In vitro modelling of Lesch-Nyhan Disease

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Abstract

Lesch-Nyhan Disease (LND) is a rare neurodevelopmental disorder characterized by metabolic symptoms including the accumulation of uric acid crystals in the urine, hyperuricemia, and gout, and neurological symptoms including severe dystonia, intellectual disability, and chronic self-harming behaviours. The causal gene, *HPRT1*, has been known since 1967 but, despite 50 years of research, the mechanisms and pathways through which *HPRT1* mutations cause the neurological symptoms of LND remain unknown. A primary challenge hindering progress in LND research is that traditional approaches to disease modelling have not been very effective. Many *in vitro* and *in vivo* models have been used to study LND, but each comes with substantial limitations and studies in different models have yielded at times contradictory results.

This work presents the development and transcriptome profiling of three novel models of LND using short hairpin RNA knockdowns (shHPRT) in immortalized human midbrain progenitors, and patient induced pluripotent stem cell-derived forebrain-like neural (fNPCs) and midbrain-like neural progenitors (mdNPCs). These are the first human neuronal models of LND and the largest and most comprehensive transcriptomic datasets available for LND research. Using a combination of bioinformatics, targeted validation, and functional assessments, we have shown cell-type specific alterations to adenosine neurotransmission and increases of the expression of mitochondrial genes. These changes are not found in the brains of HPRT knockout mice, and emphasize the need for species and cell-type accurate models of neurodevelopmental disorders.

Sommaire

Le syndrome de Lesch-Nyhan (LND) est un trouble neurodéveloppemental rare caractérisé par des symptômes métaboliques incluant l'accumulation de cristaux d'acide urique dans l'urine, l'hyperuricémie et la goutte et des symptômes neurologiques incluant dystonie sévère, déficience intellectuelle et comportements autodestructeurs chroniques. Le gène causal, HPRT1, est connu depuis 1967 mais, malgré 50 années de recherche, les mécanismes et les voies par lesquels les mutations HPRT1 causent les symptômes neurologiques de LND restent inconnus. Un obstacle principal à la progression de la recherche sur le LND est que les approches traditionnelles de la modélisation des maladies n'ont pas été très efficaces. De nombreux modèles in vitro et in vivo de LND ont été utilisés pour étudier le LND, mais chacun présente des limites importantes et des études menées dans différents modèles ont donné des résultats parfois contradictoires.

Ce travail présente le développement et le profilage transcriptomique de trois nouveaux modèles de LND utilisant des knockdowns de petit ARN en épingle à cheveux (shHPRT) dans des progéniteurs du mésencéphale humain immortalisés, et des progéniteurs neuraux du cerveau antérieur (fNPCs) et mésencéphaliques mdNPCs) dérivés de cellules souches pluripotentes dérivés de patients. Ce sont les premiers modèles neuronaux humains de LND et les ensembles de données transcriptomiques les plus vastes et les plus complets disponibles pour la recherche LND. En utilisant une combinaison de bioinformatiques, de validations ciblées, et d'évaluations fonctionnelles, nous avons montré des altérations spécifiques de type cellulaire à la neurotransmission de l'adénosine et des augmentations de l'expression des gènes mitochondriaux clés. Ces changements ne sont pas trouvés dans le cerveau des souris knock-out HPRT, et soulignent le besoin d'espèces et de modèles précis de type cellulaire des troubles neurodéveloppementaux.

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Contributions of authors

My contributions to this thesis include designing, performing, and analyzing all of the experiments presented, with the exceptions outlined below. In cases where data was collected by a collaborator, I prepared the samples and analyzed, interpreted and presented the results. I also developed and refined the iPSC culture and differentiation protocols used throughout. Finally, I have prepared the text and figures that make up this thesis.

Science is a collaborative work, and many exceptional researchers have lent their talents to my project. Dr. Alpha Diallo and Dr. Jean Francois Theroux assisted with bioinformatic processing (chapters 2-4) Dr. Laurence Daheron, and Poornima Manavalan provided invaluable training and resources for the induction, maintenance, and differentiation of iPSCs. Dr. Brigitte Kieffer and Dr. Emmanuel Darcq provided the space and resources and assisted in data collection for the GTP-γ-S experiments (chapter 3). My labmates Scott Bell and Dr. Huashan Peng have spent many hours assisting with the daily regimens of iPSC and NPC differentiation and maintenance, assisted with data collection (chapters 2-4), and performed CRISPR genome editing (Chapter 3). Data collection for HPLC quantification of dopamine and its metabolites was conducted by Luc Moquin in the lab of Dr. Alain Gratton, and HPLC data collection for adenosine containing compounds was conducted by Dr. Thad A. Rosenberger. HPRT-knockout mouse brains were provided by Dr. Jasper Visser. Lastly, my supervisors Dr. Carl Ernst and Dr. Gustavo Turecki provided constant guidance and consultation on experimental design and the interpretation of results, not to mention innumerable resources including my stipend, the salaries of lab technicians and research associates, and all of the supplies, reagents, and connections to collaborators that were needed for the project to be successful.

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List of Abbreviations

2-Chloro-N6-cyclopentyladenosine .... CCPA
3,4-Dihydroxyphenylacetic acid ..... DOPAC
5-hydroxyindoleacetic acid 5-HIAA
6-hydroxy-dopamine 6-OHDA
Adenine phosphoribosyltransferase .... APRT
Adenosine Deaminase ..... ADA
Adenosine diphosphate ..... ADP
Adenosine mono-phosphate ..... AMP
Adenosine receptor A1 ..... ADORA1
Adenosine receptor A2A ADORA2A
Adenosine triphosphate ..... ATP
Adenylosuccinate lyase ..... ADSL
Adenylosuccinate synthetase ..... ADSS
ATP Synthase subunit alpha ATP5A
ATP Synthase Stalk Subunit D ATP5H
Base pair ..... BP
Beta Actin ..... ACTB
DOPA-decarboxylase ..... DDC
Dopamine D1 receptors DRD1
Dopamine ..... DA
Embryoid body ..... EB
Embryonic stem cells ..... ES
Equilabrateive nucleoside transporter 2 ..... ENT2/SLC29A2
Forebrain-like neural progenitor ..... fNPC
Guanosine monophosphate ..... GMP
Guanosine triphosphate ..... GTP
High-performance liquid chromatography ..... HPLC
Homovanillic acid ..... HVA
Hypoxanthine ..... HPX
Hypoxanthine-guanine phosphoribosyltransferase ..... HPRT
Induced pluripotent stem cells ..... iPSC
Inosine monophosphate dehydrogenase IMPDH1
Inosine monophosphate ..... IMP
Knockout ..... KO
Lesch-Nyhan disease ..... LNS
Lesch-Nyhan Variants ..... LNV
Midbrain-like neural progenitor ..... mdNPC
Neural progenitor cell ..... NPC
Neurodevelopmental disorder ..... NDD
Orbitofrontal cortex ..... OFC
Phosphoribosyltransferase domain-containing protein 1 ..... *PRTFDC1*
Phosphoribosyl pyrophosphate ..... PRPP
Positron emission tomography ..... PET
RNA sequencing ..... RNAseq
S-Adenosylmethionine ..... SAMe
Self-injurious behaviour ..... SIB
Sonic hedgehog ..... SHH
Substantia nigra ..... SN
Tyrosine Hydroxylase ..... TH
Ventral tegmental area ..... VTA

