UNIVERSITY OF CALIFORNIA, SAN DIEGO

Characterization of HPRT-Deficient Neuronal Development in the Human NTera2 Differentiation Model

A Thesis submitted in partial satisfaction of the requirements for the degree

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in

Biology

by

Stephen K. Hsu

Committee in charge:

Professor Theodore Friedmann, Chair
Professor Randy Hampton, Co-Chair
Professor Kathleen French

2008

The Thesis of Stephen K. Hsu is approved and it is acceptable in quality and form for the publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2008

DEDICATION

This Thesis is dedicated to my dad for encouraging my interests in science and to my family for all their love and support.

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LIST OF ABBREVIATIONS

6-TG	6-thioguanine
AADC	Aromatic L-amino-acid decarboxylase
ATCC	American Type Culture Collection
bHLH	basic-helix-loop-helix
CNS	Central Nervous System
DA	dopamine/dopaminergic
DAT	Dopamine transporter
DMEM	Dulbecco's Modified Eagle Medium
EC	Embryonic Carcinoma
ES	Embryonic Stem Cell
FoxA1	forkhead box A1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HAT	hypoxanthine-Aminopterin-Thymidine
HPRT	hypoxanthine-guanine phosphoribosyletransferase
L-DOPA	L-dihydroxyphenylalanine
LAM	laminin
Lmx1a	LIM-homeobox transcription factor 1a
LND	Lesch-Nyhan Disease
Mash1	achaete-schute homologue 1
Msx1	msh homeobox homolog 1
Ngn2	neurogenin 2
Nurr1	nuclear receptor subfamily 4 group A, member 2
NT2	NTera 2 clone D/1 teratocarcinoma cells
PDL	poly-D-lysine
Pitx3	paired-like homeodomain transcription factor 3
qPCR	Quantitative Polymerase Chain Reaction
RA	Retinoic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
shRNA	small hairpin RNA
TBP	TATA binding protein
TH	tyrosine hydroxylase
VMAT2	vesicular monoamine transporter

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Chapter 2 Materials and Methods, in part, is currently being prepared for submission for publication of the material. The thesis author will be a secondary author of the paper.

Chapter 3 Results, in part, is currently being prepared for submission for publication of the material. The thesis author will be a secondary author of the paper.

Figure 5, Table 2 and Table 3 are, in part, currently being prepared for submission for publication of the material. The thesis author will be a secondary author of the paper.

ABSTRACT OF THE THESIS

Characterization of HPRT-Deficient Neuronal Development in the Human NTera2 Differentiation Model

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Stephen K. Hsu

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Professor Theodore Friedmann, Chair
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Differentiation to the neuronal cell type requires the temporal and spatial specification of neuronal transcription factors. The dysregulation of expression of these neuronal transcription factors during differentiation may lead to neuronal dysfunction or neurological disease. In Lesch-Nyhan Disease (LND), mutations in the gene encoding the purine salvaging and purine biosynthesis enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT) result in a characteristic syndrome of hyperuricemia, severe dystonia, and self-injurious behavior. LND is associated with a defect in basal ganglia dopaminergic (DA) pathways. However, the mechanisms linking the purine metabolism defect to disorders seen in the CNS are

poorly understood. We have hypothesized that the “simple,” monogenic defect in LND is associated with a complex network of genetic and protein interactions that affect development of DA neurons. In this study, we examined HPRT-deficiency in human embryonic carcinoma cells, NTera2 cl. D/1 (NT2), which provides an *in vitro* model of neurogenesis. In these studies we used a retrovirus expressing a small hairpin RNA (shRNA) to knock down HPRT expression in NT2 cells, and the resulting cells were induced to differentiate along neuronal pathways by retinoic acid (RA) treatment.

We report here that several key neuronal transcription factors and dopamininergic neuron gene markers in the HPRT-deficient NT2 cells exhibit aberrant gene expression before and during differentiation. Differentiated HPRT-deficient neurons also showed morphological differences in neurite outgrowth, but retained wild-type electrophysiological properties. These results directly connect aberrant neurogenesis to HPRT deficiency and suggest that HPRT, a housekeeping gene, plays a role in neurodevelopment.

1. Introduction

1.1 Lesch-Nyhan Disease.

Lesch-Nyhan Disease (LND) is a rare, X-linked recessive disorder caused by mutations in a gene that encodes an enzyme called hypoxanthine-guanine phosphoribosyltransferase (HPRT), which participates in purine biosynthesis and purine salvaging [1,2]. Since HPRT is a housekeeping gene responsible for recycling and maintaining intracellular pools of purines[2], LND patients see increased *de novo* biosynthesis of purines to compensate for the lack of purine salvage pathways resulting in an overproduction of uric acid, which in turn produces the clinical symptoms of hyperuricemia and gout[1,2]. The full syndromes of LND include dystonia, mental retardation and compulsive, self-injurious behavior[1,3]. There is a spectrum of HPRT deficiency that is reflected in the severity of the symptoms: patients with an almost complete deficiency of HPRT activity exhibit the full metabolic and neurological defects; whereas patients with a partial deficiency usually exhibit only hyperuricemia and gout, but not the neurological defects[1,4].

The neurological defects in LND are associated with decreased levels of the neurotransmitter dopamine (DA) and defective dopamine uptake both in the basal ganglia and in primary cultures of dopaminergic (DA) neurons[1,2]. Midbrain DA neurons are vital components of many motor, cognitive and emotional functions and defects in these cells are responsible for a variety of neurological and psychological disorders including Parkinson's disease, depression, and schizophrenia[5]. For LND, the mechanisms connecting the defects in purine metabolism to decreased DA levels are still poorly understood, thus remaining an area of active study.

1.2 Dopamine Biosynthesis.

Transgenic HPRT-deficient mouse models of Lesch-Nyhan Disease have been shown to demonstrate decreased levels of dopamine and dopamine uptake in the basal ganglia, which has proved to be an effective model for the genetic and biochemical study for LND[6]. Although LND is a seemingly "simple" monogenic human disease, we have reported previously that the mouse model of LND is associated with aberrant expression of a number of genes and gene families in the central nervous system (CNS) and liver[7]. These results suggest transcriptional defects in a number of genes and gene networks that may be involved in the LND phenotype[7]. We thus hypothesize that the HPRT-deficiency causes a dysregulation of transcription factor genes that are essential for neuronal development, leading to disrupted DA levels and the complex LND neurological defects.

Of particular relevance to LND studies are the genes for the DA biosynthesis and DA function. These genes, which also serve as DA neuronal markers, include tyrosine hydroxylase (TH), aromatic L-amino-acid decarboxylase (AADC), dopamine transporter (DAT), and vesicular monoamine transporter (VMAT2). TH is the rate limiting enzyme of dopamine biosynthesis that catalyzes the conversion of amino acid L-tyrosine to L-dihydroxyphenylalanine (L-DOPA)[8]. L-DOPA is then decarboxylated to dopamine by AADC[9]. VMAT2, a transport protein in presynaptic cells, packages dopamine into synaptic vesicles for neuronal signaling[10]. Dopamine transporter (DAT), present in the synaptic cleft of DA neurons, regulates dopaminergic signal deactivation via re-uptake of dopamine from the synaptic cleft[11] (Figure 1). Aberrant expression of any of these DA biosynthesis genes or in genes for DA function could partially explain

the decreased levels of DA seen in LND.

