tr>
<td>Synapsin I, II, III</td>
<td>present in mature synapses; regulator in providing fusion-competent synaptic vessels at the active zone during increased rate of vesicle transport</td>
<td>(Hartley et al., 1999; Leypoldt et al., 2001)</td>
</tr>
<tr>
<td>Tau</td>
<td>type of MAP; helps maintain structure of axon</td>
<td>(Pleasure et al., 1992)</td>
</tr>
<tr>
<td>Microtubule-associated protein-2β</td>
<td>MAP protein found in dendritic branches of mature neurons</td>
<td>(Bani-Yaghoub et al., 1999; Guillemain et al., 2000a)</td>
</tr>
<tr>
<td>NF-200</td>
<td>important protein expressed in cytoskeleton supporting the axon cytoplasm of the central and peripheral nervous system</td>
<td>(Bani-Yaghoub et al., 1999)</td>
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**TABLE 1.2 Neuronal markers identified in hNT neurons.**
CHAPTER 2

Development of the Dopaminergic Phenotype in hNT Neurons Cultured with Primary Rat Cells

2.1 Introduction

As the average life span of the general population increases, so does the prevalence of neurodegenerative disease since these disorders are predominately age-related illness. Scientists are exploring many different avenues such as protecting remaining neurons and/or replacing dying neurons. Such is the case in Parkinson’s disease (PD) which is caused by the degeneration of dopaminergic (DA) neurons in the substantia nigra (Hornykiewicz, 1966). Key issues in developing cell therapies to treat PD are enhancing the survival, neuritic branching, and DA phenotype of either endogenous degenerating neurons and/or transplanted cells. Prior evidence has shown that during the development of the CNS, neuronal survival, growth, differentiation, innervation and maintenance of the DA phenotype are influenced by both the release of supporting/trophic factors and/or cell-cell interactions (Bjorklund et al., 1983; Davies, 1994).

It has been suggested that the ventral mesencephalon (VM – early DA neurons) as well as the intended target for VM cells, the striatum (STR), might provide trophic support to DA cells (Prochiantz et al., 1979; Daguet et al., 1980; Dal Toso et al., 1980; di Porzio et al., 1980b; Prochiantz et al., 1981; Denis-Donini et al., 1983, 1984; Tomozawa and Appel, 1986; Niijima et al., 1990; Engele et al., 1991; Ostergaard et al., 1996; Zhou et al., 2000). Early investigations illustrated that the survival, morphology, neuritic maturity and maintenance of DA neurons was enhanced when they were cocultured with developing VM cells and/or their intended target, the STR. In rat, the nigrostriatal pathway begins to develop from
embryonic day (E) 14 and continues until at least three weeks after birth. VM neurons start differentiating around E11-13 with DA synthesis present by E14 and detected in the STR by E16. Neurogenesis in the STR begins at E12 and occurs in two waves with cells born from E12-E17 becoming part of the striosome while cells born after E18 become part of the matrix compartment. The DA projections from the VM start arriving in the STR by E14 into the striosome compartment. VM synaptic innervation and maturation peak in the STR during the first week postnatally (Gerfen et al., 1987b; Gerfen et al., 1987a; van der Kooy and Fishell, 1987; Voorn et al., 1988; Fishell and van der Kooy, 1991; Sheth and Bhide, 1997; Tepper et al., 1998; Antonopoulos et al., 2002). Therefore, we investigated the influence of E14, E18 and postnatal day (P)1 VM and STR on the survival, morphology, neuritic branching, and expression of the dopaminergic phenotype of a human cell line in a coculture system. We used the cerebellum (CER) as the non-target cell control in the study. During development, the CER forms a distinct set of folds that are conserved across species. The CER is made of four primary cell types, the granule cell layer, Purkinje cells, Bergmann glia and the deep cerebellar nuclei (Eccles, 1970). The Purkinje cells start to migrate from the neuroepithelial layer of the fourth ventricle from E15 to E19 (Yuasa et al., 1991). All the cell types, except the granule layer cells differentiate directly from the proliferating ventricular layer of the dorsal rhombomere 1 structure. The granule cells come from the dorsal rhombomere 1 structure called the rhombic lip and continue proliferating until P14. The postmitotic granule cells migrate into the CER, past the Purkinje cells to form the inner granule layer.

In this study, we used the NT2-derived hNT neurons exposed to 3 weeks (3w), 4 weeks (4w) or 5 weeks (5w) of RA treatment to investigate its effect on the survival of hNT neurons and number of primary neurites on each cell. Second, we created a coculture system in which hNT neurons were plated with E14, E18 or P1 rat cells from the VM, STR, and CER in order to examine whether these brain regions at these developmental ages enhanced survival and/or neuritic
maturation. We showed that exposing hNT neurons to RA for longer periods of time reduced their survival and TH expression while no effect was observed on the number of primary neurites. Cocultures with embryonic cells but not postnatal cells increased the hNT cell survival up to 2 fold. Also, we demonstrated that the number of primary neurites increased in all hNT neurons regardless of their RA exposure when cultured with any pre- and postnatal neural rat cells. The TH expression in hNT neurons did not increase when they were cultured with embryonic cells from the nigrostriatal pathway. Therefore, human hNT cells can respond to different factors from the developing rat brain that can enhance their survival and neuritic maturity, however, the dissociated rat cells do not influence the dopaminergic phenotype in hNT neurons. Identifying the factors which promote the survival and neuritic maturation of human cells would have implications for the treatment of PD and other neurodegenerative disorders.

2.2 Methods

2.2.1 Dissection of embryonic and postnatal tissue

Sprague-Dawley timed-pregnant rats (Harlan, Indianapolis, IN) were anesthetized with Equithesin (3 ml/kg, ip). For the postnatal tissue, the day of birth was defined as P0, and the pups were hypothermically anesthetized (5 min on ice). In both pre- and post-natal pups, the brain was removed from the skull and all attached membranes were removed. The dissection of the STR, VM and CER tissue were performed under a dissecting microscope in a sterile environment as previously described by Dunnett and colleagues (Dunnett and Bjorklund, 1992). The dissected tissue was placed in chilled HBSS (Invitrogen, Carlsbad, CA) and 15 mM HEPES (Invitrogen), centrifuged (160 g/ 3 min) and digested for 25 min at 37°C in 0.25% trypsin (Sigma, St. Louis, MO) with EDTA (Mediatech, Inc., Herndon, VA) and 0.05% DNase (Sigma). Once digestion was complete, the tissue was mechanically dissociated with a 1 mL automatic pipetter in HBSS/HEPES solution with 0.05% DNase and incubated in a 37°C water bath for five minutes. The tissue was rinsed five times and centrifuged (160 g/ 3 min) between each rinse.
### 2.2.2 hNT neurons

The hNT neurons (also known as NT2-N cells) were obtained from Layton BioScience Inc. (Sunnyvale, CA). In order to generate hNT neurons, the NT2 precursors were plated in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) with 10% fetal bovine serum (FBS; Invitrogen), and Gentamicin (50 µg/mL, Sigma). The NT2 cells were induced to differentiate by exposure to 10 µM RA which was replenished three times a week. The different types of hNT neurons were generated by varying the length of RA treatment for 3, 4 or 5 weeks (3w, 4w, 5w). After RA induction, the hNT neurons were re-plated with mitotic inhibitors for 2 days and cryopreserved and stored at –180ºC prior to use.

### 2.2.3 Cell Culture

The 3w, 4w and 5w RA-treated hNT neurons and the primary rat tissue were resuspended in 1 mL of DMEM, 10% FBS, and Gentamicin. The cell number was assessed with the trypan blue exclusion method. The primary cells were plated on poly-L-lysine coated 24 well plates (Nunc, Naperville, IL) at a seeding density of 50,000 cells/cm² with the hNT neurons plated a day later at 50,000 cells/cm² for a total of 100,000 cells/cm² at a 1:1 ratio in the coculture system. A day after plating, the media was changed to Neurobasal (Invitrogen) with 0.01% of B27 (Invitrogen) supplement and Gentamicin. The cells were maintained at 37ºC in 5% CO₂ with 95% humidity up to 15 DIV.

### 2.2.4 Immunocytochemistry

The cultures were first washed in 0.1M phosphate-buffered saline (PBS; pH 7.4), then fixed with cold 4% paraformaldehyde for 15 minutes and washed three times with cold PBS. For immunofluorescence, slides were carefully washed with fresh cold 0.1M PBS and treated for 1 h at room temperature in a mixture of 10% normal goat serum, 0.03% Triton X-100 in PBS. Cultures were then incubated in
primary antibodies overnight at 4°C. To detect humans cells, immunostaining was performed using human nuclei marker (HuN, Chemicon, San Francisco, CA; 1:100, mouse). To identify the neuritic branches, we used monoclonal or polyclonal III β-tubulin (TuJ1, Covance, Princeton, NJ; 1:500, mouse; 1:1,000, rabbit). The dopaminergic cells were visualized by labeling with either monoclonal or polyclonal TH (Incstar, Stillwater, MN; 1:1,000, mouse; Pel-Freez, Rogers, AZ; 1:200, rabbit) antibodies.

After several PBS washes, primary antibodies were detected using appropriate secondary antibodies (Molecular Probes, Eugene, OR; Alexa Fluor 594 goat anti-rabbit, 1: 2,000; Alexa Fluor 546 goat anti-mouse 1:1,000, Alexa Fluor 488 goat anti-mouse, 1:800). The slides were washed with cold PBS and coverslipped with 95% glycerol. To visualize cell nuclei, some cultures were coverslipped using Vectashield with DAPI counterstain (Vector Laboratories, Burlingame, CA). The sections were observed under epifluorescence and photographed on the Olympus BX60 microscope.

2.2.5 Quantitative Analysis

In each condition, the cells were screened in a blind coded manner using a 20x objective and a photographic frame (field = 0.3 mm²). Counts were performed from 30 fields per condition (5 pictures per well times 6 wells per condition) from three independent platings. To quantify the number of surviving neurons, we counted the number of double-labeled HuN-positive and TuJ1-positive cells. We also used HuN / TuJ1 double-labeling to determine the number of primary neurites (neurites originating from the cell body). The number of human DA cells was evaluated using double staining with HuN and TH antibodies. The counts for each experiment were reported as mean ± SEM. Data was analyzed using analysis of variance (ANOVA). The Newman-Keuls test was employed for the post hoc comparisons.
2.3 Results

2.3.1 Morphology of hNT neurons in culture

2.3.1.1 Monoculture
As previously illustrated (Misiuta et al., 2003a), there was no difference in the morphology of the various hNT neurons with different lengths of RA exposure (3w, 4w, or 5w, FIG. 1.1). At first, hNT neurons spread out evenly throughout the well either as single cells or in clumps and started extending processes (FIG. 2.1A, asterisk). As the culture matured, the clumps started to send out thick neuritic fascicles (FIG. 2.1B, arrow) and by 7 days in vitro (DIV) extensive neuronal networks were observed as interconnected clumps (FIG. 2.1C, asterisks) and multipolar neurons (FIG. 2.4C, arrows). After 10 DIV, however, the conditions in the culture diminished rapidly with remaining cells possessing a few, short processes. By 15 DIV, most cells appeared small and round without any processes (FIG. 2.1D). These results were seen in all hNT populations regardless of their RA treatment.

2.3.1.2 Coculture
When hNT neurons were cultured with pre- and postnatal rat cells, hNT cells were highly polarized and showed extensive neuritic outgrowth. The hNT neurons cultured with E14 STR cells formed clumps with each other and with the rat cells (FIG. 2.2A, arrow) while some hNT neurons remained as multipolar single cells (FIG. 2.2A, asterisk). Similar results were observed with the E14 VM cells while in the E14 CER cultures, the hNT cells mostly remained as single multipolar cells with long neurites (FIG. 2.2B, arrowhead). The morphology of hNT neurons in the E18 cocultures was similar in all three different tissue cultures. The hNT neurons were usually observed as multipolar cells with extensive neuritic networks (FIG. 2.2C, D). Similar results were observed in all three hNT populations in all prenatal cocultures.
FIGURE 2.1. Photomicrograph of 4w hNT neurons in monoculture with increasing days *in vitro*. Initially, hNT neurons spread out evenly throughout the well either as single cells or in clumps (A, asterisks). Within a couple of days, the cells and clumps started to extend thick neuritic fascicles (B, arrow) and after a week these large clumps (C, asterisk) formed interconnected networks (C, arrows). However, after two weeks in culture, conditions deteriorated rapidly with the hNT neurons appearing small and round with few, if any, processes.
Unlike the cocultures with E14 and E18 rat cells which had extensive human and rat neuronal networks, most of the cells that were TuJ1-positive in the P1 cocultures were the human hNT neurons as shown with the double-labeling with HuN and TuJ1. Although there was no difference in the average cell number between the monocultures and P1 cocultures, nearly all the hNT neurons when plated with P1 rat cells exhibited advanced morphological characteristics. For instance, we observed long, branched hNT neurons with varicosities (FIG. 2.2E and insert, arrows). Regardless of the region in which the cells originated, the hNT neurons were either found as single cells or in large clumps (FIG. 2.2F arrow).

### 2.3.2 Survival with different lengths of RA treatment

The overall ANOVA revealed significant differences in the survival of the 3w, 4w, and 5w hNT neurons (FIG. 2.6A, $F_{2,92} = 41.0$, $p<0.0001$) as well as between the culture conditions at each time period ($F_{2, 92} = 101.7$, $p<0.0001$). There was also an interaction between the different hNT neurons and the increased days in vitro conditions and the various hNT neurons ($F_{4,92} = 11.3$, $p<0.0001$).

At 5 DIV, the 3w hNT cells had more living cells compared to the 4w ($w=8.9$, $p<0.05$) and 5w neurons ($w=35.9$, $p<0.01$). In addition, the 4w cells survived better than the 5w neurons ($w=8.2$, $p<0.01$). Similar results were observed at 10 DIV in which 3w hNT neurons survival was greater than the 4w ($w=83.9$, $p<0.01$) and 5w ($w=49.2$, $p<0.01$) hNT cells, however, there was no difference between the 4w and 5w ($w=4.4$, $p>0.05$). The 3w hNT cells maintained their survival from 5 to 10 DIV ($w=2.6$, $p>0.05$) although after two weeks in vitro, there was a significant decrease in cell survival from 10 and 15 DIV ($w=79.1$, $p<0.01$). Both the 4w and the 5w hNT cell survival were significantly lower at 10 DIV compared to 5 DIV ($4w$: $w=57.1$, $p<0.01$; $5w$: $w=7.5$, $p<0.05$), yet no further decrease was observed between 10 and 15 DIV ($4w$: $w=2.0$; $5w$: $w=6.0$, $p>0.05$). At 15 DIV, there was no difference in the survival between any of the hNT neurons, 3w and 4w ($w=2.8$, $p>0.05$), 3w and 5w ($w=0.4$, $p>0.05$), or 4w and 5w ($w=1.6$, $p>0.05$).
FIGURE 2.2. Cocultures of 5w hNT neurons with rat VM, STR and CER at various ages. All cells were visualized with DAPI (blue) staining, while neurons were recognized with TuJ1 (green) labeling and the hNT cells were identified with the human nuclei marker (HuN; red). When hNT neurons (red) were cultured with E14 STR cells, they were found either in clumps (A, arrow) or as multipolar single cells (A, asterisk), while hNT neurons plated with CER cells tended to remain as highly polarized individual cells with elongated neurites (B, arrowhead). The 5w hNT cells plated with E18 VM and CER cells remained predominantly as multipolar single cells with extensive neuronal networks (C, D). Since most of the neuronal cells in P1 cultures were the hNT neurons, we could follow long, branched neurites containing varicosities (E, insert, arrows). In some P1 cocultures, large clumps of hNT neurons were observed with protruding neurites (F, arrow).
2.3.3 Survival of hNT neurons cocultured with primary rat cells
Initially, to confirm that the correct cell types were dissected from the corresponding areas, monocultures of the primary cells were identified using classic phenotypic markers specific for that area. For example, the VM cultures contained TH-positive neurons with usual morphology of long neurites, while the CER cultures were positive for glutamic acid decarboxylase (GAD) which is an enzyme typically found in the CER. The signaling molecule, dopamine- and cAMP-regulated phosphoprotein (DARPP-32) which is almost exclusively found in striatal medium spiny neurons, was also found in our STR cultures (FIG. 2.3).

2.3.3.1 Influence of developmental stage
The overall ANOVA revealed significant differences in the survival of hNT neurons in mono- and coculture conditions (FIG. 2.4B, F_{3,316}=40.6, p<0.0001). The survival of hNT neurons in culture with E14, E18 and P1 cells was significantly higher than in the monoculture (w=17.6, p<0.01, w=80.3, p<0.01, w=6.2, p<0.05, respectively). Furthermore, the conditions in the E18 coculture increased the survival of hNT neurons compared to those in the E14 cultures (w=44.4, p<0.01) and P1 cultures (w=85.8, p<0.01).

Apart from their exposure to RA, the average number cells per field in the 3 - 5w RA treated hNT neurons in monoculture was 27.1±2.0, and the combined hNT neurons in the E14 cultures was 47.7±2.3 (w=25.8, p<0.01), while the E18 and P1 cocultures the average cell number per field was 71.2±2.8 (w=80.3, p<0.01) and 39.2±2.6 9 (w=6.2, p<0.05), respectively.

2.3.3.2 E14 coculture
The overall ANOVA on the survival of hNT neurons in E14 cocultures revealed significant differences in the survival between the various hNT neurons (FIG. 2.4C, F_{2,126}=23.6, p<0.0001) as well as within the STR, VM, CER cocultures (FIG. 2.4C). (F_{3,126}=14.0, p<0.0001). Furthermore, there were interactions
FIGURE 2.3. Phenotypic markers of primary rat cells. Light (A) and fluorescent (B) microphotograph of VM cells immunostained for TH (red, asterisks). The CER cultures contained GAD-positive cells (C, red) while DARPP-32-positive (D, green) were found in the STR cultures.
between each E14 tissue and the different types of hNT neurons \( (F_{6,126}=6.4, \ p<0.0001) \).