1. Original Scholarship and Contributions to Knowledge

1. The creation and banking of three new models of LND: 1) a short hairpin mediated HPRT knockdown in immortalized human fetal ventral midbrain neurons, 2) Patient-derived forebrain-like neural progenitor cells (fNPCs) and 3) patient-derived midbrain like neural progenitor cells (mdNPCs). These are the first available human neuronal models of LND, and are all available to other research groups and collaborators.

2. The creation of the first transcriptome-wide RNA sequencing datasets from Lesch-Nyhan patient-derived neurons, the first RNAseq datasets from mouse brain, and the first transcriptome level data from multiple brain regions from the same mice.

3. We have shown that HPRT deficiency reduces the expression and translation of the adenosine signalling related genes ADORA1 and ENT2 in both immortalized fetal neural progenitors and patient-derived fNPCs, but not in patient-derived mdNPCs. In fNPCs this decrease is associated with a decreased ADORA1 signalling in response to direct agonists.

4. We have shown that there are dramatic differences in the effects of the absence of HPRT across cell types and brain regions. The transcriptome-wide impacts have minimal overlap between mdNPCs and fNPCs and are similarly divergent in the substantia nigra and striatum in adult $HPRT^{y/-}$ mice.

5. We have shown that both fNPCs and mdNPCs show substantial reductions in levels of Adenosine, AMP, ADP, and ATP.

6. Throughout my Ph.D. I have contributed to the following publications:

Bell, S., Peng, H., **Crapper, L**., Kolobova, I., Maussion, G., Vasuta, C., Yerko, V., Wong, T.P., and Ernst, C. (2016). A Rapid Pipeline to Model Rare Neurodevelopmental Disorders with Simultaneous CRISPR/Cas9 Gene Editing. *STEM CELLS Translational Medicine*

- Advised on experimental design
- Developed iPSC growth and neural differentiation protocols used as the basis for the paper
- Differentiated and maintained NPC cell lines

Bell, S., Kolobova, I., **Crapper, L**., and Ernst, C. (2016). Lesch-Nyhan syndrome: models, theories, and therapies. *Molecular Syndromology*.

- Contributed ideas, research, and editing

Merner, N., Forgeot d'Arc, B., Bell, S.C., Maussion, G., Peng, H., Gauthier, J., **Crapper, L**., Hamdan, F.F., Michaud, J.L., Mottron, L., Rouleau G.A., Ernst C. (2016). A de novo frameshift mutation in chromodomain helicase DNA-binding domain 8 (CHD8): A case report and literature review. *American journal of medical genetics*

- Conducted and analyzed qPCR experiments

Maussion, G., Diallo, A.B., Gigek, C.O., Chen, E.S., **Crapper, L**., Theroux, J.F., Chen, G.G., Vasuta, C., and Ernst, C. (2015). Investigation of genes important in neurodevelopment disorders in adult human brain. *Hum Genet*

- Grew and maintained knockdown NPCs and iPSC derived NPCs.
- Analysed BrainSpan data

Crapper, L., and Ernst, C. (2015). Comparative analysis of self-injury in people with psychopathology or neurodevelopmental disorders. *Pediatric clinics of North America*

- Formed idea, assessed literature, and wrote article

Gigek, C.O., Chen, E.S., Ota, V.K., Maussion, G., Peng, H., Vaillancourt, K., Diallo, A.B., Lopez, J.P., **Crapper, L**., Vasuta, C., Chen GG, Ernst C. (2015). A molecular model for neurodevelopmental disorders. *Transl Psychiatry*

- Advised on experimental design
- Grew and maintained neural progenitor cell lines

Maheu, M., Lopez, J.P., **Crapper, L**., Davoli, M.A., Turecki, G., and Mechawar, N. (2015). MicroRNA regulation of central glial cell line-derived neurotrophic factor (GDNF) signalling in depression. *Transl Psychiatry*

- Designed, conducted and analyzed all experiments in human neuronal culture

Chen, E.S., Gigek, C.O., Rosenfeld, J.A., Diallo, A.B., Maussion, G., Chen, G.G., Vaillancourt, K., Lopez, J.P., **Crapper, L**., Poujol, R., Shaffer L.G., Bourque G., Ernst C (2014). Molecular convergence of neurodevelopmental disorders. *Am J Hum Genet*

- Contributed to experimental design

Lopez, J.P., Lim, R., Cruceanu, C., **Crapper, L**., Fasano, C., Labonte, B., Maussion, G., Yang, J.P., Yerko, V., Vigneault, E. El Mestikawy S., Mechawar N., Pavlidis P., Turecki G (2014). miR-1202 is a primate-specific and brain-enriched microRNA involved in major depression and antidepressant treatment. *Nat Med*

- Designed, conducted and analyzed all experiments treating human neuronal cultures with antidepressants

Talkowski, M.E., Maussion, G., **Crapper, L**., Rosenfeld, J.A., Blumenthal, I., Hanscom, C., Chiang, C., Lindgren, A., Pereira, S., Ruderfer, D., Diallo AB, Lopez JP, Turecki G, Chen ES, Gigek C, Harris DJ, Lip V, An Y, Biagioli M, Macdonald ME, Lin M, Haggarty SJ, Sklar P, Purcell S, Kellis M, Schwartz S, Shaffer LG, Natowicz MR, Shen Y, Morton CC, Gusella JF, Ernst C. (2012). Disruption of a large intergenic noncoding RNA in subjects with neurodevelopmental disabilities. *Am J Hum Genet*