1.3 Transcription Factor Genes in Neurogenesis.

In the past several decades, advances in molecular biology and mouse genetics have helped to elucidate the mechanisms behind neurogenesis, particularly, DA neuron development in the mouse brain. These studies have shown that the differentiation and specification of midbrain DA neurons depends upon several families of transcription factors expressed at critical moments in the differentiation process[12]. Of particular interest are neuronal developmental factors called LIM-homeodomain transcription activators encoded by homeobox genes[12]. Homeobox genes encode patterning factors that are crucial for the establishment of cellular polarity or cellular identity[12]. Homeodomain proteins contain homeodomain folds capable of binding DNA to regulate gene networks in a regional and temporal fashion[12,13].

Previous studies have identified a family of up-stream LIM-homeodomain transcription activators such as Lmx1a and Msx1 that are specific for differentiation to the neuronal phenotype[13,14]. LIM-homeodomain transcription factors up-regulate and interact with a downstream family of basic-helix-loop-helix (bHLH) transcription factors such as Ngn2, which has been shown to be required for the induction of immature neural progenitor cells[13,14,15]. Ngn2 has also been shown to interact with a family of forkhead/winged helix transcription factors such as FoxA1 that in turn regulates development of immature DA neurons at several phases of differentiation[13,16,17]. During early and late phases of neuronal differentiation, FoxA1

induces Nurr1 expression, which cooperates with Ngn2 synergistically to induce functional midbrain DA neurons through expression of TH and AADC[18]. Mash1 is another bHLH, proneural transcription factor implicated in general neuron maturation through interactions with Nurr1[13,14]. Pitx3 has been shown to be expressed with Nurr1 and is a specific marker for terminal differentiation to midbrain DA neurons[13,14,17,18] (Table 1 and Figure 2).

1.4 NT2 Cell Culture Model.

To test and characterize the putative developmental defects of DA neurons in HPRT-deficiency, we used human NTera2 (NT2) clone D/1 teratocarcinoma cell culture as an *in vitro* model of neurogenesis. One advantage of using this model of neurogenesis is the availability of powerful selection methods to isolate HPRT-deficient variants. Cells carrying intact HPRT enzyme function can be selected by growth in hypoxanthine-Aminopterin-Thymidine (HAT), a drug cocktail that blocks *de novo* purine biosynthesis so cells require intact salvaging pathways (functional HPRT) to survive[19,20]. Conversely, cells deficient in HPRT enzyme function can be selected by growth in 6-thioguanine (6-TG), a drug metabolized by HPRT to produce a toxic metabolite[19,20].

NT2 cells are pluripotent embryonic carcinoma (EC) cells that can differentiate into a heterogeneous population of post-mitotic neurons, astrocytes, and oligodendrocytes following the treatment with all-*trans*-retinoic acid (RA)[21,22]. NT2 cells have been shown to exhibit similarities to embryonic stem (ES) cells gene expression patterns both pre-differentiation and post-differentiation[23,24]. Furthermore,

NT2-derived neurons display properties close enough to authentic neuronal function to permit transplant and correction of experimental traumatic brain injuries in animal models [25]. A number of protocols have been reported to yield differentiated NT2 cells in as short as 21 days[24,26]. Figure 3 is a schematic of such a protocol used in our study. Undifferentiated NT2 cells are plated in RA medium for 14 days on ultra-low attachment plates to produce cell aggregates similar to ES-cell-like colonies. Such aggregates are then plated on a supporting matrix of poly-D-lysine (PDL) and laminin (LAM) in medium containing mitotic inhibitors to promote differentiation. At day 18, cells are re-plated on PDL/LAM again in medium containing the mitotic inhibitors until day 21 (Figure 3; see *Materials and Methods Section* for full protocol).

In this study, we infected NT2 cells with a retrovirus expressing a puromycin selectable marker and small hairpin RNA (shRNA) to knock down HPRT gene expression via gene silencing mechanisms. A control vector containing a shRNA directed towards luciferase was used as a control. Both sets of cells were treated with puromycin to select for transduced cells. Wild-type control phenotype cells were selected with HAT and HPRT-knockdown cells selected with 6-TG. These cells were then subjected to the same retinoic acid-induced differentiation protocol. We use quantitative RT-PCR to determine the relative mRNA expression levels of various midbrain DA neuron transcription factor genes, and DA gene markers in wild-type control and HPRT-knockdown cells at various time points in the differentiation process (see *Materials and Methods Section*).

Although the thesis author was not directly involved in neurite outgrowth and neuron electrophysiology experiments, we report here that HPRT-deficient DA

neurons show action potential responses similar to wild-type neuronal cells, but demonstrate significantly impaired expression of DA neuron-related transcription factors as well as deficient neurite outgrowth morphology (personal communication from Dr. Guibinga).

2. Materials and Methods

2.1 Establishment of Wild-type and HPRT-Knockdown Cells.

The thesis author was not directly involved in oligonucleotide shRNA production, retrovirus production, and retroviral infection of shRNA procedures. The following is a summary of what was done:

> Small hairpin sequences against HPRT gene were selected using the siRNA hairpin oligonucleotide sequence algorithm (Clontech, Laboratories, Mountain View, CA) to generate potential 19mer sequences direct against target messenger RNAs, specifically at Exon 1 of the HPRT gene. Retrovirus vectors expressing anti-HPRT hairpin oligonuceotides were produced, isolated and titered on a HT-1080 cell line. Virus production and packaging was carried out in the packaging cell line, GP-293. NT2 cells were infected at a multiplicity of infection (MOI) of approximately 1 with the retroviral vectors expressing shRNA targeted either to the HPRT or to the luciferase gene. (unpublished protocol, personal communication from Dr. Guibinga).

2.2 NT2 Cell Maintenance and Differentiation Protocol.

Human NTera2 cl. D/1 (NT2) embryonic carcinoma cells were obtained from American Type Culture Collection (ATCC) and were grown to approximately 70% confluence in a 5% CO_2 atmosphere at 37^{o}C in Dulbecco's Modified Eagle Medium (DMEM) high glucose medium supplemented with 10% fetal calf serum (FCS) and 50μg/ml of penicillin/streptomycin (pen/strep) and 100μg/ml of normocin (nor) (Invitrogen, Carlsbad, CA). Medium was changed every 2 to 3 days. Before the start

of the differentiation protocol, cells were grown with medium supplemented with 3μg/ml puromycin for 5-10 days. Cells were then cultured in HAT or 6-TG medium to select HPRT-positive and HPRT-knockdown cells respectively. The differentiation protocol with RA was initiated after HAT or 6-TG selection.

2.3 Retinoic Acid (RA)-induced differentiation of NT2 cells.

HPRT-knockdown and control NT2 cell lines were subjected to an established NT2 aggregation differentiation protocol (Figure 3).