The 3w hNT cells survived better when they were cultured with either STR \( (w=33.9, \ p<0.01) \) or VM \( (w=25.8, \ p<0.01) \) cells compared to the monoculture. In addition, the 3w-STR cocultures also had more living cells compared to either the 4w-STR cells \( (w=28.1, \ p<0.01) \) or 5w-STR \( (w=27.3, \ p<0.01) \) cultures. The 3w-VM cocultures had statistically more hNT cells compared to the 4w-VM \( (w=40.8, \ p<0.01) \) or the 5w-VM \( (w=15.2, \ p<0.01) \) cultures. The 4w hNT cells survived better when cultured with CER cells \( (w=11.2, \ p<0.05) \), while more living cells were found when 5w cells were plated with either VM \( (w=12.1, \ p<0.05) \) or CER \( (w=14.0, \ p<0.01) \) cells compared to the monocultures. Finally, no difference in the survival between the hNT neurons was observed when they were cultured with CER cells \( (3w-CER \ & \ 4w-CER: \ w=0.69; \ 3w-CER \ & \ 5w-CER: \ w=0.001; \ 4w-CER \ & \ 5w-CER: \ w=0.74, \ p>0.05) \).

When the average number of cells per field were combined with all the hNT neurons in monoculture and compared to the average number of hNT cells with the E14 STR, VM and CER cocultures, the hNT & E14 STR cells had the highest average cells per field \( (50.7\pm4.2, \ w=15.3, \ p<0.01) \) while the hNT & E14 VM and hNT & E14 CER were \( 47.1\pm4.4 \ (w=11.3, \ p<0.05) \) and \( 45.8\pm3.3 \ (w=10.1, \ p<0.05) \), respectively.

2.3.3.3 E18 coculture
The overall ANOVA revealed no difference in the survival of the 3w, 4w, and 5w hNT populations \( (F_{2,121}=1.0, \ p=0.35) \) in any of the E18 cultures (FIG. 2.4D).

However, there was a significant difference between the mono- and coculture conditions \( (F_{3,121}=41.9, \ p<0.0001) \) as well as a interaction between hNT type and culture conditions \( (F_{6,121}=8.6, \ p<0.0001) \). Regardless of the hNT neuron population, all the cocultures had significantly more living cells compared to the monocultures except for the 5w hNT cells in the 5w-VM cocultures.
The only difference in survival between any of the hNT neurons in the STR cocultures was between the 5w-STR and the 4w-STR cultures (w=10.9, p<0.05). In contrast, the 5w-VM cultures had fewer surviving cells compared to the 3w-VM (w=28.0, p<0.01) and the 4w-VM (w=10.9, p<0.05). Finally, there was no difference in the number of living hNT cells when cultured with CER cells, 3w-CER & 4w CER: w=7.9, 3w-CER & 5w-CER: w=4.6, 4w-CER & 5w-CER: w=0.4, p>0.05).

When average number of cells per field were combined with all the hNT neurons in monoculture and compared to the average number of hNT cells with the E18 STR, E18 VM and E18 CER cocultures, the hNT & E18 CER cells had the highest average cell number per field (50.7±4.2, w=78.6, p<0.01) while the hNT & E18 STR and hNT & E18 VM were 47.1±4.4 (w=44.8, p<0.01) and 45.8±3.3 (w=40.5, p<0.01), respectively.

2.3.3.4 P1 coculture
The overall ANOVA revealed no difference in the survival of the various hNT populations (FIG. 2.4E; F_{2,133}=3.1, p=0.05) when cocultured with P1 rat cells or cultured alone (F_{3,133}=2.3, p=0.08). Furthermore, no interactions were observed between hNT neurons and the brain regions with P1 cells (F_{6,133}=0.17, p=0.99). The combined average number of cells per field of all hNT neurons & P1 STR cells was 40.6±3.8 while the hNT & P1 VM and hNT & P1 CER were 37.6±4.6 and 39.4±5.2 surviving cells, respectively.

2.3.3.5 Survival coculture up to 15 DIV
The overall ANOVA revealed significant differences in the survival at 5 DIV between the 3w, 4w, and 5w hNT neurons (FIG. 2.5B, F_{2,126}=23.6, p<0.0001) as well as the mono- and cocultures (F_{3,126}=14.0, p<0.0001). Furthermore, there were interactions with the coculture condition with the different E14 tissue and the various hNT neurons (F_{6,126}=6.4, p<0.0001).
FIGURE 2.4. Survival of hNT neurons in mono- or coculture with pre- and postnatal rat cells from different brain regions at 5 DIV. (A) In the hNT monocultures, the shortest RA treatment, the 3w, had the highest survival of hNT neurons compared to the 4w and 5w cells. (B) The E14, E18, and P1 rat cells all increased the survival of the hNT neurons. (C) At E14, the STR and VM were most effective at increasing the survival of 3w and 4w hNT cells. (D) At E18, survival of all hNT neurons increased in cocultures. The plus (+) indicates where significant differences exist between 3w hNT cells and the cocultures, while the number sign (#) and the at sign (@) showed significance with 4w and 5w, respectively. (E) In P1 cocultures, no statistically significant difference was observed between the various hNT cells and the specific brain regions. (*p<0.01, **p<0.05)
The number of surviving 3w hNT cells was greater when they were cocultured with either STR (w=33.9, p<0.01) or VM (w=25.8, p<0.01) cells. The 3w-STR cocultures also had more living cells compared to either the 4w-STR (w=28.1, p<0.01) or 5w-STR (w=27.3, p<0.01). In addition, the 3w-VM cocultures had significantly more hNT cells compared to the 4w-VM (w=40.8, p<0.01) or the 5w-VM (w=15.2, p<0.01) cells. Survival of the 3w hNT neurons was greater in the STR and VM cultures compared to the 3w-CER cells (w=20.2 and w=13.7, p<0.01, respectively). The 4w hNT cell survival was greater when cultured with CER cells (w=11.2, p<0.05) than in monoculture, while more living cells were found when 5w cells were plated with either VM (w=12.1, p<0.05) or CER (w=14.0, p<0.01) cells compared to the monocultures. Finally, no difference in the survival between the hNT neurons was observed when they were cultured with CER cells (3w-CER & 4w-CER: w=0.69; 3w-CER & 5w-CER: w=0.0001; 4w-CER & 5w-CER: w=0.74, p>0.05).

The overall ANOVA revealed significant differences in the survival at 10 DIV between the 3w, 4w, and 5w hNT neurons (FIG. 2.5C, F(2,122)=4.7, p<0.01) as well as the mono- and cocultures (F(3,122)=12.2, p<0.0001). Furthermore, there were interactions with the coculture condition with the different E14 tissue and the various hNT neurons (F(6,122)=8.1, p<0.0001). The 4w hNT cells had the greatest survival when cultured with CER cells compared to the 4w cell in monoculture (w=62.7, p<0.01), 4w-STR (w=44.0, p<0.01) and 4w-VM (w=26.2, p<0.01) cultures. Furthermore, the 4w-CER cells survival was higher than the 3w-CER (w=15.6, p<0.01) or 5w-CER (w=37.9, p<0.01) cocultures.

The overall ANOVA revealed significant differences in the survival at 15 DIV between the 3w, 4w, and 5w hNT neurons (FIG. 2.5D, F(2,121)=7.0, p=0.0014) as well as the mono- and cocultures (F(3,121)=20.7, p<0.0001). However, there was
FIGURE 2.5. Survival of hNT neurons cultured with E14 STR, VM and CER cells up to 15 DIV. (A) Survival of hNT neurons in monoculture with different durations of RA exposure. The 3w hNT neurons had the most living cells per field and were the only hNT cell type that maintained their survival up to 10 DIV. After two weeks in vitro, no difference was observed between the different hNT neurons. (B-D) Survival of hNT neurons cultured with STR, VM, and CER with increased DIV. (B) At 5 DIV, the 3w hNT neurons survived better when cultured with STR, VM, and CER cells. The 4w hNT neurons survived better when cultured with VM and CER cells. The 4w hNT neurons survived better when cultured with VM cells. In both the STR and VM cocultures, 3w hNT neurons had better survival compared to the 4w and 5w cells. There was no difference between the various hNT populations when cultured with CER cells. (C) In the 10 DIV cultures, the 4w hNT neurons cultured with CER cells had the greatest number of surviving cells compared to the other 4w cultures, as well as the 3w-CER and 5w-CER cultures. (D) At 15 DIV, very few cells survived in monocultures. In the cocultures, the 3w hNT cell survival was greatest in the CER, while the 4w cell survival was greater in both the STR and VM cultures. All three brain regions increased the survival of 5w hNT neurons. *p<0.01, **p<0.05
no significant difference between the conditions and the various hNT neurons (F_{6,121}=2.8, \ p=0.0145). The 3w-CER cultures had the greatest survival compared to the monocultures (w=11.5, p<0.05), while the 4w-STR and the 4w-VM cultures had the most living cells compared to the monocultures (w=18.4 and w=13.1, p<0.01), respectively. All the coculture conditions increased the survival of the 5w hNT neurons compared with the 5w monocultures (5w-CER: w=35.7, 5w-STR:w=29.6, 5w-VM:w=25.0; p<0.01). There was no difference between the 3w, 4w or 5w hNT neurons in the STR or VM cocultures, however, the 5w-CER cells had more living cells compared to the 4w-CER (w=13.5, p<0.05).

2.3.4 TH expression in hNT neurons

2.3.4.1 Monocultures

As previously stated, hNT neurons express the rate-limiting enzyme in DA synthesis, TH. The TH expression in hNT neurons was observed in the cell body (FIG. 2.6A, asterisks) and neurites (FIG. 2.6A, arrows). By 10 DIV, fewer TH-positive neurons were seen with an occasionally strong TH-positive clump (FIG. 2.6B, arrow). After two weeks in vitro, very few cells stained for TH, and staining was very light and predominantly in the cell body (FIG. 2.6C, asterisk). Similar results were observed in all hNT neurons regardless of their exposure to RA.

The overall ANOVA revealed significant differences in TH expression between the 3w, 4w, and 5w hNT neurons (FIG. 2.8A, F_{2,92}=8.5, p<0.004) as well as the age of the cultures (F_{2,92}=36.1, p<0.0001). In addition, there was also an interaction between hNT type and time in culture (F_{4,92}=3.2, p<0.0179).

Initially, the 3w and 4w hNT cells had significantly more TH expression than the 5w cells (FIG. 2.8A; w=16.2 and w=8.0, p<0.01, respectively). At 10 DIV, the TH-expression in the 3w and 4w hNT cells significantly dropped to the same level as observed in the 5w hNT population (w=0.59 and w=1.24, p<0.01). Furthermore,
FIGURE 2.6. TH expression in 4w RA-treated hNT neurons with increasing
days in culture. The hNT neurons were immunostained for TH (red) and DAPI
(blue) which identified the cell nucleus. TH-positive staining was observed in the
cell body (A, asterisks) and neurites (A, arrows) of hNT neurons. As days in vitro
increased, TH-staining became less intense with few neurons staining for the
rate-limiting enzyme in dopamine production.
by 10 DIV, no difference was found between the different types of hNT neurons, 3w and 4w (w=0.13, p>0.05), 3w and 5w (w=0.06, p>0.05), or 4w and 5w (w=0.36, p>0.05). After two weeks in culture, the 4w and 5w hNT neurons had a further decline in TH expression (4w, w=4.39, p<0.01; 5w, w=12.3, p<0.05). The 3w cells maintained their TH expression from 5 to 10 DIV (w=0.59, p>0.05). In addition, at 15 DIV, there were more TH-positive 3w hNT neurons compared to the 5w hNT cells (w=11.7, p<0.05).

2.3.4.2 E14 coculture

The hNT neurons could be easily distinguished from the rat cells by immunostaining for the human nuclei (HuN) antigen. When 3w hNT neurons were cultured with E14 STR cells, TH staining in hNT neurons was primarily observed in the cell body and axon hillock with an occasional short neurite positive for TH. Sometimes both human and rat cells in the STR cultures formed clumps that were TH-positive (FIG. 2.7A, arrow). These STR and hNT clumps had TH-positive neurites protruding outwards (FIG. 2.7A insert, double-headed arrow). In the VM-hNT cocultures, TH-positive staining was more intense in the VM cells (FIG. 2.7B, asterisks), however, individual TH-positive hNT neurites were found throughout the cultures. In the E14 CER cocultures, TH-positive staining was light and predominantly observed in the cell body with only a few TH-positive neurites (FIG. 2.7C, asterisks). Similar results were observed regardless of hNT neuron type.

The overall ANOVA revealed significant differences in TH expression in the 3w, 4w, and 5w hNT neurons at 5 DIV (FIG. 2.8B, F_{2,126}=61.2, p<0.0001) as well as the conditions between the mono- and cocultures (F_{3,126}=22.3, p<0.0001). Furthermore, there was also a significant interaction between culture conditions and the type of hNT neuron (F_{6,126}=3.2, p<0.0065).
FIGURE 2.7. Fluorescent photomicrographs of TH-positive hNT neurons cultured with E14 primary rat cells. Human nuclei marker (green) was used to identify the hNT neurons while TH (red) and DAPI (blue) identified the dopaminergic phenotype and all cell nuclei, respectively. (A) E14 STR cells tended to form TH-positive clumps (asterisks) and some clumps contained TH-positive hNT neurons (insert, double-headed arrow). (B) TH-positive staining was much stronger in VM cells (asterisks) compared to the hNT neurons (arrow) however, TH-positive and HuN-positive (red/green) individual hNT neurons were observed with long neurites. (C) Fewer TH-positive hNT neurons were visualized when cultured with the CER cells. Furthermore, the TH staining was usually seen in the cell body (asterisks) with an occasional TH-positive process stained for TH.
At 5 DIV, there was no difference in TH expression between hNT neurons in monoculture and those cultured with E14 STR (w=2.6, p>0.05). There was a decrease in TH expression in all three hNT cell types regardless of their exposure to RA when cultured with CER, 3w (w=14.5, p<0.01), 4w (w=17.8, p<0.01), and 5w (w=54.2, p<0.01) compared to the monoculture. The 4w hNT neurons maintained their TH expression when cultured with VM cells (w=2.4, p>0.05) compared to the monoculture, while the 3w and 5w hNT showed a decrease in TH expression when cultured with E14 VM cells (w=39.4, p<0.01; w=15.9, p<0.05, respectively). In the STR cultures, the 5w hNT neurons had the least TH expressing cells compared to the 3w and 4w hNT neurons (w=36.6, p<0.01 and w=41.6, p<0.01 respectively). The 4w hNT neurons in culture with VM cells had more TH expression compared to the 3w cells (w=9.6, p<0.05) and 5w hNT cells (w=33.6, p<0.01). In the CER cultures, the TH expression was highest in the 3w and 4w cells compared to the 5w hNT cells (w=24.3, p<0.01 and w=11.4, p<0.01).

The overall ANOVA revealed no difference in the TH expression in the 3w, 4w, and 5w hNT neurons (FIG. 2.8C, F2,123=2.49, p=0.088) or in the interaction between the hNT cells and conditions (F6,134=0.94, p=0.47). However, there was a significance difference in the number of TH-positive hNT neurons in monoculture between different prenatal tissue cocultures (F3,123=23.8, p<0.0001). The TH expression in the 5w hNT neurons was significantly lower in all coculture conditions compared to the monocultures (STR: w=34.0, VM: w=19.8, CER: w=22.0, p<0.01). The 3w hNT TH expression was lower in the STR and VM compared to the monocultures (STR: w=11.3, p<0.05; VM: w=23.1, p<0.01). Only exposure to STR cells decreased the TH expression in 4w hNT cells compared to the monocultures (w=15.6, p<0.01).

The overall ANOVA revealed no difference in the TH expression in the hNT monocultures (FIG. 2.8D, F2,120=0.16, p=0.86) or within the coculture conditions (F 3,120= 2.8, df, p<0.0418). However, there was a significant interaction between
FIGURE 2.8. TH expression in hNT neurons when cultured with E14 STR, VM, and CER cells. (A) TH expression of hNT neurons in monocultures with increasing days in vitro. At 5 DIV, over 50% of the 3w and 4w hNT neurons were TH-positive while only 30% of the 5w hNT neurons expressed TH. By 10 DIV, there was no difference in the TH expression between the various hNT neurons. After 15 DIV, only the 3w hNT cells had significantly more TH expression than the 5w cells. (B) In the cocultures at 5 DIV, only STR cells allowed all the hNT neurons to maintain their TH expression while the CER cultures decreased the TH expression in all hNT neurons. The 4w hNT neurons maintained their TH expression in the STR and VM cultures, while the 3w hNT cells maintained their dopaminergic phenotype in the STR cultures. (C) On the other hand, at 10 DIV, the CER cells permitted hNT neurons to maintain their TH expression compared to the monocultures, with the exception of the 5w hNT cells which showed a decrease in TH expression in all the cocultures. In addition the VM cultures also permitted the 4w hNT cells to maintain their TH expression but only around 20% were TH-positive. (D) By 15 DIV, TH expression remains unchanged in the 4w and 5w hNT neurons regardless of the condition, while the TH expression in 3w hNT neurons in monoculture had more TH-positive human cells than in all the coculture conditions. *p<0.01, **p<0.05
the different culture conditions and the type of hNT neuron (F_{6,120}=5.6, p<0.0001). After two weeks in monoculture, the 3w hNT neurons express significantly more TH than the 4w and 5w cells (w=13.8, p<0.01 and w=23.7, p<0.01 respectively). However, the TH expression in 3w cells decreased in all of the coculture conditions: the STR (w=23.2, p<0.01), VM (w=23.7, p<0.01), or CER (w=22.6, p<0.01).

2.3.5 Neurite outgrowth

2.3.5.1 Monoculture

We investigated the number of primary neurites extending from the soma of hNT neurons after different lengths of exposure to RA (FIG. 2.10A). The overall ANOVA revealed significant differences between the various hNT populations (F_{2,191}=4.9, p>0.001). There was no difference in the number of neurites between the 3w and 4w hNT neurons (w=2.8, p>0.05). The 5w hNT neurons had significantly more primary neurites compared to the 4w hNT cells (w=9.9, p<0.01). The 3w hNT cells had an average of 2.2±0.59 neurites per cell, while the 4w and 5w neurons had an average of 1.98±0.37 and 2.42±0.86 primary neurites per cell, respectively. The overall average of primary neurites per hNT neurons with 3-5w of RA treatment was 2.23±0.059 per cell.