- Analyzed ORF transcription and splicing of long non-coding RNA

Ernst, C., Marshall, C.R., Shen, Y., Metcalfe, K., Rosenfeld, J., Hodge, J.C., Torres, A., Blumenthal, I., Chiang, C., Pillalamarri, V., **Crapper L,** Diallo AB, Ruderfer D, Pereira S, Sklar P, Purcell S, Wildin RS, Spencer AC, Quade BF, Harris DJ, Lemyre E, Wu BL, Stavropoulos DJ, Geraghty MT, Shaffer LG, Morton CC, Scherer SW, Gusella JF, Talkowski ME. (2012). Highly penetrant alterations of a critical region including BDNF in human psychopathology and obesity. *Arch Gen Psychiatry*

- Analysed sequencing data

2. Introduction & Literature Review

2.1. Lesch-Nyhan Disease

Lesch-Nyhan disease (LND) is a rare X-linked neurodevelopmental disorder (NDD) characterized by metabolic symptoms – including a buildup of uric acid crystals in the urine, hyperuricemia, and gout – and neurological symptoms – including severe dystonia, intellectual disability, and chronic self-injurious behaviours (SIB). LND was first identified by Lesch and Nyhan in 1964 (Lesch and Nyhan 1964) and is estimated to occur in 1:380,000 births (Crawhall, Henderson, and Kelley 1972). The causal gene, *HPRT1,* has been known since 1967 (Seegmiller, Rosenbloom, and Kelley 1967), however, despite 50 years of research, the mechanisms and pathways through which HPRT1 mutations cause the neurological symptoms of LND remain unknown.

HPRT1 encodes the protein hypoxanthine-guanine phosphoribosyltransferase (HPRT), a critical enzyme in the purine salvage pathway that restores hypoxanthine and guanine to their monophosphate forms inosine monophosphate (IMP) and guanosine monophosphate (GMP), respectively (Figure 1). Purine reuptake is a critically involved in normal cellular metabolic processes, as up to 90% of the purine pool is maintained through recycling under baseline conditions (Lehninger 1978).

The severity of a patient's disease is strongly correlated with the level of remaining HPRT function (Fu et al. 2015). Typically, patients with less than 1% HPRT function display chronic self-harming and aggressive behaviours, hypertonia, hyperuricemia, and gout, while patients with between 1.5% and 8% residual HPRT activity show metabolic and sometimes motor problems, but do not show the self-injury phenotypes (Fu and Jinnah 2012). These milder forms of the

disease are sometimes called Kelley-Seegmiller syndrome or Lesch-Nyhan Variants (LNV), and often express mild behavioural and neurological abnormalities intermediate between LND patients and controls (Schretlen et al. 2005; Schretlen et al. 2015). It is important to note that LND does not show a perfect phenotype-genotype correlation and that there is variation in disease expression including self-harming behaviours and motor problems even in members of the same family with identical *HPRT1* mutations (Hladnik, Nyhan, and Bertelli 2008). This suggests that compensatory pathways and environmental or epigenetic factors may play a role in shaping LND phenotypes or that other genes may modulate the effects of the absence of HPRT through epistatic effects, but the nature of these factors is unknown. It is also likely that HPRT regulatory elements play a role in defining the severity of HPRT patients, as some patients have been found displaying classical LND clinical and enzymatic phenotypes without any identified mutations in the coding region of the gene (Dawson, Gordon, and Keough 2005; García et al. 2008). In rare cases, female patients have been identified with full LND, but only have one mutated copy of the gene, in these cases the disease is likely caused by non-random X-inactivation (Rinat et al. 2006; De Gregorio et al. 2000).

2.1.1. Clinical features of LND

LND is often diagnosed following the appearance of uric acid crystals in the urine of infants, indicating hyperuricemia. This symptom, along with the gout and liver and kidney failure that are common as LND and LNV progress, has a clear and direct relationship to the loss of HPRT function: The absence of HPRT causes a buildup of hypoxanthine that cannot be converted to inosine and is instead degraded to uric acid through an alternate pathway (Kelley 1972). The resulting hyperuricemia causes uric acid crystals to develop, which then accumulate in the urine, joints, and liver, causing painful urination, sandy diapers, gouty arthritis, and, if left untreated,

liver failure (Srivastava et al. 2002). These symptoms are all effectively managed by treatment with allopurinol which prevents the breakdown of hypoxanthine to xanthine and uric acid (Torres, Prior, and Puig 2007). Allopurinol treatment substantially extends life expectancy for LND patients by preventing liver failure and increases the quality of life by alleviating arthritis and painful urination. Patients who take allopurinol may still have decreased life expectancy, but causes of death are varied. One of the more common causes of death in treated LND patients is sudden death from respiratory failure (Neychev and Jinnah 2006). The causes of respiratory failures are unknown, but it was recently suggested that they may result from respiratory acidosis following a subclinical seizure which is also believed to cause sudden unexpected death in epilepsy (Christy, Nyhan, and Wilson 2016).

Beyond these physical manifestations, patients with LND show a wide variety of behavioural problems. The most striking of these behaviours is self-harm, which is seen in almost all patients with less than 1% HPRT function (Schretlen et al. 2005). LND patients exhibit a wide range of self-injurious behaviours. Among the most common are biting of the lips and fingers, sticking out an arm, leg or head when passing through a doorway, and head banging (Anderson and Ernst 1994), but people with LND appear to use whatever form of SIB is available to them, and it has been said that the variety of self-injury is only limited by the imaginations of the patients and the nature of their motor disabilities (Nyhan 1997).

Parents of LND patients report that self-harm increases during times of stress and boredom, and independent observation of boys under 5 with LND documented an increase in the incidence of self-harm during times of reduced social contact (Hall, Oliver, and Murphy 2001). These challenging behaviours typically start around three years of age but have been reported as early as one year (Anderson and Ernst 1994). Self-harm does not improve with punitive learning, but may

be slightly improved through timeouts and positive reinforcement of positive behaviours (Anderson, Dancis, and Alpert 1978; Anderson et al. 1977). Importantly, Allopurinol treatment does not affect the development of behavioural problems, motor problems, or self-harm (Torres, Prior, and Puig 2007) even when treatment is started in infancy, well before behavioural symptoms develop (Marks et al. 1968). This dissociation between metabolic and neurological symptoms suggests that LND associated behavioural problems are not a result of uric acid buildup and must result from another aspect of HPRT dysfunction.

LND is also associated with increased aggression towards others, expressed through hitting, spitting, scratching, swearing and defiant or vindictive behaviour (Schretlen et al. 2005). Although it is less commonly brought up in the LND literature, outward aggression occurs in LND patients at a similar rate to self-harm (Anderson and Ernst 1994; Schretlen et al. 2005). Interestingly, patients with cri-du-chat syndrome – caused by 5p monosomy – also display aggressive behaviours towards others, including hair pulling, hitting, and throwing objects. These behaviours are more prevalent in LND patients and cri-du-chat syndrome patients do not self-harm at the same rate or with the same compulsivity as LND patients (Collins 2002).