Differentiation Procedure:

1. Attached NT2 cells from HPRT-knockdown and control were trypsinized and plated at 10^4 cells/ml on 6-well ultra-low attachment plates in 10% FCS DMEM complete medium containing nor and pen/strep. Cells at this undifferentiated stage were designated as day 0 of differentiation.
2. Cells were incubated overnight at 37 ^oC in a 5% CO_2 atmosphere to allow aggregation of the suspended NT2 cells.
3. The next day, medium from the aggregated cell cultures was replaced with 10% FCS DMEM complete medium containing nor and pen/strep and supplemented with 10μM all-*trans*-RA (Sigma, St Louis). Cell aggregates were prepared by low speed centrifugation (≤ 2000rpm) for 5mins at room temperature. Media were aspirated, and cell pellets were re-suspended in RA medium and re-plated onto ultra-low attachment plates. This day was designated as day 1 of NT2 differentiation.
4. Under the same incubation conditions, RA-containing medium was replaced every

2 to 3 days for 14 days using the same media changing procedure.

5. On day 14, cells were transferred onto 10cm cell culture plates pre-coated the night before with 10µg/ml poly-D-lysine (PDL) (Chemicon, Temecula CA), 10µg/ml murine laminin (LAM from Sigma) and 0.1% gelatin (Sigma). Cells were maintained in complete DMEM medium containing nor and pen/strep, supplemented with a mitotic inhibitor cocktail of D-arabinofuranoside (1µM araC, Sigma) and uridine (10µM, Calbiochem). Between day 14 and day 18 in these conditions, NT2 cells undergo morphological changes, characterized by the appearance of a few neuron-like cells.
6. On day 18, cells were again re-plated onto new PDL/LAM/gelatin pre-coated cell culture plates and maintained in complete DMEM containing the same mitotic inhibitor cocktail until day 21, at which time more cells displayed neuron-like morphology.
7. The resulting cells were maintained in complete DMEM with mitotic inhibitors for up to 6 weeks.

2.4 RNA isolation and quantitative RT-PCR analysis.

At pre-differentiation (day 0) and various days of differentiation (day 18 and day 21), total RNA was isolated from control and HPRT-knockdown cells as described below:

1. A Qiagen RNeasy miniprep was used according to manufacturer's protocols. Cells were lysed and homogenized with Qiagen homogenizer/cell shredder, and Qiagen RNA columns used to bind and purify total RNA from cell samples (Qiagen,

Hilden Germany).

2. RNA concentration and purity was determined by spectrophotometric absorption at OD260 and OD280. A total of 2µg of RNA from control and HPRT-knockdown cells was obtained for subsequent synthesis of complementary DNA by reverse transcription.
3. cDNA was produced from 2µg RNA in a final volume of 100µl (TaqMan RT buffer containing 5.5mM $MgCl_2$, 500uM dNTP mixture, 2.5 uM Random Hexamer, 0.4 U/ml RNase inhibitor; 1.25 U/ml reverse transcriptase, Applied Biosystems). Reverse transcription was carried using the following thermal cycling parameters: activation at 25°C for 10 min; reverse transcription at 48°C for 30 min; inactivation at 95°C for 5 min.
4. A Qiagen Quantitech SYBR green qPCR kit was used to quantify relative expression levels of synthesized cDNA samples (Qiagen, Hilden Germany). Primers for the various transcription factors were designed and prepared using the web-based software program, OligoPerfect (Invitrogen, Carlsbad, CA) (see Table 2). qPCR and fluorescence quantitation analysis was carried out on Opticon 2 System DNA Engine (BioRad, Hercules, CA) in a total reaction volume of ~2ml (1x Quantitect Sybr Green Master Mix, 200 nM of specific primers, ≤ 500 ng of template/reaction)
5. Expression for two housekeeping genes TATA box binding protein (TBP) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize expression of other genes (Table 2). TBP was used to normalize to genes that exhibit low expression such as transcription factor genes and DA neuron markers.

HPRT, a housekeeping gene, was normalized to GAPDH gene expression in wild-type (control) and HPRT-knockdown cells.

2.5 Data Analysis.

Statistical analysis of relative expression levels was carried out using Kaleidagraph graphing and data analysis software package (Synergy Software, Reading Pa). The data are reported as means ± standard error. Student paired t-test was performed in the normalization genes expression in wild-type and HPRT-knockdown cells. Statistical significance was set at $P<0.05$.

2.6 Neurite Outgrowth.

The thesis author was not directly involved in neurite outgrowth experiments. The method is as communicated:

> Images of HPRT-deficient and control differentiating cells at 18, 21 and 49 days after onset of RA-induced differentiation were captured at 20X in a phase-contrast digital format (TIFF) and imported into Metamorph-Imaging and Morphometric software (Molecular Devices, Downingtown PA) for neurite length measurements. Neurites less than 5 pixel length (~ 5 µm) or those for which starting and ending points were not clearly evident were excluded from the quantification. Neurite lengths were measured in a minimum of 10 to 15 acquired images using an Arcturus microscope/camera (Mountain View, CA) and Matrox IntelliCam software (Matrox Imaging, Quebec) (personal communication from Dr. Guibinga).

2.7 Electrophysiology Measurements.

The thesis author was not directly involved in electrophysiology measurements. We would like to thank Dr. Xia and Dr. Zhang at the Burnham Institute's National Electrophysiology Laboratory for conducting the electrophysiology measurements. A summary of the protocol is as follows:

> Control and HPRT-deficient cells differentiated for a minimum of 21 day were used for sodium (Na) current measurements. Using whole-cell patch clamp methods, individual cells were clamped down with electrodes and maintained at -60 mV and patch-probe attached to the cells. The current was elicited by stepping voltages from -60 to + 80 mV until an action potential was generated and detected by the patch-probe (personal communication from Dr. Guibinga).

3. Results

3.1 HPRT Knockdown and Differentiation of NT2 Cells.

The HPRT knockdown was verified by quantitative RT-PCR. We demonstrate that the shRNA retrovirus produces a stable reduction of HPRT activity of approximately 94% relative to the shRNA control vector expressing the luciferase shRNA (Figure 4A).

Phase-contrast microscopy photos were taken of the differentiation at several stages of the differentiation process, including undifferentiated cells at day 0, day 18 (pre-differentiation), and day 21 (mature neural cells) NT2 cells (Figure 4B). The undifferentiated NT2 cells grow tightly packed on a monolayer with generally cuboidal cell morphology. However, no neurite outgrowth is evident. After plating onto PDL/LAM plates, distinct neurite outgrowth and cell body elongation characteristic of neurons can be observed by day 18. By day 21, neuron-like cells have become plentiful and complex neurite networks form between the neuron-like cells.

3.2 Aberrant Expression of Neuronal Transcription Factors.

Markers vital for neuronal differentiation were quantified for wild-type and HPRT-knockdown NT2 cells prior to RA exposure day 0 (undifferentiated) and day 18 (mid-differentiation) when neuron-like cells begin to appear. As expected, prior to RA-induced differentiation, all of the transcription factor gene expression levels (Lmx1A, Msx1, Ngn2, Mash1, Nurr1, Pitx3, and FoxA1) at day 0 were not significantly different between wild-type and HPRT-knockdown cells (Figure 5A-G). However, by day 18, both Lmx1A and Msx1 were significantly up-regulated in

HPRT-knockdown compared to wild-type cells (Figure 5A, B). Ngn2 also exhibited significant up-regulation on day 18 of gene expression in HPRT-knockdown compared to gene expression in wild-type cells though much more moderately (Figure 5C). On the other hand, the degree of up-regulation of Mash1 in HPRT-knockdown cells was markedly impaired compared with that of wild-type cells at day 18 (Figure 5D). Transcription factors Nurr1 and Pitx3 are up-regulated in wild-type controls, but HPRT-knockdown show a markedly reduced degree of up-regulation of both genes (Figure 5E, F). At day 18, wild-type cells showed little change in expression of FoxA1 compared to pre-differentiation, whereas HPRT-knockdown cells at day 18 showed a significantly increased expression of FoxA1 (Figure 5G). These results imply that HPRT-deficiency markedly impairs the timing as well as amount of expression of certain transcription factors important for neuronal development. Figure 6 is a summary diagram of all the changes in transcription factor gene expression demonstrated in HPRT-knockdown relative to wild-type cells on day 18.