2.3.5.2 Coculture

When hNT neurons were cocultured with pre- and postnatal rat cells, regardless of area of brain, their neuritic maturity as observed by the number of primary neurites, varicosities and neurite length was more advanced than was observed in the monocultures. In the cocultures of the hNT and VM cells, it was not possible to trace fibers from single hNT neurons in the clumps (FIG. 2.9A, arrow) so counts were obtained from individual cells around the edges or between the clumps. Both multipolar cells with up to 7 primary neurites (FIG. 2.9A, asterisks) as well as mono- and bipolar cells (FIG. 2.9A, arrowhead) were found throughout the cocultures. The hNT neurons had neurites containing varicosities (FIG. 2.9B, arrows) as well as elongated processes (FIG. 2.9C, arrowheads). Other neuritic growth and maturity markers were also observed such as growth cones (FIG.
2.9D, arrows) and cytoplasmic extensions at the end of neurites (FIG. 2.9D, arrowhead).

The overall ANOVA revealed there was no difference in the number of neurites between the various hNT populations ($F_{2,1220}=0.90$, $p=0.08$). However, there was an effect of the developmental age of the rat cells used ($F_{2,1220}=84.5$, $p<0.0001$) as well as an interaction between the type of hNT neuron and the rat developmental age ($F_{4,1220}=3.4$, $p=0.0097$).

All three hNT neuron populations had significantly more neurites in cocultures compared to the monocultures (FIG. 2.10B), the 3w and E14 ($w=27.4$, $p<0.01$), the 3w and P1 ($w=20.3$, $p<0.01$), the 4w and E14 ($w=82.7$, $p<0.01$), the 4w and P1 ($w=30.8$, $p<0.01$), the 5w and E14 ($w=30.1$, $p<0.01$) or the 5w and P1 ($w=23.7$, $p<0.01$). In addition, only the 4w hNT neurons had significantly more neurites compared to the 3w and 5w cells in the E14 cultures, $w=11.1$ ($p<0.01$) and $w=7.4$ ($p<0.01$) respectively. Furthermore, the only difference between the number of neurites found in E14 and P1 cocultures was found between the 4w-E14 and 4w-P1 cultures ($w=25.0$, $p<0.01$).

The average number of primary neurites for RA treated hNT neurons as a whole in monoculture was 2.23$\pm$0.059 per cell, while in the E14 and P1 cocultures, the average number of neurites was significantly higher ($3.50\pm0.062$, $w=130$, $p<0.01$ and $3.18\pm0.055$, $w=71.3$, $p<0.01$, respectively). The average number of primary neurites in hNT cells was also greater in E14 cultures compared to P1 ($w=16.4$, $p<0.01$). The 4w hNT neurons cultured with the E14 CER had the greatest average number of neurites with 4.38$\pm$0.20 per cell.
FIGURE 2.9. Neurite morphology of 4w hNT neurons in pre- and postnatal rat cultures at 5 DIV. When hNT neurons were cultured with E14 cells, the human cells were seen in clumps intermingled with rat cells (A, arrows) and as bi- or multipolar individual cells (A, arrowhead, asterisks, respectively). Advanced neuritic maturity such as varicosities (B, arrows) and elongated neurites (C, arrows) were evident when hNT neurons were cocultured with the P1 CER cells. On closer inspection, other indicators for growing neurites were observed such as growth cones (D, arrow) and extension of neuronal cytoplasm (D, arrowhead). Up to 7 neurites could easily be distinguished under higher magnification on some cells (E).
In the E14 cocultures, the overall ANOVA revealed significant differences between the hNT populations (FIG. 2.10C, $F_{2,725}=5.5$, $p=0.0044$) and the type of E14 cells ($F_{3,725}=41.4$, $p<0.0001$). There was also an interaction between the various hNT neurons within each coculture condition ($F_{6,725}=5.1$, $p<0.001$).

All three hNT neurons population had significantly more neurites in E14 cocultures compared to the monocultures (FIG. 2.10C), the 3w and STR ($w=28.1$, $p<0.01$), the 3w and VM ($w=11.1$, $p<0.01$), 3w and CER ($w=11.7$, $p<0.01$), 4w and STR, ($w=43.7$, $p<0.01$), 4w and VM ($w=42.5$, $p<0.01$), 4w and CER ($w=86.1$, $p<0.01$), 5w and STR ($w=27.0$, $p<0.01$), 5w and VM ($w=16.5$, $p<0.01$), or the 5w and CER ($w=8.0$, $p<0.01$). In addition, the 4w hNT neurons had significantly more neurites compared to the 3w and 5w cells in the CER cultures ($w=21.9$, $p<0.01$ and $w=25.9$, $p<0.01$, respectively). Furthermore, there was no significant difference between the number of neurites in STR or VM co-cultures; however there was more neuritic outgrowth in the 4w CER coculture compared to either the 4w-VM ($w=10.5$, $p<0.05$) or 4w-STR ($w=6.2$, $p<0.05$) cocultures.

To compare whether a specific brain region at E14 increased the overall number of primary neurites extending from the hNT neurons regardless of RA treatment, we combined the number of primary neurites in all the hNT neurons (3w, 4w, and 5w) in monoculture and then compared with the E14 STR, E14 VM and E14 CER cocultures. The hNT & E14 STR cells had the most neurites ($3.64\pm0.12$ per cell, $w=105.0$, $p<0.01$) while the hNT & E14 VM and hNT & E14 CER had $3.36\pm0.095$ ($w=70.3$, $p<0.01$) and $3.53\pm0.111$ ($w=86.4$, $p<0.01$) neurites, respectively.

In the P1 cocultures, the overall ANOVA revealed no significant differences between the hNT populations (FIG. 2.10D, $F_{2,707}=2.3$, $p=0.1035$). There was a difference between the monocultures and coculture conditions ($F_{3,701}=31.0$, $p<0.0001$). However, there was no interaction between the hNT neurons and the various conditions ($F_{6,701}=1.1$, $p=0.3839$).
FIGURE 2.10. Number of primary neurites of hNT cells in mono- or coculture with E14 and P1 cells from different rat brain regions at 5 DIV. The significant differences (p<0.01) in coculture are denoted as (+) for the 3w cells, (#) for the 4w and (@) for the 5w hNT neurons. (A) In hNT monocultures, the 5w hNT neurons had significantly more neurites per cell compared to the 4w cells, while there was no difference in number of primary processes between the 3w and 4w neurons. (*p<0.05) (B) Regardless of the hNT neurons exposure to RA, both E14 and P1 co-cultures increased the number of neurites per human cell significantly. Furthermore, in 4w-E14 coculture the hNT neurons had substantially more neurites compared to the other hNT neurons at E14 or 4w neurons cultured with P1 rat cells. (*p<0.01) (C) In the E14 cocultures, the 4w-CER cultures had the most neurites compared to 3w and 5w hNT neurons in CER, as well as 4w-VM and 4w-STR cultures. (*p<0.01 **p<0.05) (D) In P1 cocultures, all the different brain regions increased the number of primary neurites in all the hNT cells. The only difference between the number of neurites in hNT cocultures with VM, STR, and CER was between 5w-STR and the 5w-VM cells. (**p<0.05)
We also combined the data for all of primary hNT neurites regardless of RA treatment in monoculture and compared them with P1 cocultures from different areas. The hNT and P1 STR had the highest average number of neurites (3.33±0.089, w=68.6, p<0.01) while the hNT & P1 VM and the hNT & P1 CER had 2.97±0.10 (w=25.7, p<0.01) and 3.20±0.096 (w=46.6, p<0.01) neurites per cell respectively.

2.4 Discussion

In the monocultures, the length of RA exposure in hNT neurons had a profound effect on the survival of the hNT neurons with the shortest RA treatment, 3 weeks, allowing for the greatest survival. Coculture with prenatal (E14 and E18) environment increased survival of all hNT cells up to two-fold under specific conditions. Second, cells from P1 rat had no influence on the survival of any of the hNT neurons. The hNT neuron survival was greater in the long term cocultures (15 DIV) with E14 cells compared to monocultures however, the survival was never as great as observed at 5 DIV. The duration of RA treatment had no obvious effect on the number of primary neurites per hNT cell with the exception of 5w and 4w RA-treated neurons in which the 5w cells had significantly more neurites per cell than the 4w hNT neurons. Both E14 and P1 coculture environments increased the number of primary neurites for all hNT neurons regardless of the brain tissue used. However, as observed with the monocultures, the extent of RA exposure did not influence the neuritic outgrowth. In terms of the dopaminergic phenotype, none of E14 rat neural cells increased the expression of TH in hNT neurons.

2.4.1 RA influence on hNT neurons

RA acts within the cell nucleus to change the pattern of gene activity in the cells. RA is involved in processes as diverse as the specification of spatial pattern during morphogenesis of the CNS and limbs, cellular differentiation, and control
of cell proliferation (Edwards and McBurney, 1983; Heyman et al., 1992; Kliewer et al., 1992; Kliewer et al., 1994; Mangelsdorf, 1994; Chambon, 1996; Cheung et al., 1997; Kastner et al., 1997b; Morriss-Kay and Ward, 1999). Furthermore, the effects of RA varies from cell type to cell type as well within a cell type. For instance, when RA was added to embryonal carcinoma (EC) cells, the type of differentiated cell induced depended on the concentration of RA applied. At low doses the EC cells developed into cardiac cells, and at intermediate doses skeletal cells appeared, while high doses of RA produced neurons and astroglia (Edwards and McBurney, 1983).

The influence of RA on gene, enzyme and receptor expression, and differentiation of the NT2 cells, has been studied for over 20 years. Most recently, cDNA arrays have been used to detect many up and down regulated genes in the NT2 cells before and after RA-treatment and RA’s effects are thought to be time dependent (Cheung et al., 1997; Satoh and Kuroda, 2000; Bani-Yaghoub et al., 2001; Leypoldt et al., 2001; Freemantle et al., 2002; Przyborski et al., 2003). RA produced at least two induction phases, the immediate early phase which occurs within hours of exposure and a long term phase (Cheung et al., 2000). In the early phase, RARγ2 is up regulated in NT2 cells within 3 hours of RA exposure while microtubule associated protein (MAP2β) expression is not detected until at least two weeks of RA treatment (Pleasure et al., 1992; Borghi et al., 2003). Furthermore, increased exposure to RA down-regulated neural progenitor markers such as nestin, vimentin, and stage specific antigen-3 while up-regulating neuronal markers such as synaptophysin, TuJ1, tau and a repertoire of neurotransmitters and their synthetic enzymes (Andrews, 1984; Andrews et al., 1984; Lee and Andrews, 1986; Pleasure et al., 1992; Pleasure and Lee, 1993; Zeller and Strauss, 1995; Iacovitti and Stull, 1997; Guillemain et al., 2000a; Saporta et al., 2000; Zigova et al., 2000a; Newman et al., 2002).
2.4.1.1 Survival

The shorter the exposure to RA, the greater the survival of hNT cells at 5 and 10 DIV. This finding suggests that the protocol to derive hNT neurons which classically involved at least 4 weeks of RA treatment might not be the ideal method to generate the hardiest neurons. For instance, Berg and colleagues reported that only four hours of RA treatment was sufficient to induce the P19 cell line to differentiate (Berg and McBurney, 1990). Previously, we showed that exposure to RA increased expression of survival-promoting factor Bcl-2 and down-regulated the pro-apoptotic marker Bax (Daadi et al., 2001). Guillemain and colleagues showed similar increased expression of Bcl-2 up to 3w of RA treatment, but Bax remained constant (Guillemain et al., 2003). Since Bcl-2 is an important regulator during embryogenesis, both studies suggest Bcl-2 might be important during hNT RA-inducted differentiation. However, long-term RA treatment might eventually increase pro-apoptotic markers in hNT neurons. For instance, RA is known to induce apoptosis in various tumors and is currently being investigated for its therapeutic potential to treat cancer (review (Garattini et al., 2004). Moreover, researchers have found that RA regulates receptors for neurotrophins and therefore increases the cell dependence on neurotrophins for survival (Haskell et al., 1987; Zheng and Goldsmith, 1990; Rodriguez-Tebar and Rohrer, 1991; Kaplan et al., 1993; Stephens et al., 1993).

2.4.1.2 TH expression

RA is one of the signaling molecules present during the development of the CNS including the midbrain and is known to influence multiple pathways involving the expression of TH (Durston et al., 1989). For example, RA receptors are known to heterodimerize with the transcription factor Nurr1 which is essential for the proper development of midbrain DA neurons (Perlmann and Jansson, 1995; Zetterstrom et al., 1996). Nurr1 binds the promoter region of TH gene (Sakurada et al., 1999). RA also induces transcription factor AP-2 which binds to an upstream region on the TH gene (Kim et al., 2001). We report that short term exposure to RA (3 weeks) produced the most TH-positive hNT neurons and
maintained the greatest TH expression up to 15 DIV compared to the 4w and 5w hNT neurons. Therefore, the standard protocol of exposing NT2 cells to RA for 4w or longer might not be the ideal protocol to generate DA neurons. However, the ideal length of RA exposure has yet to be determined. For instance, treatment with RA for 3 days, and then phorbol esters for an additional 3 days increased the expression of TH by 3 fold on day 6 in a human neuroblastoma cell line (Pennypacker et al., 1989). Furthermore, NT2 cells were shown to express TH after 3 DIV when they were cocultured with Sertoli cells (Saporta et al., 2004).

2.4.1.3 Neuritic outgrowth
The length of RA exposure had a more varied effect on neuritic outgrowth in hNT neurons. Although the number of primary neurites was greatest in 5w hNT neurons (2.42±0.86), significance was only found between the 4w (1.98±0.37) and the 5w hNT neurons. Even though exposure to RA induces proteins for neuronal growth and differentiation, the length of RA exposure might not influence the number of primary neurites. The number of primary neurites might be influenced by other factors such as neurotrophins or cell adhesion molecules. RA exposure did influence the expression of RARγ and it was previously shown that the RARγ receptor is involved in the neuritic outgrowth in hNT cells (Moasser et al., 1995). Furthermore, recent evidence has shown that Bcl-2 enhances neuritic outgrowth (Eom et al., 2004) and as stated previously, Bcl-2 expression is influenced by exposure to RA in hNT cells (Guillemain et al., 2000b; Daadi et al., 2001).

2.4.2 Influence of specific brain regions on survival of hNT neurons
The survival of hNT neurons was increased when they were cultured with prenatal tissue but not postnatal tissue with the E18 cocultures increasing the survival of hNT cells to the greatest extent. At specific time periods during development, various events are occurring, with STR and VM developing in parallel, while the CER develops a short time later (Altman and Bayer, 1981; van der Kooy and Fishell, 1987; Voorn et al., 1988; Bayer et al., 1995). For instance,
At E14, neurogenesis is occurring in all areas of the brain. This time period is ideal for neuronal survival since there is an abundance of factors which influence the birth, growth, differentiation and migration of neurons. At E18, several unique circumstances are occurring in the rat brain. First, there is a second burst of neurogenesis with an actual overproduction of neurons, so once again the environment is favorable for survival. Second, gliogenesis starts to occur in most structures of the brain including the STR and VM (Das, 1979). Thirdly, the neurons from the first wave of neurogenesis start maturing and establishing connections with the intended target tissue. All these conditions combined may provide the ideal environment to enhance survival. In contrast, during the first week postnatally, no new neurons are born in the STR or VM. Instead many neurons undergo programmed cell death, believed to be a result of the overproduction of neurons and the limited supply of neurotrophic factors. However, recently, it was shown that high concentrations of neurotrophic factors can actually decrease survival of hNT cells (Piontek et al., 1999). It still remains to be determined if the decrease in survival is an in vitro phenomenon or if it is a result of an inverse relationship between the high concentration of neurotrophic factors and the down-regulation of their neurotrophic receptors (Frank and Rushlow, 1996; Knusel et al., 1997).

The 3 types of hNT neurons responded differently to factors provided by specific brain tissues. For instance, in the E14 cocultures, the 3w hNT neurons survival was increased by STR and VM cells, while the 4w cell survival was influenced by CER cells. The 5w hNT cell survival responded to both VM and CER cells. In the E18 cocultures, the survival of all the hNT neurons was increased by coculturing with different tissue with the exception of VM cells for 5w hNT neurons. Furthermore, the E18 STR conditions had the greatest effect on the survival of the 5w hNT cells, while the VM had the greatest effect on the survival of the 3w hNT cells. None of the rat brain regions increased the survival of hNT cells in P1 cultures.
The different hNT neurons might have responded to certain brain regions because the varying lengths of RA exposure influenced their expression of neurotrophic receptors and hence their ability to react to certain neurotrophic factors (NFs). The hNT neurons with 4w RA treatment, express high levels of TrkB and TrkC and very low levels of TrkA and the expression of these neurotrophin receptors varied in NT2 cells according to their level of RA exposure (Cheung et al., 1997; Piontek et al., 1999; Satoh and Kuroda, 2000).

The increase in survival in the E14 and E18 cocultures and not the P1 cocultures might be the result of the NT-3 expression in the developing rat brain (Maisonpierre et al., 1990a). NT-3 is highly expressed in most prenatal brain areas where proliferation, migration and differentiation are occurring and therefore may be an environmental cue to favor cell survival. NT-3 decreases substantially at birth and this may explain why the P1 cocultures at 5 DIV do not have an effect on the survival of hNT neurons. Furthermore, the fact that none of the regions of P1 rat brain increased the survival of hNT neurons might be due to the events that are occurring in the brain. The predominant activity in most areas of the brain is neuritic outgrowth and innervation. Furthermore, the brain is going through a restructuring phase in which cells that make the appropriate contact survive, and the rest die off (Buss and Oppenheim, 2004). Therefore, the NFs that are down-regulated after birth, might be survival-promoting, while the NFs that are up-regulated after birth might be important for neuritic outgrowth and innervation.

If NFs continues being expressed after birth in a specific region, suggests its importance for the maturation of that area. For instances, both GDNF and NT-4/5 are highly expressed in the prenatal STR and CER cells up to E21. After birth, the NT-4/5 expression decreased substantially in all areas of the brain while GDNF expression in the CER decreased but remained constant in the STR up to 3 weeks postnatally (Stromberg et al., 1993; Nosrat et al., 1996). This suggested that GDNF has additional importance in the STR and may influence
survival. This is consistent with the observation that GDNF is up-regulated in injured STR and has neuroprotective effects in parkinsonian rat models (Schmidt-Kastner et al., 1994; Borlongan et al., 2001).