There have been widely varying reports on the degree of intellectual disability associated with LND. Early reports suggested severe intellectual disability (Lesch and Nyhan 1964), but these reports were predominantly drawn from inferences from the patients' behaviours. Formal IQ testing can be challenging in ID populations, particularly when patients have not received traditional schooling (Fuchs et al. 1987; Neisworth and Bagnato 1992). To further complicate matters, the LND behaviours may lead patients to intentionally provide incorrect answers to questions (Anderson, Ernst, and Davis 1992). Two larger studies examining IQ in LND and LNV patients have been conducted; Schretlen et al. (2001) identified an IQ range of 39–81 (mean 59 ±

15) in 15 LND patients, and 49–96 (72 ± 15) in 9 LNV patients. This data was backed up by another larger study finding an IQ range of 39–81 in 19 LND patents (median=60) and from 42-118 in LNV (median=82) (Jinnah et al. 2010)

Although self-injurious behaviours have been observed in other neurodevelopmental disorders the behaviours seen in LND are quite distinct (Crapper and Ernst 2015). When SIB occurs in other NDDs there are usually specific forms or locations of injury. For instance, the occurrence of self-harm also approaches 100% in patients with Smith-Magenis Syndrome (Sloneem et al. 2011), but behaviours are predominantly limited to biting, hitting, picking at fingernails or toenails (onychotillomania), the insertion of foreign objects into body orifices (polyembolokoilamania), and a characteristic "self-hugging" tightly wrapping their arms around their torso and squeezing, which appears to be benign, and exacerbated by happiness (Finucane et al. 1994; Finucane, Dirrigl, and Simon 2001). In cri-du-chat syndrome, estimates of SIB prevalence range from 76.8% (Arron et al. 2011) to 92% (Collins 2002) and are predominantly expressed as hitting the head with another body part, vomiting and rumination, and hitting the head with an object.

While the absence of HPRT disrupts many neurological pathways, the lack of HPRT function specifically appears to be required for the SIB phenotype. For instance, dopamine (DA) is significantly reduced in the brains of LND patients, and dopamine system has been implicated in SIBs. However, dopaminergic dysfunction is not sufficient to cause SIB, as is evidenced by the absence of SIBs in patients with tyrosine hydroxylase deficiencies or mutations in DA receptors.

2.1.2. HPRT1

The human *HPRT1* gene is made up of 9 exons and spans approximately 40.5 kb on the X chromosome q26.2-26.3 (hg38). Ensembl indexes 138 mutations listed as pathogenic or likely pathogenic (version 91, (Zerbino et al. 2017), while the patient-sourced Lesch-Nyhan.org indexes 461 distinct mutations associated with LND and 142 associated with LNV. These mutations are distributed across the gene and, while there are areas of mutational hot-spots, mutations are not restricted to any one domain. In general, mutations that are predicted to reduce catalytic activity are more likely to cause LND; but this too is not a hard rule and there is not a clear causal relationship between each mutation and loss of protein function. (Jinnah et al. 2000)

HPRT1 is commonly perceived as a "housekeeping gene," i.e. a gene that is expressed consistently in most cell types, and is frequently used in the normalization of gene expression data but *HPRT1* expression is substantially higher in the brain than in other tissues. Even within the brain, *HPRT1* expression is different across regions and is much higher in the basal ganglia (Howard, Kerson, and Appel 1970). This differential expression is governed by both positive and negative cis-regulatory elements in the promoter and immediate upstream regions (Rincón-Limas, Krueger, and Patel 1991; Rincón-Limas et al. 1994). It is noteworthy that *HPRT1* expression is highest in the basal ganglia which is also the region most impacted by dopaminergic phenotypes (Song and Friedmann 2007). This raises the possibility of an association between the variable expression across tissues and the specificity of the neurological deficits in LND. This possibility has not been directly assessed.

The human genome has several non-coding pseudogenes to *HPRT1* (Nicklas 2006), in addition to one coding gene phosphoribosyltransferase domain-containing protein 1 (*PRTFDC1*) which has 68% nucleotide identity with *HPRT1*. This pseudogene is expressed and translated, and it can

bind the substrates of HPRT, but it has a catalytic efficiency of less than 0.05% of that of HPRT – an amino acid substitution in the catalytic domain limits nucleotide conversion – and therefore appears not to be involved in purine recycling and its function remains unknown (Welin et al. 2010). *PRTFDC1* is not found in mice, and it has been suggested that ectopic expression of *PRTFDC1* in *HPRT1* knockout (KO) mice increases their aggression and sensitivity to amphetamine (Keebaugh et al. 2011) but the mechanism for this has not been explored.

2.1.3. Purine metabolism

The purines metabolized by HPRT have many uses in the cell. Purines are used as substrates for metabolic processes, as second messengers in signalling cascades, are major contributors to energy metabolism, and can signal through direct binding to dedicated receptors.

The purine pool is maintained through *de novo* synthesis or by nucleotide recycling. In *de novo* purine synthesis, a series of 10 enzymes convert phosphoribosyl pyrophosphate (PRPP) into inosine and consumes glutamine, glycine, aspartate, N^{10}-Formyl-THF, and 4 ATP in the process. The first enzyme, glutamine PRPP amidotransferase, is the rate-limiting step, adding an amide group to the PRPP.

Purines can also be produced through catabolism, also known as purine recycling or purine salvage. In this process, enzymes add a phosphoribosyl group to the nucleoside form of the purine, which more simply means the dephosphorylated form. HPRT converts hypoxanthine (HPX) and guanine to inosine monophosphate (IMP) and guanosine monophosphate (GMP), respectively, while adenine phosphoribosyltransferase (APRT) converts adenine to adenosine monophosphate (AMP).

It is important that both purine *de novo* synthesis and purine recycling can directly lead to the production of IMP, which can be further metabolized either to AMP by adenylosuccinate synthetase (ADSS) and adenylosuccinate lyase (ADSL), or to GMP by inosine monophosphate dehydrogenase (IMPDH 1) and GMP synthase (GMPS). Additionally, GMP and AMP can be metabolized into IMP, allowing a mutual regulation and replenishment of the nucleotide pools, although the guanosine and adenosine do not always exist in equilibrium. There is also variability in the ratio of purine synthesis and reuptake between cell types, and one can often compensate for the other to maintain the purine pool (Tullson et al. 1988).