3.3 Dysregulation of DA Biosynthetic Genes.

To determine the effects of aberrant transcription factor expression on the generation of DA neurons, we characterized the expression of genes indicative of DA neuronal function at day 0 (undifferentiated state) and day 21 (differentiated neuron-like morphology) (see Figure 1 for the gene markers). Table 3 summarizes the time-dependent changes in DA gene marker expression pre-differentiation and post-differentiation that is relative to both wild-type and HPRT-knockdown NT2 cells. Prior to differentiation, both wild-type and HPRT-knockdown exhibited similar levels

of TH gene expression. As expected, after differentiation to the neuronal phenotype at day 21, both wild-type and HPRT-knockdown cells exhibited increased gene expression of the DA biosynthesis enzyme, TH. However, HPRT-knockdown cells exhibited only an approximate 2-fold up-regulation of TH gene expression from day 0 to day 21 compared to over a 5-fold up-regulation of TH gene expression in wild-type cells, suggesting impaired up-regulation of TH expression in the HPRT-knockdown cells. Expression of AADC was also up-regulated in both wild-type and HPRT-knockdown cells by day 21. Similar to TH expression, the up-regulation of AADC in the HPRT-knockdown was much less robust. On day 21, AADC demonstrated only an approximate 4-fold up-regulation in gene expression from day 0, compared to wild-type cells that demonstrated over a 12-fold up-regulation in gene expression from day 0 (Table 3). Reduced TH and AADC expression in differentiated DA neurons could result in overall reduced levels of DA biosynthesis, a finding consistent with the LND phenotype.

At day 0, DAT demonstrated higher gene expression in wild-type cells compared to HPRT-knockdown cells. At day 21, DAT demonstrated a marked down-regulation of DAT gene expression in wild-type cells, a fraction of the gene expression compared to day 0. On the other hand, DAT gene expression in HPRT-knockdown cells demonstrated a minimal up-regulation in gene expression compared to day 0. VMAT2 gene expression in wild-type cells demonstrated a less than 2-fold up-regulation on day 21. On the other hand, HPRT-knockdown cells paradoxically displayed a robust 6-fold up-regulation of VMAT2 gene expression at day 21 (Table 3). To summarize, HPRT-knockdown NT2 cells demonstrated impaired

expression of three DA neuronal markers indicative of DA biosynthesis (TH, AADC, and DAT) during RA-induced differentiation, and VMAT2 demonstrated up-regulation of expression on day 21.

3.4 Impaired Neurite Outgrowth in HPRT-deficient Neurons.

The neurite outgrowth experiment was not carried out by the thesis author (see *Materials and Methods* for the summary of the experiment). However, these collaborative results were relevant to the NT2 HPRT-deficient neurogenesis model so the results have been summarized and interpreted.

On day 18, neurite morphology and neurite numbers were indistinguishable between wild-type and HPRT-knockdown cells. However, starting at day 21 and more markedly evident in advanced day 49 neurons, HPRT-knockdown cells demonstrated significantly shorter neurite outgrowth and exhibited a scarcity of longer neurites compared to wild-type cells (data not shown, personal communication from Dr. Guibinga).

3.5 Electrophysiology of Differentiated Neurons.

The electrophysiology experiment was not carried out by the thesis author (see *Materials and Methods* for the summary of the experiment). Again these collaborative results were relevant to the NT2 HPRT-deficient neurogenesis model so the results have been summarized and interpreted.

To study neuron function, individual RA-induced differentiated NT2 cells were collected on day 21 and 49 from wild-type and HPRT-knockdown cells,

respectively, and patch clamp methods were used to measure the ability of these cells to generate action potentials. The patch clamp experiment revealed that wild-type and HPRT-knockdown cells exhibited indistinguishable neuronal generation of action potentials at day 21 and 49 (data not shown, personal communication from Dr. Guibinga). Thus RA-induced differentiation of NT2 cells produced functional neurons.

4. Discussion

Even after decades of study, the mechanism linking the inborn error of purine metabolism to the neurological dopamine dysfunction in Lesch-Nyhan Disease remains poorly understood. This study presents the first experimental evidence for disturbed genesis and differentiation of neurons in HPRT-deficient cells, suggesting that DA impairment may be partly due to aberrant transcription factor gene expression during the development of DA themselves.

Our results demonstrate that HPRT-deficiency affects many of the DA neuronal development pathways at a number of points, supporting our working model of the intrinsic DA neuron defects in HPRT deficiency. Since most transcription factors are expressed at relatively low levels compared to most cellular proteins, even slight changes in transcription factor expression could have drastic effects on timing, activation, deactivation, or interaction with downstream transcription factors. The LIM-homeodomain transcription activator genes interact with bHLH transcription factors, so over-expression of early LIM-homeodomain transcriptional activator genes could lead to aberrant expression of downstream transcription factors, as was demonstrated in the up-regulation of Lmx1a, Msx1, and Ngn2 gene expression in our HPRT-knockdown cells (Table 1 and Figure 6). FoxA1 also interacts and up-regulates Ngn2 expression[13,16,17], so the aberrant up-regulation of FoxA1 expression could be reinforcing up-regulation of Ngn2 in our HPRT-knockdown cells (Figure 5C,G. and Figure 6). Since Ngn2 and Mash1 are in same bHLH family of transcription factors and Ngn2 cooperates synergistically with Nurr1[13,18], the up-regulation of Ngn2 could inversely down-regulate Mash1 and therefore down-regulate Nurr1 expression, which

was markedly seen in our HPRT-knockdown cells (Figure 5C,D,E and Figure 6). Also consistent with previously reports that Pitx3 is expressed with Nurr1[13,14,17,18], the impaired up-regulation of Nurr1 in our HPRT-knockdown cells could also impair up-regulation of Pitx3 gene expression (Figure 5E,F and Figure 6).

The aberrant expression of transcription factor genes could be disrupting normal neuronal maturation signals during neurogenesis, leading to defects in DA metabolism as documented in this study by the marked impaired up-regulation of the DA biosynthesis genes TH and AADC in HPRT-knockdown cells, which is consistent with LND. However, in contrast to previous published reports, we found VMAT2 strongly up-regulated and DAT moderately the same in HPRT-knockdown cells compared to wild-type cells late in the differentiation process. A possible explanation for this discrepancy may come from the up-regulation of Ngn2 in HPRT-knockdown cells since Ngn2 over-expression has been shown to induce VMAT2 expression[14].