2.4.3 DA phenotype of hNT neurons in E14 cocultures

Previous research from our laboratory as well as others has suggested that hNT neurons might be a potential cell source for the treatment of PD since these human cells have properties similar to midbrain DA neurons (Iacovitti and Stull, 1997; Willing et al., 1999; Zigova et al., 1999; Zigova et al., 2000a; Zigova et al., 2000b; Iacovitti et al., 2001; Willing et al., 2002; Misiuta et al., 2003b). Earlier investigations showed that coculturing DA neurons, with either developing VM cells or their intended target the STR, might influence the DA phenotype (Prochiantz et al., 1979; Dal Toso et al., 1980; Prochiantz et al., 1981; Niijima et al., 1990; Zhou et al., 2000). We investigated whether coculturing the hNT neurons with VM cells and the STR would increase the percent of TH-positive hNT cells. Our results illustrated that neither the VM, STR or non-target CER cells increased the TH expression in hNT neurons. Furthermore, there was a substantial decrease in TH expression in hNT neurons after 5 DIV in coculture with cells from all dissected brain regions. These observations were similar to previous findings which showed that there was no increase in the proportion of TH-positive VM cells when they were cocultured or cotransplanted with primary STR cells (Lauder and Bloom, 1974; di Porzio et al., 1980a; di Porzio et al., 1980b; Tomozawa and Appel, 1986; Yurek et al., 1990; Sladek et al., 1993a; Sladek et al., 1993b).

The E14 VM or STR cells may not have induced TH expression because at E14 the necessary inducing factors might no longer be present or the length of RA exposure might make the hNT neurons non-responsive to these signals. In fact, at E14, most cells in the VM have already committed to the dopaminergic phenotype since these cells already express TH and essential transcription factors specific for VM cells. The induction of TH is one of the key components
in the differentiation into the DA phenotype and is detected in post-mitotic VM neurons at E12.5 (Lauder and Bloom, 1974; Riddle and Pollock, 2003).

Furthermore, recent sensitive detection methods find TH expression as early as E10 (review(Riddle and Pollock, 2003).

A more thorough analysis of the development of midbrain DA neurons reveals that events that drive neural precursor cells down the dopaminergic pathway occur as early E9 in rat (Hynes et al., 1995; Ye et al., 1998; Liu and Joyner, 2001). Numerous inducing factors are expressed in the region at this time which are known to cause VM precursors to proliferate and/or differentiate such as fibroblast growth factor 8 (FGF8), sonic hedgehog (SHH) (Ye, Shimamura et al. 1998), bone morphogenetic proteins (BMP) (Lee and Jessell, 1999) and RA (Durston et al., 1989; Lee and Jessell, 1999). During the induction phase (E10 to E12.5) the VM cells are proliferating, migrating and starting to differentiate into post-mitotic neurons. During this phase, the Wnt1 pathway is activated and at the same time there is an induction of transcription factors Engrailed-1 (En1) and Engrailed-2 (En2) which are essential for VM cell development (Wurst et al., 1994; Liu and Joyner, 2001; Simon et al., 2001). Another homeobox gene implicated during this early specification of VM cells is Lmx1b (Smidt et al., 1997). En1, En2 and Lmx1b all continue being expressed in the adult DA midbrain neurons (Joyner, 1996; Wurst and Bally-Cuif, 2001; Burbach et al., 2003). During the differentiation phase (E11.5 –E12.5), two more critical transcription factors are expressed, Nurr1 (NR4A3) and Ptx3 (Pitx3), which is almost exclusively expressed in VM cells in the CNS (Smidt et al., 1997) while Nurr1 was shown to be critical for the development, differentiation, maintenance and survival of midbrain DA neurons (Zetterstrom et al., 1997; Castillo et al., 1998; Saucedo-Cardenas et al., 1998; Zetterstrom et al., 1999).

In order to induce TH expression in hNT neurons in a coculture system, it might be necessary to dissect cells from younger embryos such as E7 to E9. A second method to induce TH expression might involve exposing the hNT
neurons and/or the NT2 precursors to various known inducing factors such as SHH, FGF8, and BMP. Stull and colleagues (Iacovitti et al., 2001) illustrated only a 2% increase in TH when hNT neurons were exposed to SHH and FGF8. However, the addition of coactivators such as isobutylmethylxanthine (IBMX), forskolin, phorbol 12-myristate 13-acetate (PMA) and DA increased the DA phenotype by nearly 30%.

Another approach to determining the DA potential of the hNT neurons is to determine if the hNT neurons and their NT2 precursors express some of the previously stated essential transcription factors for DA induction. In chapter 3, we investigated the expression of these transcription factors, En1, Nurr1, and Ptx in NT2 cells and the hNT neurons. The importance of this investigation as well as future studies with hNT neurons would provide key insights into the use of this cell line as a cell source for the treatment of PD. In addition, this cell line could also provide a human \textit{in vitro} model system to examine the effects of various molecules on pathways essential for the dopaminergic phenotype.

2.4.4 Effect of developing rat neural cells on hNT neurite outgrowth

The prenatal (E14) and postnatal (P1) cells increased the number of primary neurites in all hNT cells. Both time periods support neuritic outgrowth since migration and innervation are occurring at E14 and P1 while neuritic sprouting becomes evident during the first week after birth (Voorn et al., 1988; Antonopoulos et al., 2002). Although there is no substantial difference in the number of primary neurites with levels of RA exposure, RA does induce proteins and receptors important for neurite growth. RA treatment increases MAP2, growth associated protein and tau, which are all important proteins for neurite outgrowth (Lee and Andrews, 1986).

The increase in the number of primary neurites of hNT neurons in the E14 and P1 cultures regardless of whether they were cultured with VM, STR or CER might be a result of a combination of specific NFs released by these brain
regions. First of all, GDNF is expressed prenatally in the VM, STR and CER (Schaar et al., 1993; Choi-Lundberg and Bohn, 1995; Trupp et al., 1995) and GDNF was shown to increase neuritic outgrowth in midbrain DA neurons (Lin et al., 1993; Hou et al., 1996). Interestingly, the greatest number of primary neurites was observed in the 4w hNT and E14 CER cocultures. This is consistent with reports that GDNF expression is greatest in the E14 CER (Schaar et al., 1993; Choi-Lundberg and Bohn, 1995). The 4w RA treatment might have produced hNT cells that have the greatest response to factors provided by the E14 CER such as the GDNF.

Recently, Piotnek and colleagues (Piontek et al., 1999) analyzed the effect of specific NTs (BDNF, NT-3, and NT-4/5) on the survival and neuritic maturation of 5w hNT neurons. All three NTs significantly increased the survival of hNT neurons up to three weeks in vitro. All three factors also increased the neuritic maturation as assessed by the number of primary neurites and branches. Surprisingly, there was no difference in survival in hNT neurons between the factors used, however, hNT cells exposed to BDNF had significantly more neuritic branches and outgrowth compared to NT-3 and NT-4/5. This result is consistent with the expression of BDNF during development. Initially, BDNF expression in low in most brain regions prenatally, however after birth, BDNF expression increased throughout the brain coinciding with events in the brain such as increased neuritic branching (Maisonpierre et al., 1990b). Furthermore, although there are more primary hNT neurites in E14 cocultures, this measurement is just one method of measuring neuritic maturity and does not necessarily address other factors involved such as number of branches, length of the neurites, presence of synaptic markers and/or synaptic function which may be induced by BDNF.

Neuritic outgrowth may also have been enhanced by the presence of extracellular matrix molecules on the surface of the primary cells (Engele et al., 1991; Goodman, 1996; Gopinath et al., 1996; Ostergaard et al., 1996; Kiryushko
et al., 2004). For example, neural cell adhesion molecule (NCAM) and laminin are highly expressed in primary cells (Letourneau et al., 1988) which we confirmed in our primary STR, VM and CER cultures using immunocytochemical detection with the antibody for NCAM (data not shown). NCAM was shown to increase neurite outgrowth by activating the fibroblast growth factor receptor (Hall et al., 1996). RA treatment increased expression of fibroblast growth factor receptors in hNT neurons (Campbell et al., 1992). Furthermore, NCAM was shown to activate the GDNF receptor which not only influences neuritic outgrowth but survival as well (Paratcha et al., 2003). This is a primary example of how intercellular communication involves direct cell-cell contact. Chao and colleagues showed that subchronic GDNF administration in the substantia nigra of rats increased the expression of NCAM (Chao et al., 2003). Therefore, there seems to be an interplay not only between the NFs and their receptors but also with cell adhesion molecules which are critical for neuronal outgrowth.

Neurotransmitters have also been shown to increase both survival and neuritic outgrowth (Lipton and Kater, 1989; Knipper et al., 1994; Lindholm, 1994; Zheng et al., 1994; Marty et al., 1996; Zetterstrom et al., 1999; Kuppers and Beyer, 2001; van Kesteren and Spencer, 2003). For example, DA, glutamate, and γ-aminobutyric acid have been shown to increase the neuritic outgrowth in various cells types such as cultured hippocampal neurons (Lipton and Kater, 1989; Zheng et al., 1994; Gaiarsa et al., 1995) while the same neurotransmitters inhibited the neurite outgrowth in other cells types such as the retinal neurons (Lankford et al., 1988; Lipton et al., 1988). The hNT neurons have been shown to express a variety of neurotransmitter receptors such as dopamine (Zigova et al., 2000a; Sodja et al., 2002), GABA (Matsuoka et al., 1997; Neelands et al., 1998; Neelands et al., 1999), acetylcholine (Newman et al., 2002), opioid, N-methyl-D-aspartate (Munir et al., 1996; Beczkowska et al., 1997), and glutamine receptors (Younkin et al., 1993; Dunlop et al., 1998). We found various neurotransmitter synthesizing enzymes in our primary cultures such as TH and
glutamic acid decarboxylase (FIG. 2.3), and some of these neurotransmitters could be influencing the survival and neuritic outgrowth in the hNT neurons.

2.5 Conclusion

This investigation illustrated that certain periods during the development of the rat CNS can increase the survival and neuritic outgrowth of a human cell line. These influences are probably due to either soluble factors and/or cell to cell contact. The hNT neurons provide an excellent human model system to examine factors influencing survival and neuritic outgrowth. Since many neurodegenerative diseases including PD are associated with the death of neurons, it will be critical to find factors that can provide the appropriate environment to induce survival, neuritic outgrowth, and differentiation of specific phenotypes. Second, factors that influence the specific phenotype are established early during development and involve the intrinsic properties of the cell which are determined by inducing factors in the local environment. RA is an inducing agent, however, the level of RA exposure in terms of concentration and time period that is effective at producing the ideal DA phenotype in hNT neurons is not yet fully understood and most likely involves other inducing factors as well.
CHAPTER 3

Expression of Transcription Factors Essential for the Induction of the Dopaminergic Phenotype

3.1 Introduction

The NT2 precursor cells have properties similar to neural precursors and differentiate into all three major lineages – neurons, astrocytes and oligodendrocytes (Andrews, 1984; Andrews et al., 1984; Lee and Andrews, 1986; Pleasure et al., 1992; Langlois and Duval, 1997; Bani-Yaghoub et al., 1999; Sandhu et al., 2002). The 99% pure neuronal population, the hNT or NT2-N neurons can be generated from NT2 cells through exposure to RA (10 µM). A subpopulation of hNT neurons express a constellation of enzymes and receptors that are found in midbrain DA neurons including the rate-limiting enzyme for DA synthesis, TH, DAT, D2 receptor, and AHD-2 (Zigova et al., 1999; Zigova et al., 2000a). These neurons were shown to form functional synapses and when exposed to potassium chloride, these cells released DA confirming the function as DA neurons (Hartley et al., 1999; Iacovitti et al., 2001).

How many of these neurons become dopaminergic depends upon the length of RA exposure with 3 weeks (3w) exposure inducing TH expression in up to 60% of the cells while the continued exposure to RA (5w) drops TH expression down to 25%. RA is a differentiating factor present during the development of VM cells which acts in the cell nucleus to change the pattern of gene expression (Edwards and McBurney, 1983; Heyman et al., 1992; Kliewer et al., 1994; Mangelsdorf, 1994; Chambon, 1996; Cheung et al., 1997; Kastner et al., 1997a; Kastner et al., 1997b; Morriss-Kay and Ward, 1999; Wallen-Mackenzie et al., 2003). RA is known to effect many pathways which influence TH expression (Perlmann and Jansson, 1995; Zetterstrom et al., 1996; Sakurada et al., 1999; Kim et al., 2001). For example, RA receptor (RXR) was shown to heterodimerize with Nurr1 and
therefore affect the transcription of TH (which can bind to the promoter region of the TH gene) (Perlmann and Jansson, 1995; Zetterstrom et al., 1996; Sakurada et al., 1999; Kim et al., 2001; Airaksinen and Saarma, 2002).

Recently, numerous transcription factors important for the induction and differentiation of DA neurons have been identified. Among the early transcription factors expressed during the induction of early midbrain DA neurons, also called VM precursor cells, are two homologues, Engrailed-1 (En-1) and Engrailed-2 (En-2), which are controlled by signaling molecules in the Wnt pathway (McMahon and Bradley, 1990; Danielian and McMahon, 1996). In mice null for En-1/En-2 there was a reduction in the size of mid- and hindbrain (Liu and Joyner, 2001; Simon et al., 2001). Although TH expression is induced in Engrailed-1/-2 null mice, its expression soon diminishes and no TH-positive neurons are found at birth. This suggests that Engraileds may not be essential for the initial induction of TH but are necessary for the maintenance of the DA phenotype.

During differentiation, two more transcription factors critical for DA development are expressed, Nurr1 and Ptx3. Nurr1 is an orphan steroid receptor shown to be critical for the development, differentiation, maintenance and survival of midbrain DA neurons since DA neurons failed to develop in Nurr1 null mice (Zetterstrom et al., 1997; Castillo et al., 1998; Saucedo-Cardenas et al., 1998; Wallen et al., 1999). Additionally, Nurr1 was shown to regulate genes for TH, VMAT2, and DAT (Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998; Le et al., 1999a; Sakurada et al., 1999; Wallen et al., 1999; Iwawaki et al., 2000; Sacchetti et al., 2001; Wallen et al., 2001). Ptx3 is almost exclusively expressed in VM cells in the CNS with some Ptx3-positive cells observed in the tongue (Smidt et al., 1997). Ptx3 is even more specific to midbrain neurons since Ptx3-deficient aphakia mice failed to develop DA neurons of the substantia nigra pars compacta (Semina et al., 1997; Smidt et al., 1997) and Ptx3 was shown to increase
expression of TH by binding the promoter region of the TH gene (Smidt et al., 2000; Lebel et al., 2001).

Since Engrailed-1, Nurr1 and Ptx3 are important in the expression and maintenance of the DA phenotype, a cell source that expresses these three transcription factors could have the potential to be used as a model system to study the DA phenotype. Therefore, we examined Engrailed-1, Nurr1 and Ptx3 expression in NT2 precursors using immunocytochemical techniques and/or western blot analysis. Furthermore, we investigated the expression of these three transcription factors after 3 – 5 weeks of RA treatment. We report that NT2 precursors endogenously express the three essential VM transcription factors En1, Nurr1, and Ptx3. RA-treatment (3 – 5 weeks) up-regulates Nurr1, however, there is a down-regulation of Ptx3 and Engrailed-1 in the differentiated hNT cells.

3.2 Methods

3.2.1 Cell culture

The NT2 cells were obtained from American Type Culture Collection (Manassas, VA) while the hNT neurons were obtained from Layton Bioscience, Inc. (Sunnyvale, CA). To produce the three different types of hNT neurons, NT2 cells were exposed to 10 \( \mu \text{M} \) RA treatment for either 3, 4 or 5 weeks (3w, 4w, and 5w). After RA induction, the hNT neurons were re-plated with mitotic inhibitors for 10 days. All cells were cryopreserved and stored at –180°C prior to use. The cells were thawed rapidly at 37°C and transferred into a 15-cc tube containing Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen), 10% fetal bovine serum (FBS; Invitrogen), and Gentamicin (50 \( \mu \text{g/mL} \), Sigma). The cells were centrifuged (160 g/ 7 min) and resuspended in 1 ml of fresh media and the cell number and viability assessed with the tyrant blue exclusion methods. For western blot analysis, protein samples were prepared immediately after thawing.

For immunocytochemical analysis the cells were plated in triplicate on poly-L-lysine coated 8-well chamber slides (Nunc, Naperville, IL) at concentrations of 100,000/cm\(^2\) for the hNT neurons and 75,000/cm\(^2\) for the NT2 cells. The cells
were maintained at 37°C in 5% CO₂ at 95% humidity. After 24 h the plating media was switched to DMEM:F12 (Invitrogen) containing 0.1% insulin-transferrin-selenium (ITS; Sigma, St. Louis, MO) and Gentamicin (50 µg/mL, Sigma). The cultures were maintained up to 4 DIV for NT2 cells and up to 5 days for the hNT neurons.

3.2.2 Immunocytochemistry
The cultures were first washed in 0.1M phosphate-buffered saline (PBS; pH 7.4), then fixed with cold 4% paraformaldehyde for 15 minutes and washed three times with cold PBS. The slides were treated for 1 h at room temperature with 10% normal goat serum, 0.03% Triton X-100 in PBS. Cultures were then incubated in primary antibodies overnight at 4°C. To identify the transcription factor Nurr1 we used either polyclonal anti-Nurr1/NOT1 (Chemicon, San Francisco, CA; 1:100, rabbit) or monoclonal anti-Nurr1 (BD Bioscience, Mansfield, MA; 1:200, mouse). To detect healthy neurons, immunostaining was performed using either monoclonal or polyclonal III β-tubulin (TuJ1, Covance, Princeton, NJ; 1:500, mouse; 1:1000, rabbit). Monoclonal or polyclonal TH (Diasorin, Stillwater, MN; 1:1,000, mouse; Pel-Freez, Rogers, AZ; 1:200, rabbit) antibodies were used to identify DA cells. The primary antibody was omitted from the control slides.