2.1.4. Purine neurotransmission

Purine nucleotides and nucleosides are most commonly discussed as metabolic substrates for cellular processes and are well known in their roles as the backbone of DNA and RNA or as energy and phosphate donors as adenosine triphosphate (ATP) and guanosine triphosphate (GTP), but they also serve an important role in neurotransmission in the PNS and CNS. ATP and Adenosine (Ade) in particular have strong neuromodulatory roles and signal through dedicated receptors (Table 1) and by co-signalling with other neurotransmitters. Purine receptors arepresent throughout the brain and are involved in many distinct processes including wakefulness regulation, neuromodulation, and neuron-glia communications (Abbracchio et al. 2009; Fields and Burnstock 2006).

P2X receptors operate as homomeric or heteromeric Ca^{2+} permeable ion channels. All P2X receptors are expressed in neurons, and they are expressed in a mosaic throughout the brain in ways that remain poorly understood (Collo et al. 1997). While some studies have suggested glial expression of P2X receptors, they are primarily expressed in neurons (Li et al. 2000). P2Y

receptors are metabotropic receptors expressed in both neurons and glia throughout the brain (Abbracchio et al. 2009).

The P1 receptor family, also known as adenosine receptor A (ADORA) family, are metabotropic adenosine receptors that signal through a variety of G-proteins (Table 1). Although they are found throughout the brain expression is not even. ADORA1 is expressed highly throughout the brain but is most expressed in the cortex and spinal cord, ADORA2A expression is very concentrated in the caudate, putamen and nucleus accumbens (GTEx Browser). These receptors are involved in a myriad of neurological functions including modulating neurotransmitter release (Prince and Stevens 1992; Quarta et al. 2004), inflammation (Ohta and Sitkovsky 2001), vasodilation (Hardebo, Kåhrström, and Owman 1987), synaptic plasticity (Moore, Nicoll, and Schmitz 2003), and sleep (Porkka-Heiskanen et al. 1997). Many of these depend on the interactions between ADORA1 and ADORA2A signalling (Sebastião and Ribeiro 2000; Cunha 2001).

2.1.5. Nucleotide metabolism in LND

Studies examining nucleotide metabolism in LND paint a complex picture that emphasize the tissue and species differences in response to HPRT deficiency. HPRT KO mouse brains have approximately normal levels of purines, and a 4-5 fold increase in purine synthesis (Jinnah, Page, and Friedmann 1993). However, purine levels are reduced in mouse neuroblastoma cell line models of LND, (Wood et al. 1973) and in patient fibroblasts (Willis and Seegmiller 1980), and neither model show an increase in purine metabolism. NAD metabolism and concentration are increased in mouse liver but not brain or blood (Micheli et al. 2009). Moreover, HPRT deficiency decreased the V_{max} of low-affinity nucleoside 5′-triphosphatase (NTPase) activities by up to 7-fold in membranes from human fibroblasts, rat B103- neuroblastoma cells, and mouse Neuro-2a

cells, but increased activity by up to 4 fold in membranes from mouse fibroblasts (Pinto et al. 2005). These discrepancies appear to be caused by complex and poorly understood changes in the regulation and expression of different NTPDase family enzymes (Lorenz, Pinto, and Seifert 2007).

Table 1 *Major classes of purine receptors*

Receptor	Ligand	Receptor type
P1 Receptors		
ADORA1	Adenosine	$G_{i/o}$
ADORA2A	Adenosine	G_s
ADORA2B	Adenosine	G_s
ADORA3	Adenosine	$G_{i/o}$
P2X Receptors		
P2X1	ATP	ion channel
P2X2	ATP	ion channel
P2X3	ATP	ion channel
P2X4	ATP	ion channel
P2X5	ATP	ion channel
P2X6	ATP	ion channel
P2X7	ATP	ion channel
P2Y1	ADP	$G_{q/11}$
P2Y Receptors		
P2RY2	ATP,UTP	$G_{q/11}$
P2RY4	UTP	$G_{i/o}$ and $G_{q/11}$
P2RY6	UDP	$G_{q/11}$
P2RY8	orphan receptor	
P2RY10	orphan receptor	
P2RY11	ATP, NAD+, NAADP+	G_s and $G_{q/11}$
P2RY12	ADP	$G_{i/o}$
P2RY13	ADP	$G_{i/o}$
P2RY14	UDP	$G_{i/o}$