The human NT2 embryonic carcinoma differentiation system is a well established model system for neuronal differentiation. Despite the fact that wild-type and HPRT-deficient cells demonstrate similar electrophysiological properties and seem to represent functional neurons after RA-induced differentiation, we found significant morphological differences between wild-type and HPRT-knockdown cells in terms of neurite outgrowth. Interestingly enough, Pitx3-knockout mice have been reported to be associated with reduced neurite outgrowth[14]. Our HPRT-knockdown cells demonstrated reduced expression levels of Pitx3 (Figure 5F), consistent this mouse model and blunted neurite outgrowth. Although the detailed mechanism behind abnormal neurite outgrowth has yet to be fully elucidated, our data suggest that

blunted neurite outgrowth in HPRT-deficient cells may play a role in the neurological phenotype of LND. This is also consistent with the fact that DA reduction does not result from a loss of midbrain DA neurons since cell numbers have been shown to be relatively normal in human LND patients and in the HPRT-knockout mouse model[1,6].

Perhaps the most significant result in these studies is experimental documentation for the conclusion that a housekeeping gene such as HPRT can play a key role in developmental pathways; i.e., in the temporal specification of a number of transcription factors crucial for the development and identity of DA neurons and the DA neurotransmitter pathway. We suggest that other housekeeping genes could play similar roles in many other important developmental pathways.

Appendix

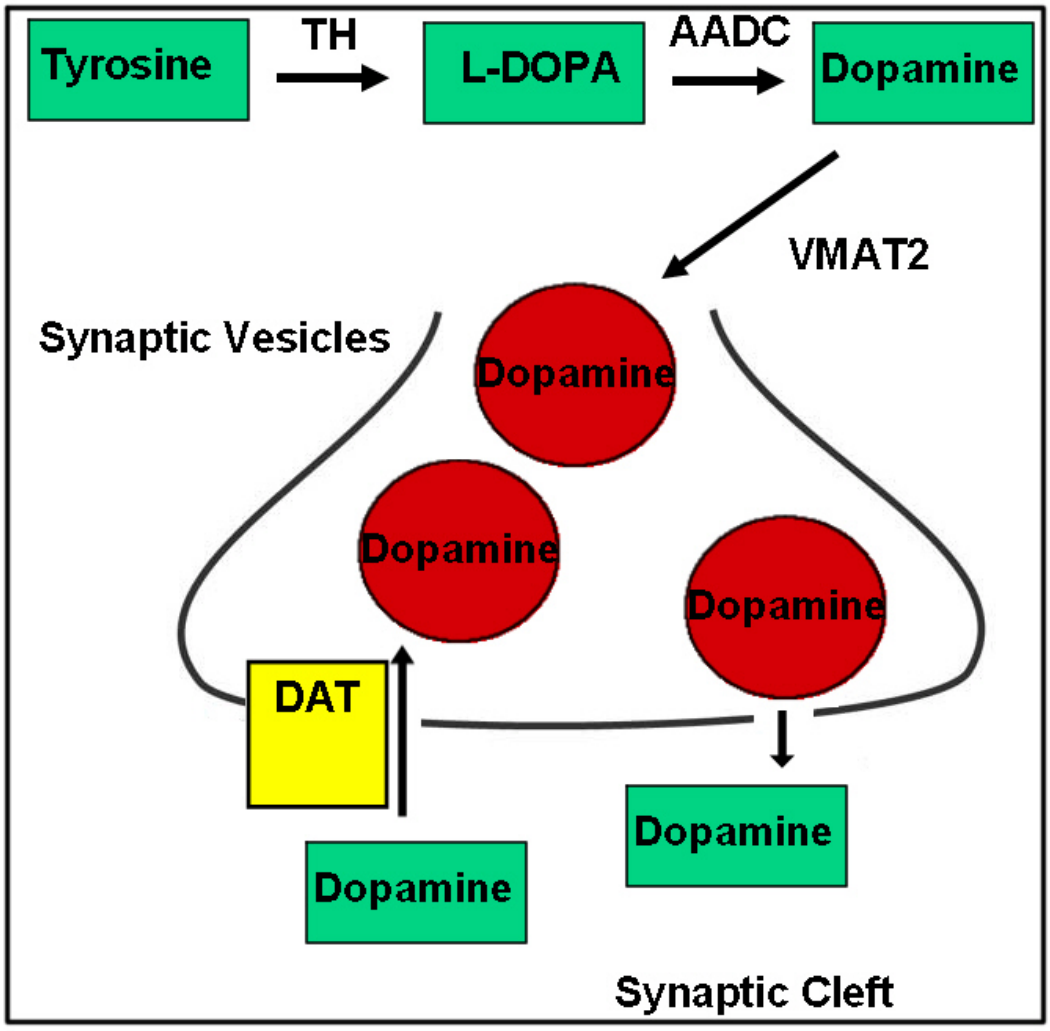

Figure 1. Pathway of Dopamine Biosynthesis and Function. In the rate-limiting step, L-tyrosine is converted to L-dihydroxyphenylalanine (L-DOPA) by tyrosine hyroxylase (TH). L-DOPA is converted to dopamine by aromatic L-amino-acid decarboxylase (AADC). Vesicular monoamine transporter (VMAT2) is vital for packaging dopamine into synaptic vesicles for DA signaling. Dopamine transporter (DAT) regulates DA signal deactivation via re-uptake of dopamine from the synaptic cleft.