After several PBS washes, the slides were incubated in appropriate secondary antibodies (Molecular Probes, Eugene, OR; Alexa Fluor 594 goat anti-rabbit, 1:2,000; Alexa Fluor 546 goat anti-mouse 1:1,000, Alexa Fluor 488 goat anti-mouse, 1:800). The slides were washed with cold PBS and coverslipped with 95% glycerol. To visualize cell nuclei, some cultures were coverslipped using Vectashield with DAPI counterstain (Vector Laboratories, Burlingame, CA). The sections were observed under epifluorescence and photographed on the Olympus BX60 microscope.
3.2.3 RNA Isolation
The relative levels of Nurr1 RNA expression were determined for NT2 and hNT cells using standard RT-PCR assay. Total RNA was isolated from cell samples using Trizol reagent (Invitrogen) according to manufacturer’s recommendations and cDNA was prepared using the SuperScript RT-PCR kit (Invitrogen). PCR primers were designed using the MacVector software package to minimize primer self-annealing and PCR artifacts. Both the forward and reverse primers for Nurr1 were designed as 25mers that gave a specific band at 480 bp. PCR was performed for 30 cycles using a Robocycler (Stratagene, La Jolla, CA) with commercially available enzymes and TAQ polymerase according to the manufacturer’s recommendations. PCR products were run on 6% Tris-Borate Ethylenediaminetetraacetic acid sodium (TBE) polyacrylamide gels, stained with ethidium bromide and visualized under UV illuminator. All samples had negative control reactions that did not contain reverse transcriptase to demonstrate that cDNA was necessary to produce the PCR band and a positive control using PCR primers for Glyceraldehyde 3-phosphate dehydrogenase.

3.2.4 Western Blot
The samples were prepared from freshly thawed cells. Protein samples were suspended in lysis buffer and 1 μM dithiothreitol and sonicated. Protein samples (20 μg) and full range molecular weight markers (Amersham Bioscience) were resolved on 10% SDS-PAGE gel, and transferred to Invitronol PVDF membranes (Invitrogen). The membranes were incubated in Tris-buffered saline (TBS) containing 5% non-fat milk and 0.1% Tween-20 for 1 hour at room temperature to block non-specific binding, and then incubated overnight in appropriate antibody at 4°C. The membranes were washed three times, 10 minutes each, in TBS with 0.1% Tween-20 and incubated in peroxidase-conjugated anti-mouse IgG (1:30,000, Jackson ImmunoResearch ) or peroxidase-conjugated anti-rabbit (1:20,000) for 1 hour at room temperature. Primary and secondary antibodies were diluted in TBS, 5% non-fat milk and 0.1% Tween-20. Immunoreactivity was visualized using a West Pico Chemiluminescent Kit (Pierce Biotechnology).
Digitized images of the films were analyzed using Image Pro-Plus (Media Cybernetics, Silver Springs, MD) software. The primary antibodies were Nurr1 (1:1,000, mouse, BD Biosciences), Ptx3 (1:500, rabbit, Abcam), and Engrailed-1 (1:500, rabbit, Chemicon).

3.2.5 Quantitative Analysis
For the immunocytochemical analysis, the cells were screened in a blind coded manner using a 20x objective and a photographic frame (field = 0.3 mm²). Counts were performed from 30 fields per condition (5 pictures per well times 6 wells per condition) from three independent platings. For western blot analysis, three different protein samples were used and Image Pro-Plus was used to determine the optical densities of the different samples. The values for each experiment were reported as mean ± SEM. Data was analyzed using analysis of variance (ANOVA). The Newman-Keuls test was employed for the post hoc comparisons.

3.3 Results
3.3.1 Viability and morphology of NT2 precursors and hNT neurons
After thawing, initial viability of NT2 cells was between 75-85%. Within 2-3 hours these cells attached to the plates and were rapidly expanding. At 1 DIV, the NT2 precursors had irregular, flat, large cell bodies and prominent nuclei. Blue-counterstained slides revealed many of these cells at different mitotic stages (FIG. 3.1A, B; asterisks). By 2 DIV, almost the entire culture well was covered with a tightly packed monolayer of NT2 cells of polygonal shape bearing large nuclei, prominent nucleoli, and occasional short processes (FIG. 3.1B).

The post-thaw viability of hNT neurons was consistently within the range of 55-65% regardless of length of RA exposure. After 1 DIV, prior to media change, the hNT neurons were small and round with few short processes. In many instances we found numerous small clumps throughout the well. As DIV increased, many neurons showed elongated perikarya with either bi-or multipolar...
processes that formed extensive neuritic branches interconnecting with other multi-layered clusters of neurons. Many hNT neurons migrated from the small clusters and established a very complex network (FIG. 3.1C-H). The soma of these developing neuron-like cells was approximately four times smaller in comparison to their NT2 precursors. Occasionally, we found darkly stained, round pyknotic nuclei in all three cultured groups resembling cells undergoing apoptosis as was observed in previous studies (Zigova et al., 2001).

3.3.2 Immunocytochemical expression of Nurr1 in NT2 cells and hNT neurons

Short-term (1-2 DIV) cultures of NT2 cells were used for immunocytochemical detection of the Nurr1 transcription factor. The signal was localized within the nucleus (FIG. 3.2A1-3). Within the sample, the overall intensity of nuclear staining varied from very intense to weak with similar results seen using either monoclonal or polyclonal antibodies. In hNT neurons we detected Nurr1 expression in all three RA-treated hNT neurons. In both 1 DIV and 5 DIV hNT cultures, Nurr1 immunoreactivity was strictly confined to the nucleus (FIG. 3.2B1) as confirmed with DAPI counterstaining (FIG. 3.2B2).
FIGURE 3.1. Light microscopy photomicrographs showing blue counterstained NT2 cells and hNT neurons in short-term culture. (A) By 2 DIV, NT2 precursors almost completely covered the well. Their irregular shaped bodies had more cytoplasm compared to the differentiated hNT neurons. At this time many of the cells were in different stages of mitosis (asterisk). Bar= 50 µm (B) NT2 precursors viewed at higher magnification. Bar = 25 µm (C-H) Bright-field photomicrographs of cultured hNT neurons previously exposed to RA for 3, 4, or 5 weeks. (C-E) hNT neurons formed tightly packed clumps (C, 3w) or loose networks (D, 4w; E, 5w). Their elongated cell bodies sent out numerous processes. Occasionally we found darkly stained, round, pyknotic nuclei in all three experimental groups (asterisk, C, E). The 3w (F), 4w (G), and 5w (H) hNT neurons at higher magnification. The scale bar represents 25 µm for (C-E) and 10 µm for (F-H).
The healthy hNT neurons are identified by immunostaining with TuJ1 and therefore only double-labeled TuJ1-positive / Nurr1-positive hNT neurons were included in the counts. All TuJ1-positive hNT neurons were also Nurr1-positive (TABLE 3.1). Furthermore, only a few Nurr1-positive hNT neurons did not show any TuJ1 labeling (FIG. 3.2C1-3). These "seemingly" Nurr1-positive cells were strikingly similar to the pyknotic, darkly blue-counterstained cells (FIG. 3.2E) as we previously observed (Zigova et al., 2000a) and therefore were excluded from the quantitative studies. The total of number of NT2 cells that were Nurr1-positive was approximately 12% (FIG. 3.2A).

3.3.3 Co-expression of Nurr1 and TH in hNT neurons

To determine whether the transcription factor Nurr1 was present in dopaminergic hNT neurons, we double-labeled several sister cultures with both Nurr1 and TH. Independent of the exposure period to RA treatment, every hNT cell expressing TH was also Nurr1-positive (FIG. 3.2D3). In addition, TH-negative hNT neurons were also Nurr1-positive. This finding suggests that either the immature hNT neurons are at different stages of differentiation and have yet to reach their final phenotype and/or Nurr1 has a function other than TH activation in hNT neurons.

<table>
<thead>
<tr>
<th>Percent of Nurr1-positive NT2 cells and hNT neurons</th>
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<tbody>
<tr>
<td>Plating 1</td>
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<td>Plating 2</td>
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<td>Plating 1</td>
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<td>3</td>
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<td>12.5 ± 4.36 (776)</td>
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TABLE 3.1. Number of Nurr1-positive NT2 and hNT cells. Data was called from two separate platings and counts represent number of Nurr1-positive cells vs. total number of cells (number in parentheses) ± standard deviation in NT2 cells at 2 DIV or hNT at 5 DIV.
FIGURE 3.2. Nurr1 expression in NT2 cells and hNT neurons. (A) Fluorescent photomicrographs showing several NT2 cells (2 DIV) with Nurr1-positive nuclei (arrows, A1,A3; asterisks, A2). (A4) Control. A1, A3 bar = 10 µm and A2, A4 bar = 25 µm. (B1) Nuclear localization of Nurr1 antigen (red) in hNT neurons (1 DIV, 3 w RA). (B2) The same group of neurons as in B1 counterstained with DAPI (blue) to confirm the overlap of two nuclear signals. Bar = 10 µm (C) Double immunostaining for transcription factor Nurr1 and neuronal marker TuJ1 in hNT neurons. (C1-C3) The fluorescent photomicrographs of cultured hNT neurons (5 DIV, 3 w RA) expressing Nurr1 (red, C1) and TuJ1 (green, C2). Dual filter confirms the presence of double labeled (Nurr1+/TuJ1+) hNT neurons (C3). (C4-C6) Similar results were obtained in hNT cultures treated with RA for 5 weeks. (C4) Nuclear Nurr1-immunoreactivity (red), (C5) TuJ1 expression (green), and double labeled (Nurr1+/TuJ1+) in hNT neurons (C6). Asterisk in C4 and C6 point to Nurr1-positive but TuJ1-negative cells. These single-labeled (red) cells were not included in quantitative analysis. Bar = 10 µm. (D) Co-expression of Nurr1 and tyrosine hydroxylase in hNT neurons. (D1) Nuclear Nurr1-positivity (red, arrow), (D2) cytoplasmic TH expression (green, arrow) and co-localization of both signals under a dual filter (D3). Bar = 10 µm
3.3.4 RT-PCR analysis of Nurr1 expression in NT2 and hNT cells

The relative level of Nurr1 expression was determined using standard RT-PCR assay. Since earlier studies analyzing the time course of the TH expression had shown that the level of TH decreases in hNT neurons as the time in RA increased to 5 or 6 weeks (Zigova et al., 1999; Zigova et al., 2000a) we wanted to determine if the expression of Nurr1 correlates with down regulation of TH in this developmental model system. The expression of Nurr1 was compared in the hNT neurons and their NT2 precursors. RNA was prepared for the NT2 cells and the hNT neurons. cDNA was synthesized from each RNA sample and PCR analysis performed using Nurr1 specific primers. The expected Nurr1 specific band of about 480 bp in size can be clearly seen in both NT2 precursors and the different hNT neurons samples (FIG. 3.3). Although this assay is not quantitative, the Nurr1 band from each sample was the same apparent size and of comparable intensity, confirming the presence of Nurr1 RNA in NT2 cells, as well as the 3, 4, and 5 week hNT neurons.

FIGURE 3.3. RT-PCR analysis of Nurr1 in hNT neurons and their NT2 precursors. The results show that both NT2 and hNT cells have a comparable Nurr1 specific band at about 500bp in size. Lane 1) NT2, 2) NT2 without reverse transcriptase, 3) 100 bp DNA marker, 4) 3 w RA-treated, 5) 4 w RA-treated and 6) 5 w RA-treated hNT neurons.
3.3.5 Expression of dopaminergic transcription factors, Engrailed-1 and Ptx3, in NT2 and hNT cells

We used western blot analysis to examine if NT2 precursor cells and the pure neuronal population, the hNT cells, express two essential midbrain DA transcription factors, Engrailed-1 and Ptx3. The correct band size of both Engrailed-1 and Ptx3 were detected in the NT2 precursors, however, the 3, 4 or 5 week RA-treated hNT cells did not express either of the two proteins (FIG. 3.4).

FIGURE 3.4. Expression of Engrailed-1 and Ptx3 in NT2 and hNT cells. The correct band size of 55 kDA for Engrailed and 32 kDA for Ptx3 was observed in NT2 cells. Once hNT neurons were exposed to 3 – 5 weeks of RA treatment, they no longer expressed Engrailed-1 or Ptx3 protein.
3.4 Discussion

Our immunocytochemical and western blot analysis revealed that NT2 cells express three transcription factors, Engrailed-1, Nurr1, and Ptx3, essential for the proper development of midbrain DA neurons. Even though the RA treatment increased the expression of Nurr1 in hNT neurons, 3 weeks or greater exposure to RA decreased the expression of Engrailed-1 and Ptx3. Therefore, NT2 cells can be used for development of DA neurons, however exposure to 3 weeks or more of RA treatment does not drive the NT2 precursors down the DA pathway.

Nurr1 is essential for the induction of the DA phenotype since the VM cells in Nurr1 knockout mice never express TH (Zetterstrom et al., 1996; Baffi et al., 1999). However, Nurr1 expression might be more important to direct the precursor cell down the neuronal pathway with the potential to become dopaminergic if exposed to other appropriate factors. For instance, Nurr1-overexpression in a neural stem cell line induced a neuronal fate in the majority of the neural cells, however there was no increase in the number of TH-positive neurons. Conversely, once these Nurr1-overexpressing cells were exposed to type I primary astrocytes from the VM, their TH expression increased to 70%, suggesting that supplementary factors were necessary for dopaminergic differentiation (Wagner et al., 1999). Furthermore, Nurr1 is expressed in other areas of the brain such as the hippocampus and cerebral cortex (Xiao et al., 1996; Zetterstrom et al., 1996) and therefore TH-positive and TH-negative neurons express Nurr1, as observed in our analysis with hNT neurons.

In addition to Nurr1, both Ptx3 and Engrailed-1 are critical to the proper development of midbrain DA neurons. Even though TH expression is induced in Ptx3 and Engrailed-1 knockouts, TH expression is reduced with no TH-positive cells found after birth with death following within a couple of days (Smidt et al., 1997; Liu and Joyner, 2001; Simon et al., 2001; Smidt et al., 2004a). Although Nurr1 is required for the induction of TH in VM cells, Ptx3 expression is highly specific to VM cells (Smidt et al., 1997). In addition, both Nurr1 and Ptx3 were
shown to directly bind to the promoter region on the TH gene (Sakurada et al., 1999; Smidt et al., 2000; Lebel et al., 2001).

All three transcription factors are expressed into adulthood suggesting their importance in maintaining the DA phenotype (Saucedo-Cardenas and Conneely, 1996; Zetterstrom et al., 1996; Smidt et al., 1997). Furthermore, mutations in any of these three genes as well as others have been implicated in various neurological diseases including Parkinson’s disease (PD) (Xiao et al., 1996; Kruger et al., 1999a; Kruger et al., 1999b; Piccini et al., 1999; Oliveri et al., 2000; Ramsden et al., 2001; Xu et al., 2002; Carmine et al., 2003; Klein et al., 2003; Le et al., 2003; Zimprich et al., 2003; Huang et al., 2004; Zimprich et al., 2004). For instance, reduced expression of Nurr1 increased the vulnerability of midbrain DA neurons to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced injury which is used as a model for PD (Le et al., 1999a). Furthermore, a polymorphism in the Nurr1 intron was found in patients with PD (Xu et al., 2002). In addition, two heterozygous mutations on Nurr1 were revealed in 10 out of 107 individuals with familial PD (Le et al., 2003). Finally, genetic mutations in α-synuclein have been implicated in PD and En1 and En2 have been shown to regulate α-synuclein (Polymeropoulos et al., 1997; Simon et al., 2001).

Moreover, all three critical transcription factors, Engrailed-1, Nurr1, and Ptx3, must be present in the neuron to be considered a true dopaminergic cell. The fact that RA increases the expression of Nurr1 and not Ptx3 or Engrailed-1 suggests it does not drive the NT2 cells towards the DA phenotype. Multiple pathways are activated in parallel for the proper development of VM cells (FIG. 3.5). The earliest known transcription factor expressed in proliferating VM precursors cells Lmx1b is known to act on the Wnt pathway as well as increase the expression of Ptx3 (review (Burbach et al., 2003). The Wnts are highly conserved glycoproteins important for development (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Nusse, 1992; Nusse and Varmus, 1992; Salinas and Nusse, 1992; Parr et al., 1993; Fritzsch et al., 1995; Danielian and
McMahon, 1996). Three specific Wnt proteins, Wnt1, Wnt3a, Wnt5a, have been implicated in the development of midbrain DA neurons and were shown to influence the expression of Engrailed-1, Ptx3, and Nurr1 (Castelo-Branco et al., 2003). Wnt3a promoted the proliferation of precursor cells expressing Nurr1 but did not increase the number of TH-positive neurons. Wnt1 and Wnt5a increased the number of TH positive neurons. Wnt1 predominantly increased the proliferation of Nurr1 precursors while Wnt5a increased the proportion of Nurr1-positive precursors that acquired the DA phenotype and up-regulated Ptx3. Recently the expression of various Wnts in NT2 cells after 72 hours of RA treatment was reviewed (Katoh, 2002a). RA exposure decreased Wnt3a and slightly down-regulated Wnt5a (Katoh, 2002b; Saitoh and Katoh, 2002). Our observations are consistent with those of Mena and colleagues who showed that RA suppresses the DA phenotype and includes chorine acetyltransferase activity in fetal rat midbrain neurons and a human neuroblastoma cell line (Mena et al., 1994).

In summary, since the NT2 cells express three essential transcription factors for midbrain DA development and were previously shown to express Wnts, these human neural-progenitor like cells could be used as an in vitro model system to examine which agents might drive these cells toward the DA phenotype, although RA does not appear to be that agent.
FIGURE 3.5. Events important for the survival, induction, maintenance and maturity of midbrain DA neurons. VM cells are generated in the mid-hindbrain boundary under the influence of numerous inducing factors at various concentrations such as sonic hedgehog (SHH), fibroblast growth factor 8 (FGF8), retinoic acid (RA), and bone morphogenetic protein (BMP). One of the earliest transcription factors expressed during this proliferation phase is Lmx1b which in turn stimulates the Wnt1 glycoprotein and the downstream expression of Ptx3. The Wnt1 pathway has broad influence in that it is essential for the development of both the mid- and hindbrain. More specifically, Wnt1 increases the expression Wnt3a and Wnt5a. Wnt3a increases the proliferation of Nurr1-positive cells. Wnt5a increases Ptx3 expression as well as TH-positive cells by enhancing Nurr1 maturity. The Wnt pathway was also shown to affect the expression of Engrailed-1/-2. The next two critical transcription factors for dopaminergic differentiation that are expressed are Nurr1 and Ptx3. Both Nurr1 and Ptx3 increase the expression of TH and aldehyde dehydrogenase 2 (AHD2). The interrelationship between these multiple pathways allows for the proper development of midbrain DA neurons.
CHAPTER 4
The Effect of Lithium Treatment on the Proliferation, Survival, and Dopaminergic Transcription Factors in NT2 Precursor Cells

4.1 Introduction
The NTera2/D1 (NT2) cell line has been used as a human in vitro model system to examine neural development since these cells have numerous unique features similar to neural precursor cells (Andrews, 1984; Andrews et al., 1984; Lee and Andrews, 1986; Pleasure et al., 1992; Langlois and Duval, 1997; Bani-Yaghoub et al., 1999; Sandhu et al., 2002). A neural progenitor cell is identified as a cell that can self-replicate, give rise to all three neural lineages, and express proteins specific to neural stem cells. The NT2 cells meet these criteria. In addition the NT2 cells express Engrailed-1, Ptx3 and Nurr1 transcription factors as shown in Chapter 3 and therefore suggest that these cells have the potential to become DA with the correct stimuli. Thus NT2 cells can be used as a model to examine which pharmacological agents increase essential DA markers. Clinically, the degeneration of midbrain DA neurons is the cause of the symptoms in Parkinson’s disease.