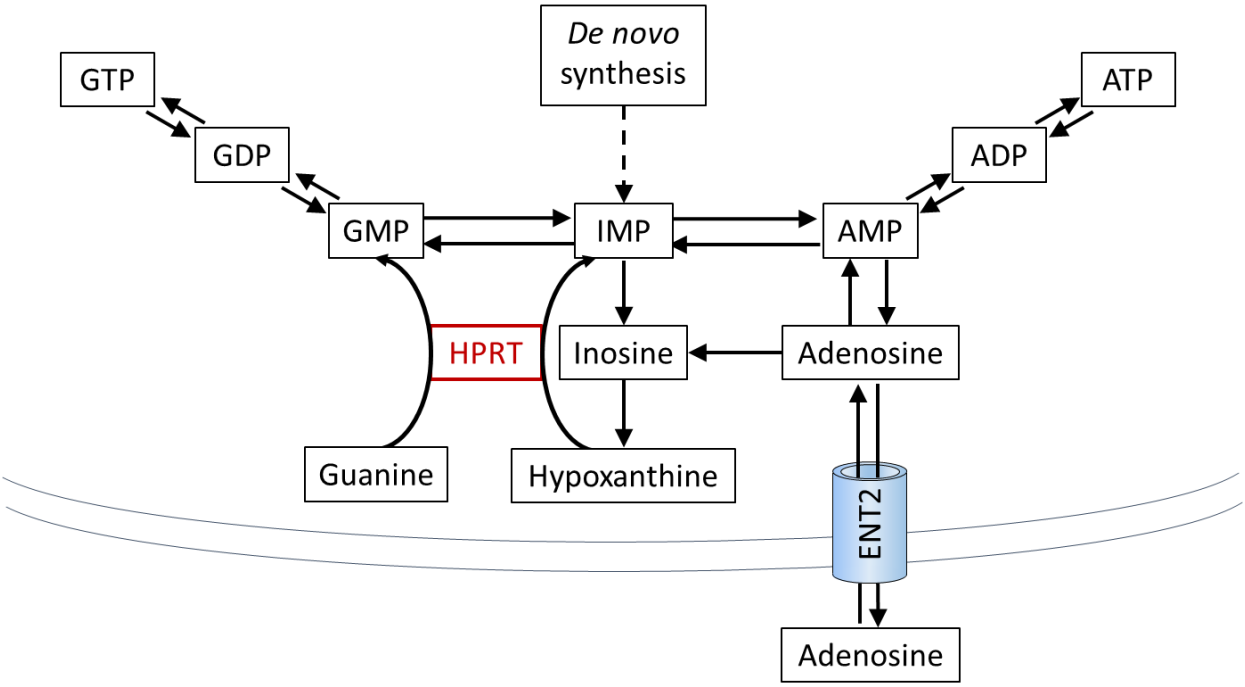

Figure 1 Schematic representation of purine reuptake. *HPRT indicated in red.*

2.2.Neurological features of LND

2.2.1. Dopamine

The most consistent finding across models of LND has been the involvement of the dopamine system. Post-mortem brains from patients with LND show a 60% decrease in striatal DA concentrations compared to controls, and 20-50% reductions throughout the brain (Lloyd et al. 1981). This decrease appears to be the result of a deficiency in DA synthesis rather than a decrease in the number of dopaminergic neurons. The HPRT knockout mouse (Wu and Melton 1993), rat (Isotani et al. 2016), and LND patient brains (Gottle et al. 2014) have shown reductions in Tyrosine Hydroxylase (TH) expression and TH immunoreactivity without significant changes in TH-expressing cell bodies (Jinnah et al. 1994; Gottle et al. 2014). Positron emission tomography (PET) scans in LND patients have revealed 30-50% decreases in DOPA-decarboxylase activity – metabolising L-DOPA to dopamine – and dopamine storage in the

putamen, caudate, and frontal cortex ventral tegmentum (Ernst et al. 1996), and 50-75% reduction in DA transporter binding in the caudate, and putamen of LND (Wong et al. 1996). These studies are complemented by postmortem work showing substantial increases in cell surface expression of D1 and D2 receptors in the putamen and caudate (Saito et al. 1999), increased D1 and D2 receptor sensitivity to amphetamine in HPRT KO mice indicated by a reduced stereotypy breakpoint (Jinnah, Gage, and Friedmann 1991), and persistently increased sensitivity of dopamine receptors in neonatally 6-OHDA lesioned rats (Leslie et al. 1991; Dewar et al. 1990). Though sample sizes are small for each of these studies, the magnitude and consistency of the effects make it clear that DA depletion is a reproducible finding in LND.

One of the enduring mysteries of LND is the cause of the dopamine deficiencies. Although there are several lines of evidence pointing to different causes, none are conclusive. Several groups have identified alterations in the developmental programming of dopaminergic neurons although sometimes with contrasting results. Table 2 shows the results of several studies that have looked at gene dysregulation in *in vitro* models of LND that are presumed to represent early developmental time points. There are very few overlaps in the findings between studies. This is in part because these studies only targeted individual systems and did not look genome-wide, however even in cases where the same genes were examined they results are inconsistent. The only replicated finding is that EN1 and lmx1a are decreased two papers (Guibinga et al. 2012; Kang et al. 2011), but a third paper in a different model finds a significant increase in EN1 (Ceballos-Picot et al. 2009), making interpretation difficult. Others have hypothesized that a broad deregulation of G-proteins ultimately resulting from an overabundance of hypoxanthine leads to changes in the dopamine receptors and dopamine hypersensitivity leads to reduced DA

production but have not provided direct evidence of this hypothesis (García, Puig, and Torres 2012).

2.2.2. Serotonin

There is also some evidence of serotonergic dysfunction in LND. In one study, some patients showed increased levels of 5-hydroxyindoleacetic acid (5-HIAA), the primary metabolite of serotonin, in their CSF (Jankovic et al. 1988). However, in two others 5-HIAA levels were within (Silverstein et al. 1985; Lloyd, Hornykiewicz, and Davidson 1981). Work in patient lymphocytes had demonstrated a decrease in the expression of serotonin receptors (García, Puig, and Torres 2012), and patient brains have increased serotonin concentrations (Breese et al. 1995), and serotonin appears to mediate some behaviours in the 6-hydroxydopamine lesioned rat (Allen and Davis 1999).

2.2.3. Nucleotides

Because HPRT is involved in nucleotide metabolism, it seems clear that nucleotides should play an important role in the pathogenesis of LND. Considering the directness of this relationship surprisingly little work on the connection of nucleotides in the brain and LND has been carried out. Research on purine metabolism in LND was prominent after HPRT was identified as the causal gene in LND, and revealed many of the biochemical aspects of the disease, including greatly elevated levels of hypoxanthine in the CSF and urine of LND patients (Harkness, McCreanor, and Watts 1988; Sweetman and Nyhan 1970), in increased dependence on de novo purine synthesis in HPRT deficient cells (Upchurch and Gabridge 1983), an that HPRT deficient cells have low levels of GTP (Simmonds et al. 1988) and high levels of hypoxanthine (Palmour, Heshka, and Ervin 1989). Other suggestions of the importance of purine levels in LND came from animal models. Chronic administration of high levels of caffeine, which is a broad

Table 2 Significant gene expression changes in in vitro models of LND. *Genes that have been reported as significantly increased (Up) or decreased (Down) in each model of LND. Due to the large number of genes in Dammer et al. increased and decreased GO terms have been listed with the number of significant genes in each cluster indicated below.*

Paper	Lewers et al. 2008	Ceballos-Picot et al. 2009	Cristini et al. 2010	Kang et al. 2011	Guibinga et al. 2012	Mastrangelo et al. 2012	Guibinga, Murray, and Barron. 2013	Guibinga et al. 2013	Dammer et al. 2015
Model	MN9D	MN9D	fetal NPC	SH-SY5Y	SH-SY5Y	ESCs/iPSCs	MN9D	SH-SY5Y	PC6-3 (rat)
Up	COMT GCH1 NOS1 SOD2	Chrnb2 En1 Hoxa5 Jund1 Arrb2 Camk4 Nos1	PPARD BMP2 Notch1 myst GCN5l2			NTPase	PDE10A		RNA splicing (N=19) RNA binding (N=33) Mitochondrial (N=54)
Down	DAT MAOB parkin TH DDC VMAT a-syn	Chrna6 Gria3	B-Tub FLT4 CD44 NCAM1 Neurog2 TUBB3 ALDH1	P-B-catenin EN1 lmx1a PS1 metabolites	En1 EN2 BRN2 LMX1a	p2y1 P2X3 b-catenin	CREB Synapsin PKA signalling	epac1 rap1 epac2 prex tiam1 vav2	tRNA Aminoacylation (N=19) Translation initiation (N=12 Proteasomal proteins (N=10) Aminopeptidases (N=8) Ribosomal proteins and biogenesis (N=7) Amine biosynthesis (N=9) Amine binding (N=11 Neurotransmitter transport (N=10) Neurotransmitter regulation (N=10) Neurotransmitter secretion/release (N=9) mitochondrial function (N=114)