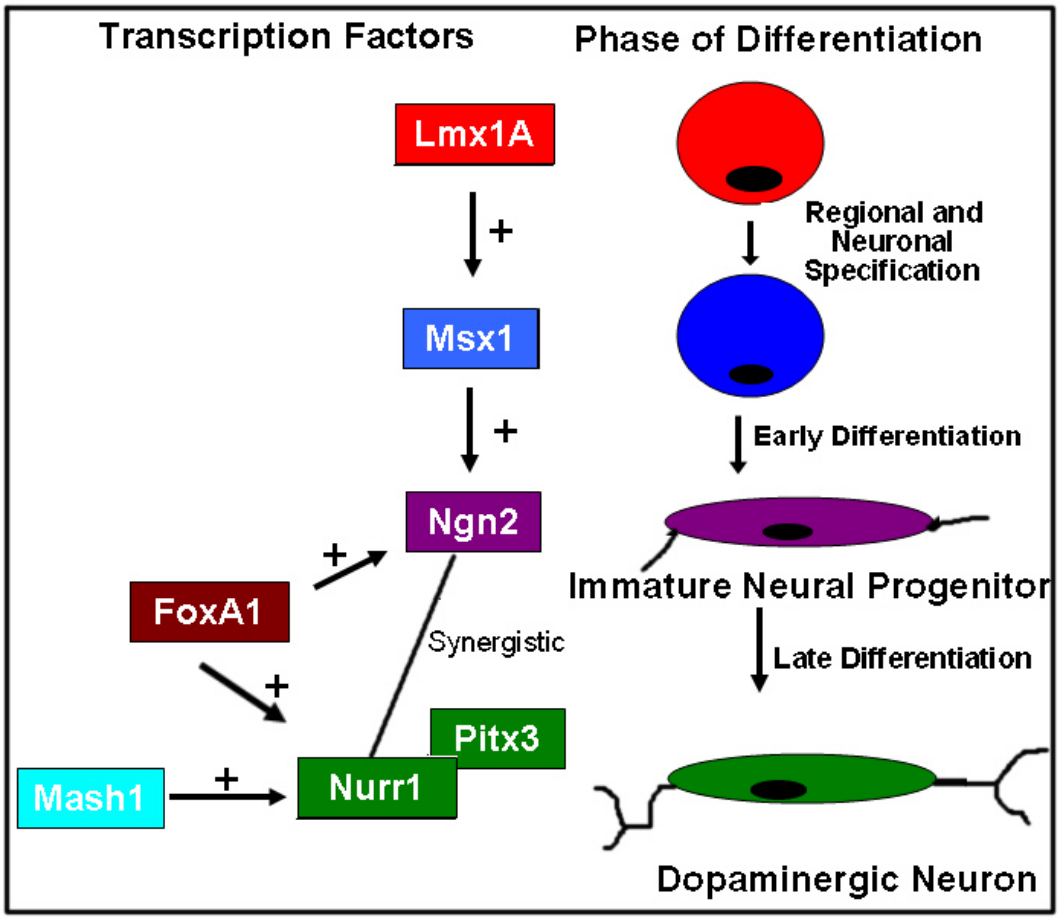

Figure 2. Simplified Model of Gene Interactions in DA Neurogenesis. The left side is a diagram of transcription factor gene interactions in this study: Lmx1A, Msx1, Ngn2, FoxA1, Mash1, Nurr1, and Pitx3 (See Table 1 for transcription factor functions and interactions). + indicates transcription factor can up-regulate expression of interacting transcription factor. The right side is a schematic of the phases of neuronal differentiation corresponding to the expression of various transcription factor genes.

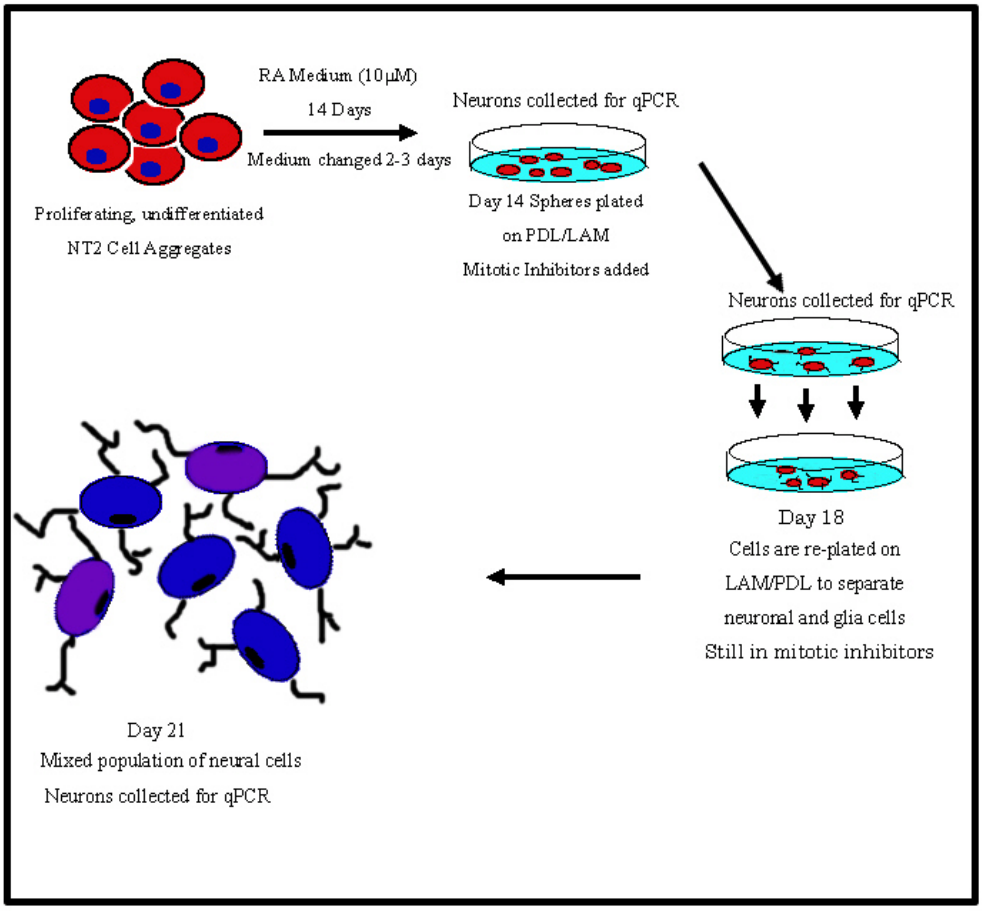

Figure 3. Schematic for Retinoic acid-induced Differentiation of NT2 Cells. NT2 cells plated on ultra-low-attachment plates form cell aggregates that are then treated with 10μM of RA for 14 days and then plated on poly-D-lysine (PDL) and laminin (LAM) plates with a mitotic inhibitor medium (1μM D-arabinofuranoside and 10μM uridine, no RA). Day 18, cells are re-plated again on LAM/PDL with the same mitotic inhibitor cocktail. Day 21, neuron-like cells may be observed. Differentiated cells are maintained in mitotic inhibitor medium for up to 6 weeks for subsequent analysis.