In this chapter, we will examine the ability of lithium to induce expression of the transcription factors Nurr1, Ptx3, Engrailed-1 which are critical for the DA phenotype. Our laboratory and others have recently shown that treatment of the post-mitotic hNT neurons with lithium increased their TH expression and survival in vitro and in vivo (Zigova et al., 1999; Baker et al., 2000; Willing et al., 2002). In addition, Chen and colleagues illustrated lithium exposure (1mM) increased TH levels in human SH-SY5Y neuroblastoma cell line after 24 hours in culture (Chen et al., 1998). Furthermore, chronic lithium treatment increased the number of TH-positive neurons in culture and increased the TH protein levels in rat frontal cortex, hippocampus, and striatum (Otero Losada and Rubio, 1985; Chen et al., 1998).
Lithium is widely used as a mood-stabilizing drug to treat bi-polar (manic depressive) disorders (Goodwin and Jamison, 1990; Price and Heninger, 1994; Manji and Lenox, 2000; Manji et al., 2000a). Although it has been used to treat this psychiatric disorder for over fifty years, only recently have the advances in the cellular and molecular biology started to open the door on some of its mechanisms of actions. Lithium has dramatic effects on the morphogenesis in early development in vivo as well as embryonic stem cells differentiation in vitro (Lallier, 1952; Maeda, 1970; Kao et al., 1986; Van Lookeren Campagne et al., 1988; Livingston and Wilt, 1989; Hansen et al., 1990; Livingston and Wilt, 1990; Stachel et al., 1993; Schmidt et al., 2001; Kim et al., 2004). For instance in Xenopus, lithium causes a duplication of the dorsal axis (Kao et al., 1986). In vitro, lithium inhibited the differentiation of mouse ES cells into cardiac and myogenic cells in a concentration dependent manner (Schmidt et al., 2001). Recently, some developmental abnormalities have been reported in children whose mothers were treated with lithium for bipolar disease during pregnancy such as thyroid dysfunction, hypoglycemia, hypotonia and Ebstein’s anomaly which is a defect in the tricuspid valve (review (Pinelli et al., 2002).

In addition lithium affects expression of various genes and proteins (Dixon and Hokin, 1994; Hyman and Nestler, 1996; Ozaki and Chuang, 1997; Jope, 1999; Manji et al., 2001; Williams et al., 2002). It can influence numerous genes by the activation of transcription factors such as AP-1 (Bullock et al., 1994; Williams and Jope, 1995; Hedgepeth et al., 1997; Yuan et al., 1998). Lithium can affect neurotransmitter release and metabolism of monoamines (Baptista et al., 1990; Gilman et al., 1990; Goodwin and Jamison, 1990; Dixon and Hokin, 1997). For instance, lithium treatment induced the clustering of synapsin I, a presynaptic protein involved in the formation of synapses and neurotransmitter release, and therefore increased neurotransmitter release (Higashitani et al., 1990; Dziedzicka-Wasylewska et al., 1996; Hall et al., 2002). On the other hand, lithium has also been shown to inhibit the release norepinephrine and DA but may stimulate the release of serotonin (Hesketh et al., 1978; Smythe et al., 1979;
Treiser et al., 1981; McIntyre et al., 1983; Silverstone, 1985). Lithium exposure was shown to inhibit neuritic outgrowth in various in developing but not mature neurons by inhibiting the phosphorylation of various neurofilament proteins such as MAPs (Burstein et al., 1985; Bennett et al., 1991; Lenox and Hahn, 2000). For example, Lithium also provides neuroprotection and hence increases survival by increasing the levels of brain derived neurotrophic factor (BDNF) and B-cell lymphoma protein-2 (Bcl-2) (Grignon and Bruguerolle, 1996; Centeno et al., 1998; Manji et al., 2000a; Wei et al., 2000; Fukumoto et al., 2001). Clinically, because of lithium’s characteristic delay in therapeutic response, sometimes up to a month or more, it has been suggested that Li treatment might alter gene expression (Manji and Lenox, 1994; Manji et al., 1995). Long-term Li administration might alter transcription factors and/or post-translation modifications which might affect neural plasticity and neuronal synapses. For example, lithium was shown to induce morphological changes in axons and expression of synaptic proteins by inhibiting GSK-3β and thereby activating Wnt-7a pathway (Lucas and Salinas, 1997).

One reason why the molecular mechanism underlying the therapeutic effects of lithium have been difficult to characterize is that the lithium affects multiple cellular targets. It has been proposed that the lithium targets key components of signal transduction pathways (Manji et al., 1995). One of the prevailing hypotheses is the inositol depletion hypothesis, which is based on the observation that lithium inhibits inositol monophosphatase (IMPase) (Hallcher and Sherman, 1980; Berridge et al., 1989; Berridge and Irvine, 1989). The inhibition of IMPase decreases inositol which is necessary for the production of various second messengers such as diacylglycerol (DAG) and inositol-1,4,5-triphosphate which activate protein kinase C and increase intracellular calcium, respectively (Majerus, 1992; Gould and Manji, 2002). Previous research showed that lithium stimulated cell proliferation in some cell types and it was suggested to involve the phosphoinositol pathway (Hori and Oka, 1979; Ptashne et al., 1980; Korycka and Robak, 1991). Recently, it was also demonstrated that lithium
treatment increased the number of neural progenitor cells both in vitro and in vivo (Hashimoto et al., 2003; Kim et al., 2004). Furthermore, proliferation was also induced by lithium in glial cells in the rat pituitary (Levine et al., 2002). Therefore, we examined the effects of lithium on the proliferation of NT2 cells.

Another pathway lithium directly inhibits is glycogen synthase kinase-3 (GSK-3) (Klein and Melton, 1996). GSK-3 has multiple roles in cellular signaling from regulating neural plasticity to neuroprotection (review (Grimes and Jope, 2001). For instance, in vivo lithium treatment was shown to increase neuroprotection by up-regulating the expression of anti-apoptotic marker B-cell lymphoma protein-2 (Bcl-2) (Manji et al., 2000b). GSK-3 is also involved in the regulation of neuronal differentiation by phosphorylation and frequently inhibiting multiple downstream substrates such as proneural marker neuroD (Marcus et al., 1998). Lithium also has affects on several signaling cascades since exposure to lithium in rat increased cyclic AMP response element-binding protein (CREB) and activator protein-1 (AP-1) (Ozaki and Chuang, 1997; Unlap and Jope, 1997).

Recently, our laboratory showed that lithium increases the expression and survival of TH-positive hNT neurons (Zigova et al., 1999). Furthermore, when hNT neurons were pretreated with lithium prior to transplantation into PD rat models, not only did we get an increase in cell survival but also an increase in TH expression (Willing et al., 2002). In addition, lithium increased TH activity and catecholamine secretion in adrenal medullary cells in vitro (Terao et al., 1992). Furthermore, systemic administration in rat increased TH concentrations in the brain, particularly in the STR (Chen et al., 1998). Lithium’s affects on TH expression is thought to involve AP-1 transcription factors (Ozaki and Chuang, 1997; Chen et al., 1998; Yuan et al., 1998) while the effect on survival might be a result of the induction of bcl-2 expression and inhibition of Bax (Youdim and Arraf, 2004).
In addition to affecting the TH expression in post-mitotic neurons, lithium might activate the signaling pathway in neural precursor cells that specify the DA phenotype. The activation of the Wnt pathway by the inhibition of the GSK-3β is particularly important in DA development (Parr et al., 1993). As currently understood, Wnt proteins bind to the Frizzled receptors on the cell surface and through several cytoplasmic relay components inhibit GSK-3β. The inhibition of GSK-3β allows for the accumulation of β-catenin, which then enters the nucleus and forms complexes to activate transcription of Wnt target genes, some of which are important during dopaminergic neurogenesis and differentiation (Danielian and McMahon, 1996; Castelo-Branco et al., 2003). Previous research showed that the activation of the Wnt pathway increased the expression of Nurr1 and Ptx3. Therefore, since lithium treatment inhibits GSK-3β, it might mimic the Wnt pathway and increase the expression of Nurr1 and Ptx3.

In this experiment, we exposed NT2 cells to lithium chloride (Li) treatment for up to 4 DIV. We examined the expression of Nurr1, Ptx3 and Engrailed after Li treatment (1 mM) and report that there was no increase in the level of these transcription factors. However, there was an increase in NT2 cell viability and proliferation after Li treatment in this human cell line.

4.2 Method

4.2.1 Cell culture

The NT2 cells were obtained from American Type Culture Collection (Manassas, VA) and stored at -180°C prior to use. To culture the cells, they were first thawed rapidly at 37°C and transferred into a 15-cc tube containing Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen), 10% fetal bovine serum (FBS; Invitrogen), and Gentamicin (50 μg/mL, Sigma). The cells were centrifuged (160 g/ 7 min) and resuspended in 1 ml of the above fresh media and the cell number and viability was assessed with the trypan blue dye exclusion method.
The cells were plated in triplicate in 100 mm plates at a seeding density of 50,000 cells/cm² and maintained at 37°C in 5% CO₂ with 95% humidity. In the experiment determining the dosage concentration of lithium chloride (Li, Sigma), the DMEM/FBS/Gentamicin media was supplemented with 0.5 mM, 1.0 mM, 3.0 mM, 6.0 mM or 10 mM of Li for 4 DIV and the viability and total cell number was determined using the Beckman Coulter Cell Counter (Beckman Coulter, Inc., CA). In the second series of experiments we opted to use the clinically applicable dose of 1.0 mM Li not only for its therapeutic relevance but also due to the fact that this dose had no detrimental influence on the viability of the cells and had a profound effect on stimulating the cells. In addition, in previous studies with hNT neurons, 0.5 – 2.0 mM Li increased the dopaminergic phenotype as assessed by the number of TH-positive neurons (Zigova et al. 2000). The NT2 cultures were treated with Li (1 mM), potassium chloride (KCl; 1 mM, Sigma) and retinoic acid (RA; 10 µM, Sigma) for 1 DIV to assess the viability and total cell number of cultured NT2 cells using fluorescein diacetate (FDA) – propidium iodide (PI) staining on the live cells. We used KCl as the salt cation positive control, and RA since it is known to be important during the induction of neuronal differentiation. For cultures that were maintained for 4 DIV, the NT2 were prepared by first removing the media and adding 0.25% trypsin (Sigma, St. Louis, MO) with EDTA (Mediatech, Inc., Herndon, VA) for 2 minutes. About 25 ml of DMEM/FBS with and 0.05% DNase (Sigma) was then added to the cultures and the cells were then aspirated from the culture wells and centrifuged for 5 min at 1000 rpm. The cells were washed and centrifuged three times in the DMEM/ FBS/ 0.05% DNase and the pellet was resuspended in cold PBS for further Western Blot analysis or in 1 ml of media for the Beckman Coulter Cell Counter.

4.2.2 Immunocytochemistry
For immunocytochemistry, the cultures were first washed in 0.1M phosphate-buffered saline (PBS; pH 7.4), then fixed with cold 4% paraformaldehyde for 15 minutes and washed three times with cold PBS. For immunofluorescence, slides were carefully washed with fresh cold 0.1M PBS and treated for 1 h at room
temperature in a mixture of 10% normal goat serum, 0.03% Triton X-100 in PBS. Cultures were then incubated overnight at 4°C with the following primary antibodies, Nurr1 (1:100, mouse, BD Biosciences), Ptx3 (1:250, rabbit, Abcam) and Ki67 (1:100, mouse, Abcam). After several PBS washes, primary antibodies were incubated with appropriate secondary antibodies (Molecular Probes, Eugene, OR; Alexa Fluor 594 goat anti-rabbit, 1:2,000; Alexa Fluor 546 goat anti-mouse 1:1,000, Alexa Fluor 488 goat anti-mouse, 1:800). The slides were washed with cold PBS and coverslipped with 95% glycerol. To visualize cell nuclei, some cultures were coverslipped using Vectashield with DAPI (Vector Laboratories, Burlingame, CA). The sections were observed under epifluorescence and photographed on the Olympus BX60 microscope.

4.2.3 Western Blot(138,773),(992,807)
The NT2 cells were trypsinized and washed as described earlier and then the sample was placed in cold PBS with 10 mM dithiothreitol and sonicated for 30 seconds (3x's). Protein samples (20 µg) and full range molecular weight markers (Amersham Bioscience) were resolved on 10% SDS-PAGE gel, and transferred to Invitrolon PVDF membranes (Invitrogen). The membranes were incubated in Tris-buffered saline (TBS) containing 5% non-fat milk and 0.1% Tween-20 for 1 hour at room temperature to block non-specific binding, and then incubated overnight in appropriate antibody at 4°C. The membranes were washed three times, 10 minutes each, in TBS with 0.1% Tween-20 and incubated in peroxidase-conjugated anti-mouse IgG (1:30,000, Jackson ImmunoResearch) or peroxidase-conjugated anti-rabbit (1:20,000) for 1 hour at room temperature. Primary and secondary antibodies were diluted in TBS, 5% non-fat milk and 0.1% Tween-20. Immunoreactivity was visualized using a West Pico Chemiluminescent Kit (Pierce Biotechnology). Digitized images of the films were analyzed using Image Pro-Plus (Media Cybernetics, Silver Springs, MD) software. The primary antibodies and their appropriate concentrations were Nurr1 (1:1,000, mouse, BD Biosciences), Ptx3 (1:500, rabbit, Abcam), Engrailed-1 (1:500, rabbit, Chemicon), β-catenin (1:5,000, rabbit, BD Biosciences),
glycogen synthase kinase-3β (1:1,000, rabbit, Cell Signaling Technology, Inc., MA), and Bcl-2 (1:500, mouse, Calbiochem).

4.2.4 Quantitative Analysis
For the immunocytochemical analysis, the cells were screened in a blind coded manner using a 20x objective and a photographic frame (field = 0.3 mm²). Counts were performed from 30 fields per condition (5 pictures per well times 6 wells per condition) from three independent platings. To quantify the viability and total cell number for NT2 cells at 4 DIV, the Beckman Coulter Cell Counter was used with 3 samples from three independent platings. For western blot analysis, three different protein samples were used and Image Pro-Plus determined the optical densities of the different samples. The values for each experiment were reported as mean ± SEM. Data was analyzed using analysis of variance (ANOVA). The Newman-Keuls test was employed for the post hoc comparisons.

4.3 Results
4.3.1 Dose effect of lithium treatment on the viability and total number of NT2 cells at 4 DIV
We exposed NT2 cells to 0.5 mM, 1.0 mM, 3.0 mM, 6.0 mM, and 10 mM of Li for 4 DIV and then determined the effect of these dosages of Li on the viability and total number of NT2 cells. ANOVA revealed a significant difference in the total cell number between the untreated and treated NT2 cells (FIG. 4.1A, F₅,₁₇=14.3, p<0.001). In addition, there was no significant difference in the viability between the Li-treated and untreated NT2 cells (FIG. 4.1B).

The 1.0 mM (w=17.2, p<0.01), 3.0 mM (w=41.0, p<0.01), 6.0 mM (w=17.0, p<0.01) and 10 mM (w=11.6, p<0.01) Li-treated cultures had more cells compared to the untreated NT2 cells. There was no difference in the total cell number between the untreated and the 0.5 mM Li-treated NT2 cells (w=0.27, p>0.05). The number of cells peaked at 3.0 mM Li. There were significantly more NT2 cells in the 3.0 mM Li than in the untreated (w=41.0, p<0.01), the 0.5
mM (w=48.0, p<0.01), and 1.0 mM Li (w=5.1, p<0.05) groups. While the cell number had a tendency to decrease at 6 mM Li (w=5.3, p>0.05) and 10 mM Li (w=9.02, p>0.05), these were not significantly different from 3.0 mM Li-treated group.

4.3.2 Expression of Nurr1 and Ptx3 in NT2 cells after 1 day of retinoic acid or lithium treatment
When we exposed NT2 cells to one day of Li, RA, or KCl, ANOVA revealed significant differences in the Nurr1 expression between the untreated and treated groups (FIG. 4.2A, F3,83=4.6, p<0.0054). After 24 h of treatment, only the RA treated group had significantly more Nurr1 expression (w=6.7, p<0.05), while no difference in Nurr1 expression was observed between the control Li- (w=1.3, p>0.05) and KCl- (w=0.8, p>0.05) treated NT2 cells. None of the treatments influenced Ptx3 expression in NT2 cells (FIG. 4.2B, F3, 39=1.0, p=0.40).

4.3.3 Lithium's effect on the expression of dopaminergic transcription factors in NT2 cells at 4 DIV
Western blot analysis revealed bands for Engrailed-1 (55 kDA), Ptx3 (35 kDA) and Nurr1 (70 kDA) (FIG. 4.3 A,C,E). When NT2 cells were exposed to 4 days of 1 mM of Li treatment, there was no change in the relative amount of Engrailed-1 (FIG. 4.3B, F1,11=1.2, p=0.031) or Nurr1 (FIG. 4.3F, F1,5=0.6, p=0.48) protein. However, there was a significant decrease in the concentration of Ptx3 protein in NT2 cells after 4 days of Li treatment (FIG. 4.3D, F1,7= 6.2, p=0.047).
FIGURE 4.1. The influence of lithium dosage on the proliferation and viability of NT2 cells after 4 DIV. (A) The total number of cells was significantly greater when NT2 cells were exposed to between 1.0 mM and 10 mM of Li treatment with 3.0 mM containing the most NT2 cells. (B) Li treatment between 0.5 mM to 10 mM had no effect on the viability of NT2 cells. *p<0.01

FIGURE 4.2. Percent of Nurr1- and Ptx3-positive NT2 cells after 1 day of lithium or retinoic acid treatment. (A) Around 12% of NT2 cells endogenously express Nurr1. Exposure to Li (1.0 mM) did not increase the Nurr1 expression while RA treatment (10 μM) increased Nurr1 expression to 20%. (B) Around 3% of NT2 cells express Ptx3 after 1 DIV, and none of the treatments increased Ptx3 expression in NT2 cells. **p<0.05
4.3.4 Lithium’s influences on the GSK-3β / β-catenin pathway in NT2 cells
Western blot analysis revealed band for GSK-3β (50 kDA) and β-catenin (90 k DA) in the NT2 cells (FIG. 4.4A,C). Four days of Li treatment did not inhibit GSK-3β (FIG. 4.4B, 20 F1,11=.00, p=0.99) or increase the β-catenin (FIG. 4.4D, F1,15=0.30, p=0.59) protein in NT2 cells.