adenosine receptor agonist, causes a self-biting behaviour in rats and has been proposed as a model for LND (Lloyd and Stone 1981), and similar behaviour is seen following chronic dopamine agonism (Mueller et al. 1982). Additionally, agonism of the ADORA2A receptor reduces dopamine-induced self-biting in neonatally 6-OHDA lesioned rats, and agonism of ADORA1 increases dopamine-induced rearing and paw treading and a decrease in grooming in these rats, although not self-biting.

Despite this early evidence of the importance of purine levels and adenosine signalling in LND, for the last two decades research on this topic has been limited and predominantly carried out by one research group. Over a series of papers they shown that hypoxanthine can reduce adenosine transport through the EN1 and EN2 equilibrative nucleoside transporters in peripheral cells (Prior, Torres, and Puig 2006).They hypothesized that this increases extracellular adenosine, leading to a decrease in adenosine receptors which help regulate the amount and activity of dopamine and serotonin receptors (García, Puig, and Torres 2012). This group has also published data suggesting that the presence of excess hypoxanthine causes changes in gene expression during neurodevelopment, however among the documented changes is an increase in the expression of TH and ADORA2A (Torres and Puig 2015) which contradicts both the decreased TH expression that would be expected in LND and data from their previous work which showed in decreases of ADORA2A in HPRT deficient peripheral cells (García, Puig, and Torres 2009).

2.3.Modeling LND

Relating the different neurological features of HPRT function and purine metabolism to the symptoms of LND has been difficult because no single LND model captures the entirety of the

disease. LND has been modelled using transgenic animals, pharmacological models, and in vitro models.

2.3.1. Transgenic models of LND

Because of its ubiquitous expression, and usefulness as a positive and negative selectable marker, *HPRT* was the among the first genes to be knocked out of a mouse (Kuehn et al. 1987). Unfortunately the *HPRT* KO mouse has no behavioural phenotypes reminiscent of LND (Finger et al. 1988). *HPRT* KO mice appear to have normal learning and memory, normal motor function and do not display any aggressive behaviours or self-harming behaviours (Kuehn, Bradley, and Robertson 1987; Jinnah et al. 1999; Finger et al. 1988; Edamura and Sasai 1998; Keebaugh et al. 2011). This remains the case if the mice are stressed by treatment with amphetamine (Jinnah, Gage, and Friedmann 1991). Additional disruption of purine recycling through either genetically knocking out (Engle, Womer, and Davies 1996) or pharmacologically inhibiting (Edamura and Sasai 1998) the function of APRT, a complementary enzyme recycling the purine adenosine, is also not sufficient to cause LND like behavioural changes. Despite this, some neurological disruptions have been identified in HPRT knockout mice, including reductions in DA concentrations throughout the basal ganglia (Jinnah et al. 1994; Gottle et al. 2014). This finding held true, though to varying degrees, across five separate strains of HPRT knockout mice (Jinnah et al. 1999). The Mouse Genome Database (Eppig et al. 2015) has described the majority of the $HPRT^{null/Y}$ mice it has examined as having at least one of abnormal motor capabilities/coordination/movement as measured by the tail suspension test, or abnormal motor coordination/balance measured as increased slips when climbing in a forced swim test but motor problems in HPRT mice have not been widely recorded in the literature. In another example, a lowered amphetamine breakpoint - one of the only behavioural phenotypes to have been reported

in the mouse (Jinnah, Gage, and Friedmann 1991) - has not been used to examine the dopamine system in other mouse strains or to examine the effects of treatments before they are given to LND patients.

Recently, two groups have generated HPRT KO Rat lines using homologous recombination (Meek et al. 2016) in rat embryonic stem cells or using through mouse←rat ES chimeras (Isotani et al. 2016). Similar to the mouse models, the HPRT knockout rat lacks immediately apparent behavioural phenotypes but also demonstrates a reduction of dopamine in the basal ganglia. Because of the newness of the HPRT-KO rat models, they have been even less substantially phenotyped. Both papers describe normal motor function but did neither detail any formal testing. While both papers report a decrease in midbrain dopamine levels, none perform any dopamine-dependent behavioural tasks or examine the effects of amphetamines on rat behaviour.

HPRT has also been the target of gene knockout in the rabbit (Yin et al. 2015) however the mutation was embryonic lethal in HPRT Knockout rabbits and female carrier rabbits did not display behavioural phenotypes. These experiments were conducted as a proof of concept for genetically engineered rabbits, however, rabbits have been shown to exhibit forms hereditary self-harm (Iglauer et al. 1995) and may, therefore, be a candidate animal for modelling LND.

2.3.2. Pharmacological models of LND

Several pharmacological models have also been used in the study of LND. The most prevalent is the neonatally 6-hydroxydopamine (6-OHDA) lesioned rat. In the model 6-OHDA, which selectively ablates dopaminergic neurons, is administered to rats neonatally. When neonatally rats are treated with L-Dopa or other dopamine agonists they are observed to bite their paws, which some have interpreted as a similar to the self-harm observed in LND (Knapp and Breese 2016),

but the self-harming behaviour is not seen in rats lesioned in adulthood. While this has been the most commonly used model to study LND, it is not the only pharmacological model of self-biting. Self-harming has also been shown following chronic high dose administration of the adenosine receptor antagonist caffeine (Minana et al. 1984; Peters 1967), the stimulants pemoline (Mueller and Hsiao 1980) and amphetamine (Mueller et al. 1982), the dopamine reuptake inhibitor GBR-12909 (Heikkila and Manzino 1984), and the calcium channel agonist Bay K 8644 (Morpurgo 1968). Although they do not all act on the dopamine system directly, dopamine appears to play an important role in self-harm across each of these models (Devine 2012). Interestingly, HPRT deficient mice do not show increased susceptibility to the self-harming caused by treatments with any of these compounds (Kasim and Jinnah 2002). It is therefore possible that the effects of HPRT loss in mice are epistatic to the effects of the drugs, that the drugs are acting on a pathway that is independent of the dopaminergic effects of HPRT loss, or that similar concentrations of the drugs are required to achieve the pharmacological effects that lead to self-biting, regardless of the reduced dopamine caused by HPRT loss.