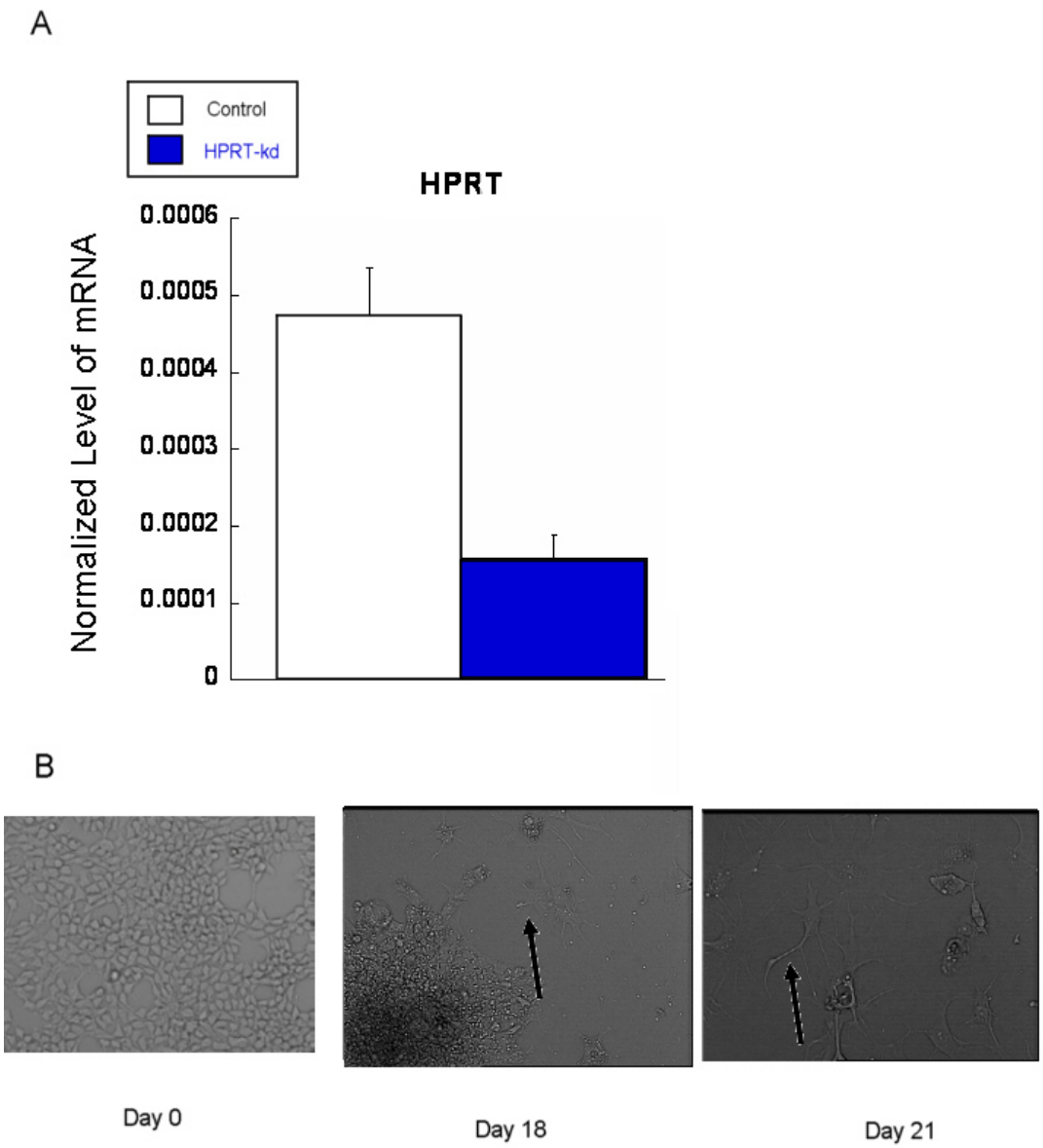

Figure 4. HPRT-knockdown of NT2 Cells and Cell Morphology. (A) Quantitative RT-PCR was used to quantify expression level of HPRT in wild-type and HPRT-knockdown cells. Results are normalized to the expression of the housekeeping gene GAPDH standard. RNA samples were obtained from wild-type (open bar) and HPRT-knockdown (solid bar) NT2 cells. (B) Representative phase-contrast photos of undifferentiated day 0, mid differentiation day 18, and differentiated day 21. Arrows show examples of neurite outgrowth.

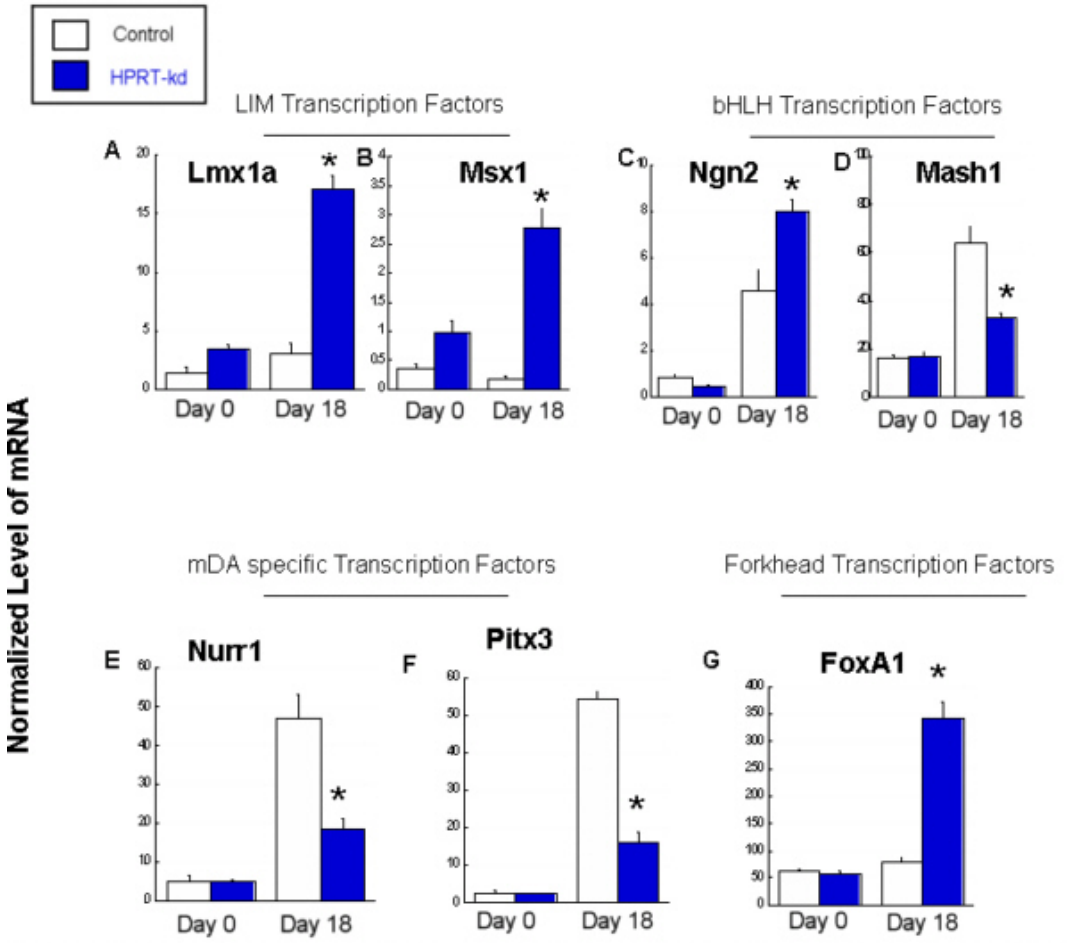

Figure 5. Transcription Factors Expression in RA Differentiation. Quantitative RT-PCR was used to quantify expression levels of transcription factors and results were normalized to the expression of the housekeeping gene, TATA binding protein (TBP). Cellular RNA was obtained from wild-type (open bar) and HPRT-knockdown (solid bar) NT2 cells at day 0 and 18 for the transcription factors (A) Lmx1a, (B) Msx1, (C) Ngn2, (D) Mash1, (E) Nurr1, (F) Pitx3 and (G) FoxA1. Significant differences in gene expression are designated by *