4.3.5 Lithium’s effect on proliferation and viability of NT2 cells after 1 DIV
We used immunocytochemical techniques to determine the influence of Li treatment on proliferation and viability of NT2 cells. The Ki67 antigen is expressed by proliferating cells in all active phases of the cell cycle (G1, S, G2 and M phase) and is absent in resting (G0) cells. The NT2 cell nuclei were visualized with DAPI staining (4.5B1, blue) and Ki67 was immunolabeled with rhodamine (FIG. 4.5B1, red). We used two controls, one group with no treatment, and the other group with 1mM of KCl. Nearly all the NT2 cells treated with Li were positive for Ki67 (FIG. 4.4B2) compared to only about half of the untreated and KCl-treated NT2 cells (FIG. 4.4B2). ANOVA revealed a significant difference between the treatment groups of NT2 cells (FIG. 4.4B, F2,59=59.8, p<0.0001). Li-treated NT2 cells had significantly more Ki67-positive cells compared to the untreated NT2 cells (w=100.8, p<0.01) and KCl-treated NT2 cells (w=76.8, p<0.01). There was no difference in the number of Ki67-positive in the untreated and KCl-treated NT2 cells (w=1.63, p>0.05). Therefore, after only 1 day of Li treatment, there was significantly more NT2 proliferating cells than in the untreated group.

When we examined the viability using fluorescein diacetate (FDA) to stain living cells green and propidium iodide (PI) which labels dead cells red (FIG. 4.5A1), the overall ANOVA revealed significant differences between the three groups (FIG. 4.5A2, F2,59=20.4, p<0.0001). The Li-treated NT2 cells had significantly more viable cells compared to the control NT2 cells (w=35.6, p<0.01) and the
FIGURE 4.3. Expression of essential dopaminergic transcription factors in NT2 cells after lithium treatment. Four days of Li treatment (1.0 mM) did not increase the expression of Engrailed-1 (A,B) or Nurr1 (E,F) in NT2 cells. Furthermore, Li treated cells showed a decrease in relative Ptx3 protein (C,D). p<0.05
KCl-treated NT2 cells (w=24.7, p<0.01). There was no difference in the number of viable cells between the untreated NT2 cells and the NT2 cells treated with KCl (w=0.99, p>0.05). Even though the viability and the proliferation of the Li-treated NT2 cells was significantly greater than observed in the control groups, there was no difference in the total number of cells after 1 DIV (FIG. 4.5C, F2, 59=0.50, p=0.61).

4.3.6 Lithium’s influence on the total cell number and viability of NT2 cells after 4 DIV
We determined the total number and the viability of NT2 cells after 4 days of Li-treatment. There was no difference in the total number of viable cells between the untreated and the Li-treated NT2 cells (F1,5=0.43, p=0.55) with both cultures being over 90% viable (FIG. 4.5D). However, there was a significant increase in the total number of cells in the Li-treated group (FIG. 4.5E, F1, 5=14.3, p=0.02) at 4 DIV. There was an average of 80 million cells in the Li-treated groups compared to 60 million in the untreated NT2 cells at 4 DIV (w=13.2, p<0.05).
FIGURE 4.4. Western blot analysis of the Gsk-3β / β-catenin pathway in NT2 cells. Four days of Li treatment (1.0 mM) did not inhibit the Gsk-3β (A,B) in NT2 cells and therefore no accumulation of β-catenin (C,D) was observed.
FIGURE 4.5. The influence of lithium treatment on the proliferation, viability, and total cell number in NT2 cultures. (A) The viability increased when NT2 cells were exposed to 1 day of Li (A1) as assessed by fluorescein diacetate (FDA) to stain living cells green and propidium iodide (PI) which stains dead cell red (A2). (B1) NT2 cells were stained a marker labeling proliferating cells, Ki67 (red), and their nuclei visualized with DAPI (blue). (B2) When the number of Ki67-positive cells was quantified after 1 DIV, around 50% of untreated NT2 cells were proliferating, while nearly 99% of Li treated NT2 cells were Ki67-positive (red). (C) The total NT2 cell number did not increase after 1 DIV. (D) After 4 DIV, the viability of NT2 cells was above 80% in the Li treated cultures and the untreated NT2 cells increased from 60% at 1 DIV to above 80% in 4 DIV as well. (E) After 4 DIV, the total cell number in the Li-treated cultures increased by approximately 20%. *p<0.01, **p<0.05
4.3.7 Lithium’s effect on the anti-apoptotic protein Bcl-2 in NT2 cells

We wanted to determine if the increase in total cell number of NT2 cells with Li treatment might involve an increase of anti-apoptotic protein B-cell lymphoma protein-2 (Bcl-2). We previously determined that 4 weeks of RA-treatment increased the Bcl-2 protein in the neuronal hNT population. However, our ANOVA revealed no difference in the relative amount of Bcl-2 protein in the untreated and Li-treated NT2 cells at 4 DIV (FIG. 4.6; $F_{1,7} = 0.35$, $p=0.57$).

![Image of Bcl-2 expression](image)

**FIGURE 4.6.** Anti-apoptotic marker Bcl-2 expression in NT2 cells after 4 DIV. A 25 kDA band was observed in NT2 cells (A), however 4 days of Li (1.0 mM) did not increase the expression of Bcl-2 (B).
4.4 Discussion

We investigated the effect of Li treatment on viability, proliferation and the expression of transcription factors important for dopaminergic development of NT2 cells and found that Li-treatment significantly increased the number of total cells compared to the control NT2 cells, although there was no difference in the viability of NT2 cells with various lithium doses at 4 DIV.

4.4.1 Lithium’s effect on dopaminergic differentiation

Neither Li nor KCl (cation control) treatment increased the expression of Nurr1 or Ptx3 in NT2 cells after 1 DIV, while RA increased Nurr1 expression in NT2 cells. In terms of Ptx3 expression, none of the treatments increased the Ptx3 expression in NT2 cells, while exposure to RA-treatment had a tendency to decrease Ptx3 expression. These results with RA treatment were in agreement with our previous observations that 3 – 5 weeks of RA treatment decreased the expression of Ptx3 in the hNT neurons (see Chapter 3). Next we investigated the effect of longer Li treatment (4 DIV) on the expression Nurr1, Ptx3 and Engrailed-1 in NT2 cells using Western blot analysis. Once again, lithium did not increase the expression of these transcription factors critical for the development of a dopaminergic phenotype, and in fact, there was a decrease in the relative amount of Ptx3 protein.

We previously showed that exposure to RA increased Nurr1 expression in all neuronal-committed NT2 cells also known as hNT neurons or NT2-N cells (Misiuta et al., 2003a). RA treatment is the only factor that increased the expression of Nurr1 after only 24 hours of treatment. The activation of Nurr1 is essential for the dopaminergic phenotype however we also showed that TH-negative hNT neurons also expressed Nurr1. Furthermore, Nurr1 is expressed in various neurons in the CNS and is not specific to DA neurons (Saucedo-Cardenas and Conneely, 1996; Xiao et al., 1996). RA might be necessary to drive the NT2 cells toward the neuronal lineage via the activation of Nurr1 but not necessarily toward the DA phenotype. This rapid increase might be a result
Nurr1 heterodimerizing with RA receptors (RXRs and RARs) which other investigators have shown to occur \textit{in vitro} (Perlmann and Jansson, 1995; Zetterstrom et al., 1996; Aarnisalo et al., 2002; Sacchetti et al., 2002; Wallen-Mackenzie et al., 2003).

Previous research showed that the activation of the Wnt1 pathway increased the expression of Nurr1 and Ptx3 and the proliferation and differentiation of DA precursors during VM development (Danielian and McMahon, 1996; Castelo-Branco et al., 2003). Li treatment is thought to mimic the activation of the Wnt1 pathway by inhibiting glycogen synthase kinase-3\(\beta\) (GSK-3\(\beta\)) (Klein and Melton, 1996; Hedgepeth et al., 1997; Phiel and Klein, 2001). The inhibition of GSK-3\(\beta\) allows for the accumulation of \(\beta\)-catenin, which then enters the nucleus and forms complexes to activate transcription of other Wnt target genes, some of which are important during dopaminergic differentiation like Wnt3a and Wnt5a. We examined the GSK-3\(\beta\) and \(\beta\)-catenin in Li-treated NT2 cells and as expected, found no difference in GSK-3\(\beta\) / \(\beta\)-catenin expression between untreated and Li-treated NT2 cells. Therefore, 1.0 mM Li for 4 days on NT2 cells does not inhibit GSK-3\(\beta\) nor does it increase Nurr1, Ptx3, or Engrailed-1 expression in NT2 cells.

Even though Banchaabouchi and colleagues suggested a close interaction between lithium and Nurr1 expression, the actual affects of lithium on the dopaminergic phenotype might vary according to the dosage and days of exposure (Al Banchaabouchi et al., 2004). The 4 days of Li treatment might be too short to affect the relative amount of transcription factors. It takes at least 7 days of RA treatment to see an up-regulation of neuronal markers such as III-\(\beta\) tubulin or microtubule associated protein (Pleasure and Lee, 1993). On the other hand, lithium might drive the NT2 cells down another pathway not involving Engrailed-1. For instance, the Li exposure decreased the expression of Engrailed-1 in mouse embryonic stem cells (Schmidt et al., 2001).
Although there is a relative decrease in Ptx3 protein after 4 DIV, long-term Li exposure might also reverse this result. However, it was recently shown that chronic treatment of lithium decreased the expression of Nurr1 in rat brain (Al Banchaabouchi et al., 2004), and therefore the ideal exposure time still needs to be elucidated.

4.4.2 Lithium’s effect on cell proliferation

Our analysis of the dose response of Li to the total cell number of NT2 cells revealed an increase in cell number after 4 DIV. We therefore decided to examine the effect Li treatment has on the viability and proliferation of NT2 cells. After 24 hours of Li-treatment, Li-treated NT2 cells were more viable compared to the untreated NT2 cells as assessed by FDA/PI staining. In addition, untreated NT2 cultures had fewer proliferating cells than Li-treated NT2 cultures as determined with Ki67 immunolabeling; this resulted in significantly more cells in Li-treated cultures after 4 days. The viability remained above 80% in the lithium treated group, while untreated NT2 cells increased from 65% at 1 DIV to 80% at 4 DIV. Once again, as was observed in the dose response curve, there was about a 20% increase in total cell number after 4 days of Li treatment.

While increased immunostaining for Ki67 suggests an increase in proliferation, the increase in cell number could also be a function of a decrease in cell death. To address this issue, we examined the effect of Li treatment on an anti-apoptotic marker B-cell lymphoma protein-2 (Bcl-2). In vivo Li treatment was shown to increase the expression of Bcl-2 by inhibiting GSK-3β (Manji et al., 2000b). We showed that Bcl-2 was not enhanced after 4 days of Li treatment as we suspected since it involves the GSK-3β pathway. Therefore we can also conclude that the increase in total cell number by Li treatment was not due to an increase in the survival-promoting anti-apoptotic protein, Bcl-2. However, long-term exposure (> 7 DIV) might be necessary to observe an up-regulation of anti-apoptotic markers. For example, it took 7 days of Li treatment to protect cultured rat cerebellar, cerebral cortical, and hippocampal neurons against glutamate-
induced excitotoxicity by the up-regulation of anti-apoptotic markers (Nonaka et al., 1998; Grimes and Jope, 2001).

One possible mechanism for lithium’s action on proliferation is by inhibition of inositol monophosphatase (IMPase) and/or related phosphomonoesterases (Berridge and Irvine, 1989; York et al., 1995). The inhibition of IMPase decreases inositol which is necessary for the production of various second messengers such as diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3) which activate protein kinase C and increase intracellular calcium, respectively (Majerus, 1992; Gould and Manji, 2002). Previous research showed that lithium stimulated cell proliferation in some cell types and it was thought to involve the phosphoinositol pathway (Hori and Oka, 1979; Ptashne et al., 1980; Korycka and Robak, 1991). Recently, it was also demonstrated that Li treatment increased the number of neural progenitor cells both *in vitro* and *in vivo* (Hashimoto et al., 2003; Kim et al., 2004). Furthermore, proliferation was also induced by lithium in glial cells in the rat pituitary (Levine et al., 2002). NT2 cells have similar properties to neural progenitor cells since they express various neural antigens, self-replicate and produce all three neural lineages, neurons, astrocytes, and oligodendrocytes (Andrews et al., 1984; Lee and Andrews, 1986; Pleasure and Lee, 1993; Langlois and Duval, 1997; Bani-Yaghoub et al., 1999; Sandhu et al., 2002). The fact that lithium stimulates proliferation in NT2 cells as it does in neural precursor provides yet another example of a similarity between NT2 cells and neural precursors. In addition, increasing cell proliferation may also be important in expanding cell lines, especially cell lines with a low percentage of progenitor cells. Recently using DNA microarrays, the Wnt pathway was shown to increase proliferation and cell adhesion of CD34+ thymocytes; CD34 is a marker for hematopoietic progenitor cells (Staal et al., 2004). Therefore, pretreatment with Li may be an effective protocol to expand stem cell lines.
4.5 Conclusion
Since the NT2 cells express three essential transcription factors for the development of midbrain DA neurons and have properties similar to neural progenitor cells, they are an excellent human cell source to study the effect of various pharmacological treatments that could drive cells down the dopaminergic pathway. Different approaches are currently being explored to try and increase the dopaminergic phenotype in various cell types such as embryonic stem cells, neural precursors and mesenchymal cell lines (De Boer et al., 2004). Various TH-inducing agents such as acidic fibroblast growth factor (FGF) and co-activating substances tissue polypeptide antigen (TPA; PKC activator), 3-isobutyl-1-methylxanthine (IMBX; phosphodiesterase inhibitor), forskolin (PKA activators), and the neurotransmitter DA, can increase TH expression dramatically especially if given in a cocktail one week after plating. In NT2 cells specifically, the combination of SHH and FGF8 only increased TH expression by 2%, however, in conjunction with a cocktail of the known activators listed above increased the expression of TH in hNT neurons up to 80% (Iacovitti et al., 2001; Stull and Iacovitti, 2001). Our laboratory has also demonstrated that the TH expression in hNT neurons can be influenced by lithium or by coculturing hNT neurons with Sertoli cells which are known to secrete several trophic factors (Othberg et al., 1998; Willing et al., 1999; Saporta et al., 2004). It might be necessary to increase the time NT2 cells are exposed to lithium in order to effect the Wnt pathway and eventually induce the expression of TH. Chen, Yang and colleagues demonstrated an increase in TH expression through the activator protein-1 (AP-1) transcription after Li treatment in vitro. (Chen et al., 1998). They also reported that chronic Li administration (1.0 mM) resulted in a significant increase in TH levels in the rat frontal cortex, hippocampus and striatum. However, lithium affects multiple pathways and therefore therapeutically speaking, it might be necessary to specifically identify the pathway by which Li induces TH expression and find more specific agonist/antagonist to produce the desired result.
CHAPTER 5. Conclusion

The Characterization of the NTera2/D1 Cell Line

The NTera2/D1 (NT2) cell line was originally derived from embryonic carcinomas (EC) cells which have strikingly similar properties to embryonic stem (ES) cells (Friedman and Moore, 1946; Dixon and Moore, 1953; Pierce et al., 1957; Martin and Evans, 1975; Damjanov et al., 1979). ES cells are defined as totipotent cells since they are capable of either symmetric or asymmetric division that build an entire organism (reviewed (Limke and Rao, 2002). As the cells differentiate further, they become more restricted to their developmental potential. A cell is called multipotent when it has the ability to differentiate into multiple cell lineages however some restrictions exist on its final fate. Neural stem cells are defined as cells which can self-replicate and generate all three major types of cells in the CNS: neurons, astrocytes, and oligodendrocytes. NT2 cells have been labeled neural progenitor cells since they possess these properties. This dissertation further examined the potential of this human neural stem cell-like precursor and their neuronal progeny in vitro in terms of their survival, proliferation, neuronal and dopaminergic development.

5.1 Potential of neural stem cells

The excitement generated in the field of stem cell research was fueled by the hope that stem cells would be able to repair or replace damaged cells. One mechanism proposed, cell replacement therapy, was concerned with replacing damaged or degenerating neurons with the progeny of neural stem cells in the hope that these cells would reduce symptoms that were created by the loss of the specific cell types (Bjorklund and Lindvall, 2000a, b). Another promising area in the stem cell field that became important in neurodegenerative disease is the ability of stem cells to increase the survival and hence neuroprotection of the remaining neurons. More recently, since the discovery that neural stem cells exist in the adult brain, scientists have been investigating possible mechanisms
to stimulate the endogenous cells to proliferate and differentiate into the appropriate cell type that can help restore some of the function that was lost in the disease state (Li et al., 2000; Roy et al., 2000; Song et al., 2002). Even more interesting is the recent data which suggests the possibility of stem-like properties in astrocytes. Astrocytes under normal conditions are quiescent \textit{in vivo}, however, \textit{in vitro} data demonstrated astrocytes were capable of dedifferentiating then re-differentiating into neurons (Kondo and Raff, 2000; Sommer and Rao, 2002). The potential of the events that can stimulate neural precursors to proliferate, differentiate and promote survival has yet to be determined.