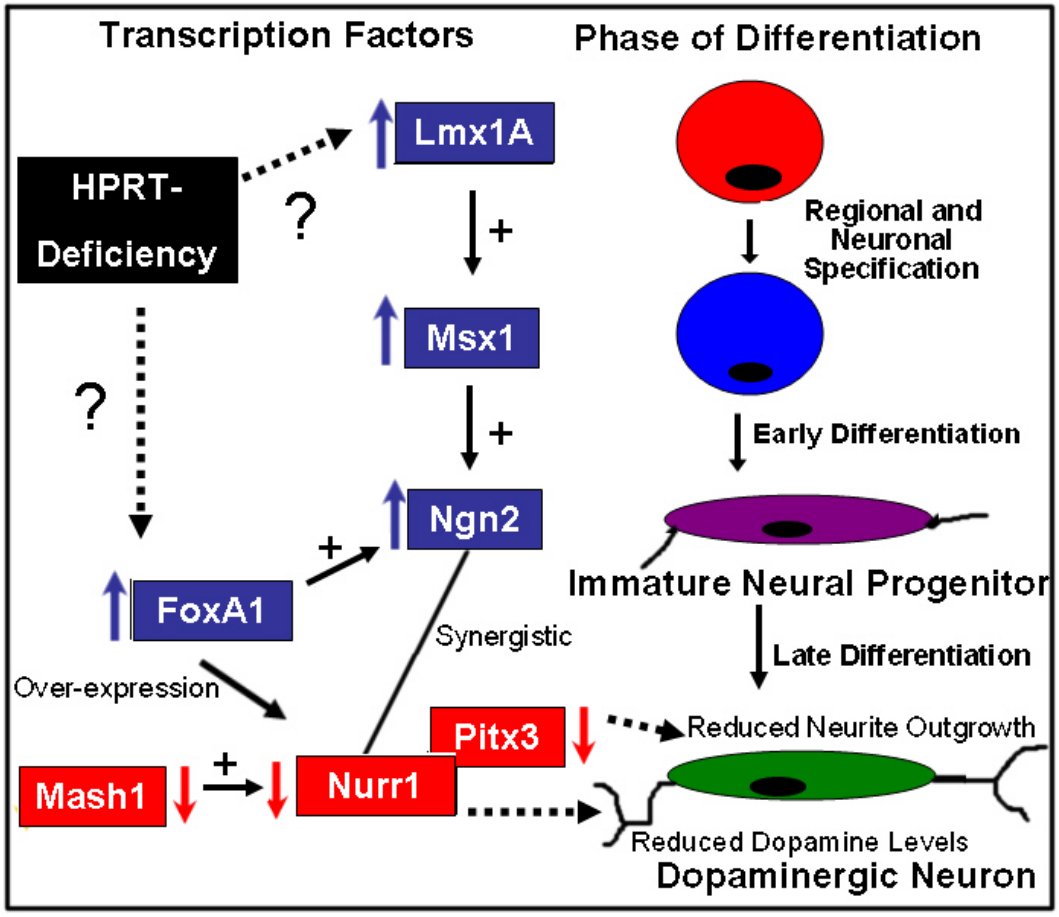

Figure 6. Model for Neurogenesis Dyregulation in HPRT-Deficieny. Transcription factors in blue indicate up-regulation of gene expression and transcription factors in red indicate impaired up-regulation of gene expression in HPRT-knockdown NT2 cells relative to gene expression of wild-type NT2 cells as seen on day 18 of differentiation. Reduced up-regulation of Nurr1 and Pitx3 may lead to impaired DA neurons.

Table 1. Summary of Transcription Factors Vital for Neurogenesis

Name of Transcription Factor	Family/Class of Protein	Function/Interactions
Lmx1a	LIM-homeodomain transcription activator	Early transcription factor crucial for neuron identity
Msx1	LIM-homeodomain transcription activator	Early transcription factor crucial for neuron identity
Mash1	basic-helix-loop-helix transcription factor	Induces Nurr1 expression and the general maturation to functional DA neurons
Ngn2	basic-helix-loop-helix transcription factor	Induces appearance of immature neural progenitor cells, up-regulated in differentiating neurons, synergistically cooperates with Nurr1 to induce DA neurons
FoxA1	forkhead/winged helix transcription factor	Expressed at various phases of DA neurogenesis, interacts with Ngn2 to induce Nurr1 expression, induces DA gene expression of TH and AADC
Nurr1	nuclear receptor subfamily 4 transcription factor	Cooperates with Mash1 and Pitx3 to induce functional DA neurons
Pitx3	homeodomain transcription factor	Terminal neuronal differentiation marker, shown to be vital for neurite outgrowth in mouse neurogenesis model

Table 2. List of Primers used in Quantitative RT-PCR Experiments

Genes	GI No	Tm (° C)	Sequences 5'→3' (F/R)
FOXA1	24497500	56	CCG TTC TCC ATC AAC AAC CT/ GTG TTT AGG ACG GGT CTG GA
DAT	133008627	56.5	TGA GCT CTT CAC GCT CTT CA / CAC CAT AGA ACC AGG CCA
FOXA2	24497503	56	TTT AAA CTG CCA TGC ACT CG/ GTT GCT CAC GGA GGA GTA GC
LMX1A	75677383	57	GTG CTG GGG TTC ATT CTG TT/AGT CTC AGC CCT GCT CAT GT
LMX1B	168740510	59	ACG AGG AGT GTT TGC AGT GCG/ CCC TCC TTG AGC ACG AAT TCG
MSX1	118582283	55.5	GCT AGA GGC CAT GTC TCC TG / CCC CAG AGC AAA TGT TTT GT
NGN1	38455395	55	CCG ACG ACA CCA AGC TCA/ GGA ATG AAA CAG GGC GTT
VMAT2	349711	55	ATG AGT TTG TGG GGA AGA CG /CTT TCG GGA ACA CAT GGT CT
NGN2	31340581	59	GGT CTG GTA CAC GAT TGC AAA C/ GCT GTT GGT GCA ACT CCA CGT
GAPDH	37551787	53	GAA GGT GAA GGT CGG AGT C/GAA GAT GGT GAT GGG ATT TC
MASH1	4757787	63	GTC GAG TAC ATC CGC GCG CTG /AGA ACC AGT TGG TGA AGT CGA
NEUROD	4505376	60	GCC CCA GGG TTA TGA GAC TAT CAC T/ CCG ACA GAG CCC AGA TGT AGT TCT T
NURR1	27894348	56	CACTCCGGGTCGGTTTACTA/ GAGACTGGCGTTTTCCTCTG
AADC	132814447	56	ACA AGT TTG TCC TGC GCT TT / CCA CAG ACA GCT GAG TTC CA
PITX3	24234713	56	CAACCTTAGTCCGTGCCAGT/ CCAGTCAAAATGACCCCAGT
HPRT	164518913	54.5	TGC TCG AGA TGT GAT GAA GG/ CTG CAT TGT TTT GCC AGT GT
HES1	4505376	53	TGC CAG CTG ATA TAA TGG AG/ TGG GAA TGA GGA AAG CAA AC
TH	88900500	57	ACTGGTTCACGGTGGAGTTC/ AGCTCCTGAGCTTGTCCTTG
TBP	88900500	54	CGGCTGTTTAACTTCGCTTC /TTCTTGGCAAACCAGAAACC

Table 3. Quantitative RT-PCR of Dopamine Marker Genes. ( ) indicates the fold change of the DA marker gene expression post-differentiation compared to that marker's gene expression prior to differentiation.

	DAY 0		DAY 21	
	Wild-type	HPRT-Kd	Wild-type	HPRT-Kd
TH	1.80±0.22	3.92±0.76	10.09±2.09 *(5.61)*	8.15±0.83 *(2.07)*
AADC	3.91±0.82	2.91±0.633	47.74±9.81 *(12.20)*	12.45±1.35 *(4.28)*
DAT	20.01±2.5	7.33±1.10	2.59±0.53 *(0.13)*	8.44±0.71 *(1.15)*
VMAT 2	13.40±2.3	14.44±4.13	21.97±0.97 *(1.64)*	92.13±14.0 *(6.38)*

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