5.2 hNT neurons as a model to study neuronal survival and growth
The hNT cells provide an excellent \textit{in vitro} model system to examine survival, neuritic outgrowth and neuronal differentiation as well as the DA phenotype of a human cell line. The pharmacological agent that has been most widely used to differentiate the NT2 cells into hNT neurons is RA. Depending on the length of RA treatment determines their eventual phenotype to neurons, astrocytes or oligodendrocytes (Andrews, 1984; Andrews et al., 1984; Lee and Andrews, 1986; Pleasure et al., 1992; Langlois and Duval, 1997; Bani-Yaghoub et al., 1999; Sandhu et al., 2002). Our collaborators, Layton BioSciences (no longer in existence) used the standard protocol for differentiation of NT2 cells into a pure neuronal population called the hNT neurons by exposure to RA for 3 to 5 weeks (10 µM). We studied the survival, neurite maturation, and dopaminergic phenotype of 3 different hNT neurons, 3 weeks (3w), 4 weeks (4w) and 5 weeks (5w) of RA treatment. We demonstrated that short-term RA exposure (3w) produced more viable and TH-positive cells, however, length of RA treatment did not alter neuritic outgrowth.

For the certain neurodegenerative disease such as PD, a possible therapy could be a combination of protecting the remaining neurons and increasing their neuritic sprouting in the striatum to provide for greater dopaminergic innervation.
The developing CNS is the ideal environment to support survival, neurite outgrowth, differentiation, and maintain phenotypes. However, it has never been elucidated if there is a specific period or brain regions during development that significantly increase the survival, neuritic outgrowth, and dopaminergic phenotype. We cocultured the human hNT neurons with various ages of neural tissue from different regions of the developing rat brain. Embryonic rat neural cells increased the survival of hNT neurons up to two fold, while both embryonic and postnatal cells increased the neuritic maturation as assessed by the number of primary neurites. Furthermore E18 cultures provided an environment that increased the survival to the greatest extent in hNT neurons. Analyses of which factors, cells or events would have a profound influence on survival could have important therapeutic implications. For instance in diseased brain, the environment is inhospitable for neuronal survival and growth since usually neuronal degeneration is followed by inflammation which has even more detrimental effects on these already vulnerable cells. Therefore, the replacing cells not only have to survive the conditions during transplantation but the conditions in the unfavorable environment itself. One way is by pre-treating cells which are about to be transplanted and/or pre-treating the degenerating environment in the brain with survival-promoting factors prior to transplantation.

The increase in hNT neurons survival in cocultures can be mediated by either cell-to-cell interactions or soluble factors released by surrounding cells. Within a cell, exposure to one factor can influence multiple pathways; therefore, one event does not just influence one activity. In addition, there is not only a redundancy in the signal pathways but there is also a cross-talk between different pathways within the cell and between neighboring cells. Development biologists are just beginning to determine how specific factors influence the downstream events. A more complete understanding of these activities will allow researchers more opportunities to use a combination of treatments to produce the desired clinical outcomes. It is clear from our results that the E18 environment provides factors which can increase the survival of hNT neurons up to 2-fold and an analysis of
which molecules or cells might lead to the discovery of new therapies to increase the survival of degenerating cells.

The expression of specific receptors on hNT neurons gives them an intrinsic mechanism to respond to the local environment which can increase their survival. The fact that certain types of hNT neurons (i.e. 3w compared to 5w) respond to local environment differently can be explained by the fact that RA exposure up-regulates various receptors and proteins such as neurotrophins and their receptors (Cheung et al., 2000). In addition, the length of RA treatment may also influence the expression of neurotrophic receptors and transcription factors. It is well established that the overall levels of NFs determine the balance between cell survival and cell death during development (Piontek et al., 1999). Primary rat neural cells express different NF in certain areas at specific times. The hNT neurons react to specific NFs by the expression of specific NF receptors.

Neurotransmitters themselves have been shown to influence the survival of neurons. For instance, the neurotransmitter $\gamma$-aminobutyric acid (GABA) significantly enhanced the survival of rat E18 STR neurons in vitro (Ikeda et al., 1997). Also the activation of glutamate receptors increased the survival of postnatal rat cerebellar granule neurons and embryonic spinal cord neurons (Balazs et al., 1988a; Balazs et al., 1988b; Monti et al., 2002). In our coculture studies, we dissected different regions of the rat brain with each area having different predominant neurotransmitters. In the STR, 90% of the medium spiny neurons are inhibitory and hence contain GABA, while VM cells predominantly synthesize DA. The CER contains cells that use both glutamate and GABA. The hNT neurons are known to express GABA, DA, N-methyl-D- aspartate, and nicotinic receptors (Beczkowska et al., 1997; Matsuoka et al., 1997; Neelands et al., 1998; Neelands et al., 1999; Zigova et al., 2000a; Newman et al., 2002; Sodja et al., 2002). Therefore, hNT neurons could be used as a model system to examine the effects of specific neurotransmitters and/or their antagonist on survival of a human neuronal cell population.
All areas of the brain at both E14 and P1 increased the neuritic outgrowth of hNT neurons. The sprouting of neurites called neuritogenesis can have extremely important implications in various neurodegenerative diseases. The neuritic outgrowth as assessed by the number of primary neurites was greatest in E14 cocultures with an average of 4 neurites per cell. The fact that hNT neurons can form such extensive neuritic networks within 5 days of plating illustrates their amazing intrinsic cytoskeletal dynamics and their ability to react to the local environment. Growth cones and microtubules are important for the maintenance of elongated neurites, their polarity and speed of growth (Zheng et al., 1991; Zakharenko and Popov, 1998). Neurobiologist believe that neuritogenesis occurs in post-mitotic neurons during migration where they extend there lamellipodia (a sheet-like extension at the edge of the cells that contains a cross-link F-actin meshwork) and filopodia (a long, thin protrusion at the periphery of cells and growth cones that is composed of F-actin bundles) (Puelles and Privat, 1977; Bourrat and Sotelo, 1988; Lambert de Rouvroit and Goffinet, 2001). RA exposure increased the number of primary neurites in hNT neurons could be due a combination of factors. First, hNT neurons have the appropriate intrinsic mechanisms such as growth associated proteins and cytoplasmic microtubules to extend neurites. Next, the neural cells themselves might release factors into the extracellular matrix which provides a suitable environment for neuron outgrowth. Finally, the contact with specific cells, such as radial glial which are known to guide axonal growth, can be excellent substrata for neuronal outgrowth (review (Steinbach and Schlosshauer, 2000).

5.3 Dopaminergic potential of NT2 cells

In terms of the DA phenotype, our laboratory has shown that hNT neurons express various markers for midbrain DA neurons such as including TH, DAT, D2 receptor, and AHD-2 (Zigova et al., 2000a). During development, the local environment as well as the intended target for DA neurons, the STR, provides signals that allow for the proper development of midbrain DA neurons. We cultured E14 rat cell from the nigrostriatal pathway, the VM and STR, and the
non-target CER with hNT neurons and assessed their TH expression. There was no increase in TH expression but rather a decrease in TH expression with increased DIV. The fact that the E14 VM or STR cells did not increase TH expression in hNT neurons could have many explanations. First, at E14 most of the VM precursor cells already express transcription factors critical of DA neurons and therefore, these signals necessary for DA induction may no longer be present. Second, removing cells from the developing CNS and putting them in vitro disrupts the three dimensional cytoarchitecture of the region which might be critical for proper development. It is known that many signaling factors are released in specific areas in a concentration dependent manner and the dissociation process might interfere with this gradient (Durston et al., 1989; Ye et al., 1998; Lee and Jessell, 1999).

Another reason why hNT neurons did not develop DA phenotype in response to coculture with VM or STR deals with the differentiation process of hNT neurons. RA exposure of 3w or greater might differentiate these cells into another neuronal phenotype such that they could not respond to DA differentiation factors provided by the developing VM. This theory is justified by the fact that longer RA exposure (5w compared to 3w) produced fewer TH-positive cells. Further, the induction of differentiation begins with cessation of proliferation. RA is known to stop proliferation and hence induce differentiation (Spinella et al., 1999). The hNT neurons in these experiments were post-mitotic due to RA exposure and the addition of mitotic inhibitors. Therefore, the hNT neurons may no longer be capable of responding to other differentiating factors released by the developing rat brain that would have increased the DA phenotype of dividing VM precursor cells.

When we examined the expression of three critical transcription factors necessary for the development of VM cells, we discovered that the NT2 cells endogenously expressed Engrailed-1, Nurr1, and Ptx3. However, exposure to RA down-regulated Ptx3 and Engrailed, although, Nurr1 was up-regulated.
These findings suggest that RA exposure (3w – 5w) does not drive the NT2 cells down the developmental pathway for DA phenotype. Even though there was an up-regulation of Nurr1, Nurr1 has been shown to be in other cell types and has actually been suggested to be more important for neuronal differentiation (Xiao et al., 1996; Zetterstrom et al., 1996). Since the NT2 cells express three transcription factors essential for the development of midbrain DA neurons and have properties similar to neural progenitor cells, they are an excellent human cell source to study the effects of various pharmacological treatments that could drive cells down the dopaminergic pathway.

5.4 Lithium's influence on the dopaminergic markers in NT2 cells

Even though our laboratory previously illustrated that lithium increased TH expression in hNT neurons but did not influence the DA transcription factors Engrailed-1, Ptx3, or Nurr1 in NT2 cells could be explained by the fact that hNT cells are post-mitotic neurons that already express various neurotransmitters and transporters. For instance, hNT neurons express TH and vesicular monoamine transporter 2 (VMAT2) (Misiuta et al., 2002) both of which are known to be influenced by Li treatment (Cordeiro et al., 2000; Cordeiro et al., 2002). For example Li increases gene expression through the activator protein-1 (AP-1) transcription factor pathway in vitro and AP-1 sites have been identified in the promoter regions of genes for TH (Kumer and Vrana, 1996; Ozaki and Chuang, 1997). On the other hand, NT2 cells are known to express various markers typical of neural progenitor cells which are important in cell division and therefore, lithium in NT2 cells might stimulate pathways important for proliferation and survival (FIG. 5.1).
FIGURE 5.1. Influences of lithium on NT2 and hNT cells. In NT2 cells, Li treatment increased proliferation and viability, while in post-mitotic hNT neurons, Li increased the expression of various proteins important for neurotransmitter function such as TH and VMAT2. Li also increased their survival by up-regulating pro-apoptotic marker Bcl-2.
However lithium exposure did not increase the relative amount of protein for the three DA transcription factors at least in short-term culture. Long-term Li treatment could increase the expression of TuJ1 and NeuroD which is necessary to drive the neural precursor cells down the neuronal. GSK-3 expression increases during development of rat brain (Takahashi et al., 1994). GSK-3 is involved in the regulation of neuronal differentiation via the phosphorylation and frequently the inhibition of multiple substrates such as neuroD (Marcus et al., 1998). Previous research has shown that GSK-3 activity increases moderately during neural differentiation of NT2 cells (Gompel et al., 2004). GSK-3 activity stops increasing in late stages of NT2 differentiation which is similar to the expression of GSK-3 in postnatal development when GSK-3 substantially decreased in the period of dendritic extension and synaptogenesis.

5.5 Lithium and NT2 cell viability
Even though Li treatment did not increase DA transcription factors, the drug did have a profound effect on the viability of NT2 cells. Many mechanisms could influence the viability of NT2 cells such as an increase in anti-apoptotic proteins or the decrease of pro-apoptotic proteins. The interpretation of lithium’s action is complicated since it affects many different pathways. Also, the apparent cellular response to Li treatment may vary from cell type to cell type. For instance the viability of thyroid follicular cells is reduced when they are treated with 0.3 – 2 mM of Li, while the viability in NT2 and hNT cells is increased with lithium exposure (Zigova et al., 1999; Gaberscek et al., 2002). In addition, lithium protects neurons from a variety of pro-apoptotic stimuli both in vitro and in vivo (review (Manji et al., 1999; Grimes and Jope, 2001). For example treatment with Li for 7 days protects cultured rat cerebellar, cerebral cortical, and hippocampal neurons against glutamate-induced excitotoxicity (Nonaka et al., 1998).
5.6 Lithium and NT2 proliferation

We found that Li treatment (1 mM) for up to 4 DIV increased the proliferation of NT2 cells as determined by labeling with a marker of proliferating cells Ki67 and increased in total cell number compared to the control. Neuronal differentiation requires the progenitor to exit the cell cycle. When a neural stem cell is in G\(_1\) phase, it is in between cell divisions and not dividing at that moment. The progression to the S-phase which begins with start of DNA synthesis leads to proliferation of the neural precursor cells. If the cell goes into the G\(_0\) phase, it enters neurogenesis which is the final differentiation phase before it turns into a neuron. Lithium can stimulate the NT2 cells to enter or remain in the G\(_1\) or S-phase. Since there is a choice between neurogenesis or proliferation, lithium at 1 mM for 4 DIV most likely influences intracellular pathways in favor of proliferation.

Not only can the lithium alter the proliferation, as we demonstrated in NT2 cells, Hashimoto and colleagues showed that various concentrations of lithium effected the proliferation of two different neural progenitors in vitro (Hashimoto et al., 2003). For instance, the greatest number of proliferating cerebellar granule cells was observed at 3 – 5 mM Li, while cerebral cortical cells proliferated at 0.5 mM Li. The duration of lithium exposure produces different effects as well. Cui and colleague showed that short term (<24 hours) pretreatment with high concentration (10 mM) of lithium increased proliferation in cerebellar granule cells (Cui and Bulleit, 1998).

Proliferation of neural progenitors cells is of primary importance both during early development as well as in the adult brain. It has recently become apparent that neural progenitor cells exist in adult mammalian brain including that of humans (review (Cameron and McKay, 1998; Gage, 2000; Jacobs and Miller, 2000; Magavi and Macklis, 2001). Various factors are known to influence the proliferation of precursor cells such as growth factors, neurotransmitters and drugs. For example, proliferation is increased by basic fibroblast and epidermal
growth factors, while glutamate, GABA and opioid peptides decrease proliferation (Gould et al., 1992; Gould et al., 1994; Cameron and McKay, 1998). To stimulate the proliferation of endogenous precursor cells could have an extraordinary impact on various diseases. For instance, depression is theorized to be associated with the decrease in progenitor cell proliferation induced by chronic stress (review (Jacobs et al., 2000). Furthermore, increasing cell proliferation is also important in expanding cell lines, especially cell lines with a low percentage of progenitor cells.

5.7 Aging and neurodegenerative diseases
The one thing in common with numerous neurodegenerative diseases is their association with age. One viewpoint is that aging is a gradual decline in the function of biological systems which negatively affects the capacity of cells to self-renewal or repair and eventually leads to a system of collapse. The cell eventually loses its ability to maintain homeostasis as a result of molecular events resulting from intrinsic metabolic processes and from a lifetime of environmental stress producing increased vulnerability to pathology and eventually cell death. Understanding the key components in the aging process can help slow down its progression. The process of aging has been extensively studied in the field of dermatology since society puts such high emphasis on a youthful appearance. Clearly, the skin’s degeneration is caused by both genetic and environmental factors, as in the case of neurodegenerative disease. The degeneration of the skin which produces side effects such as wrinkles, pigmentation and loss of collagen and hence elasticity, is partially a result of the reduced capacity for proliferation of adult skin stem cells. Consumers counteract this degeneration by applying stimulatory factors that promote proliferation such as retinoids.

The aging process in the brain has much more devastating impact on the individual, his/her family and society. One mechanism to begin to understand the aging process is to study the events that are important during development. In
addition to having an *in vitro* model system of human neural stem cells like the NT2 cells can provide a plethora of information on molecular events which effect proliferation and differentiation. One can then easily screen potential pharmacological agents, such as lithium, in terms of their potential to stimulate proliferation, differentiation and survival of the neural cells in the aging brain. Some of these novel factors could be provided by developing brain tissue as observed in our E18 cultures which had a tremendous effect on increasing the survival of hNT neurons. Therefore, factors that can stimulate the adult progenitor cells to proliferate might reduce impairments such as memory loss which occurs in normal aging due to the reduction of neurogenesis in the hippocampus (review (Prickaerts et al., 2004).

It is well documented that stem cells exist in at least two areas in the adult mammalian brain, the subventricular zone (SVZ) of the lateral ventricle and the subgranular layer (SGL) of the hippocampal dentate gyrus. Cells in the SVZ follow the rostral migratory stream to become interneurons in the olfactory bulb, while cells in the SGL migrate a short distance to differentiate into hippocampal granule neurons (Garcia-Verdugo et al., 1998; Lemaire et al., 2000). Recently neural stem cells have been identified in the adult substantia nigra which produce glia *in vivo* and neurons, astrocytes and oligodendrocytes *in vitro* (Flax et al., 1998). Interestingly, these cells can differentiate into neurons when transplanted into the hippocampus but not in the substantia nigra suggesting that local microenvironment inhibits neurogenesis of their endogenous stem cells. The stimulation of any of these adult stem cells can have significant impact on neurodegenerative disease such as PD and Alzheimer's disease. Currently, some of these adult stem cells have been induced to proliferate by exercise, exposure to an enriched living environment (Parent et al., 1997), or even by cerebral ischemia or severe seizures (Kuhn et al., 1996). In rats which received middle cerebral artery occlusion, a small population of cells proliferated in the SVZ and migrated to the site of injury, expressed neuronal markers and sent out projections to appropriate target (Lie et al., 2002).
Therefore our results illustrate that NT2 cells have extraordinary potential to study the proliferation, survival and differentiation of human neural precursor cells *in vitro*. In addition, one can examine which agents might increase their dopaminergic potential by examining the expression of essential dopaminergic transcription factors.
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ABOUT THE AUTHOR

Iwona Elizabeth Misiuta was born on November 27, 1976 in Warzsawa, Poland. Her family came to New Jersey in July 13, 1981 originally on vacation, but due to political instability (Solidarity Movement), the family decided to start a new life in America. Iwona grew up in Livingston, NJ and entered Rutgers University in 1994 where she majored in Biology. She also made the Division I Big East Varsity Volleyball Team. After graduating, she moved to New Orleans, LA to pursue her Masters in Health Administration at Tulane University.

Iwona loves to travel, sail and dance the night away. She has been to Europe many times since receiving her U.S. citizenship in 1995 – England, Scotland, Holland, France, Spain, Portugal, Germany, Hungary, Poland, Belgium, Luxemburg, Switzerland, Austria, Italy, Czech, and Slovakia. She has visited many other countries (Taiwan, Costa Rica, Mexico, Canada) as well as many islands in the Caribbean (U.S. and British Virgin Islands, Turks and Caicos, Jamaica, Bahamas, Dominican Republic, Bonaire, Puerto Rico).

After the completion of her doctorate, she is moving back to the New York City area to change the world.