2.4. Treating LND

A variety of approaches have been used to try and treat LND. Because of the observed decreases in striatal DA, Levodopa, which is metabolized to dopamine in the brain, was given to patients. Despite the efficacy of levodopa in Parkinson's disease (Cotzias, Papavasiliou, and Gellene 1969), it was not observed to have any beneficial effects in LND patients (Proctor and Mcginness 1970) and appears to make SIB worse in some patients (Visser et al. 2011). Based on the idea that LND behaviours may result from dopamine hypersensitivity, treatment with D1 antagonists has been attempted. In a single dose, open-label study, the majority of patients experienced significant off-target effects and needed to be withdrawn from the study, however one patient

showed a continued improvement of symptoms (Khasnavis et al. 2016). It is possible that LND patients are more sensitive to D1 antagonists than other patients – the dose was based on that used in several other disorders – and that with proper dosing this could be an effective treatment, but the data so far are inconclusive.

Some trials targeting serotonin to reduce self-harming behaviours in LND have been promising. Administering 5-hydroxytryptophan, a precursor to serotonin, has decreased self-harm in two small studies of LND patients (Frith et al. 1976; Mizuno and Yugari 1975), but not in all studies (Castells et al. 1979) and the effects can disappear repeated treatment (Nyhan et al. 1980). Risperidone, an atypical antipsychotic which antagonizes DA and serotonin receptors, has been shown to reduce challenging behaviours, including SIB in one patient (Allen and Rice 1996)

Through serendipity, S-Adenosylmethionine (SAMe) has become a promising potential treatment. SAMe was first administered to an LND patient to treat liver symptoms associated with fentanyl treatment but clinicians and family members were surprised to see a rapid and sustained decrease in self-injurious behaviours and motor problems (Glick 2006). Another study in five family members with LND, each with the same mutation, found a moderate improvement in SIB and motor function in all patients. A larger study with 14 LND patients found that they divided into two groups: a group of responders who improved substantially with SAMe treatment, and non-responders who either remained at baseline or in many cases got worse with SAMe treatment (Dolcetta et al. 2013). The most recent study used a single patient for a blinded, within-subject design, alternating between 50 days of placebo treatment and 50 days of SAMe, and found significantly reduced SIB during treatment (Lauber et al. 2016). Taken together it is possible that there are multiple groups of LND patients: some that respond positively to SAMe,

and some that respond to D1 antagonists. More studies will be required to determine to what extent these groups overlap, and to what extent they are determined based on mutation type.

2.5.iPSC disease modelling

2.5.1. Induced pluripotent stem cells

Stem cells, cells that can divide indefinitely and differentiate into different cell types, were first discovered in 1963 by Till and McCulloch when they identified a population of cells in bone marrow that could differentiate into numerous cell types, repopulating the hematopoietic and immune cell systems (Till and McCulloch 1961). This discovery led the way for isolating and working with stem cells, and the eventual isolation and expansion of embryonic stem cells (ES) from mouse (Martin 1981) and from humans (Thomson et al. 1998). Through studying gene expression differences between ES cells and somatic cells the group of Shinja Yamanaka identified a network of regulatory genes related to maintaining pluripotency, and that by adding four of these genes through adding combinations of these factors to fibroblasts, identified OCT4, SOX2, KLF4, and c-MYC – known as the Yamanaka factors – as master transcription factors that regulate the pluripotency network. They also found that driving ectopic expression of these genes is sufficient to convert mouse (Takahashi and Yamanaka 2006) and human (Takahashi et al. 2007) fibroblasts into induced pluripotent stem cells (iPSCs). iPSCs can differentiate into cell types from any germ layer, and will spontaneously form teratomas with all three layers if injected into a mouse. Although establishing and differentiating iPSC cultures remains a challenging procedure and requires large investments of time and resources, many groups have worked to improve the efficiency of conversion, growth conditions and differentiation protocols available (Malik and Rao 2013; Rony et al. 2015; Bell, Peng, et al. 2016).

Substantial amounts of work have been done to guide the differentiation of iPSCs to different cell types, and there has been a particularly strong focus on neural differentiation. The general principle of iPSC differentiation is to provide the cells with similar signalling cues to what they would experience during differentiation into the cell type of interest *in vivo* and to inhibit signalling that would contribute to alternate pathways. Presently the most common approaches to neural differentiation involve the inhibition of the SMAD pathways to encourage neuroectodermal differentiation, combined with the addition of signalling factors to specify the neuronal cell type of interest (Chambers et al. 2009).

2.5.2. Modelling developmental disorders with iPSCs

The ability of iPSCs to create neurons from patients, resulting in neural cultures with the genetic background of the patient, has been a very powerful tool for studying rare neurodevelopmental disorders. Historically, neurological diseases including LND have primarily been studied using immortalized cell lines and animal models (Van der Worp et al. 2010; Kaiser and Feng 2015). While these analogs of LND have been invaluable research tools, concerns about the translatability of results obtained in these models have been raised since their inception and success in translating pre-clinical findings from animal or immortalized models to the clinic has been limited (Kaiser and Feng 2015; Albani and Prakken 2009).

iPSCs have several advantages over other methods of modelling NDDs. That they are derived from patient cells eliminates both intra- and interspecies variation and prevents the confounding oncogenic effects observed in immortalized cell lines. Further, they approximate the developmental patterns human neurons *in vivo* (Marchetto et al. 2011), meaning that they may provide a better model of the early impacts of NDDs. When paired with genome editing technologies like CRISPR/Cas9, iPSCs become an even more powerful tool, since these allow for

the rapid creation of monogenic disease models, or for the creation of isogenic controls based on patient cell lines (Smith et al. 2014; Heidenreich and Zhang 2016).

2.6. The future of LND research

Because of the genetic simplicity of LND it has been a go-to disease in the use of new research methodologies including being one of the first genes knocked out of a mouse (Hooper et al. 1987) but, so far, the secrets of LND have not been forthcoming . This appears to stem predominantly from four factors 1) The rarity of LND makes well powered well designed studies in patients or patient brain tissue exceptionally challenging, 2) For reasons unknown HPRT knockout rodents do not show behavioural analogs to LND patient, 3) The presence of dopamine deficits in both patients and HPRT-KO rodents has lead to an intense focus on dopaminergic neurons system at the expense of studying other systems, and 4) Labs that focus on rare diseases like LND are historically underfunded and most lack the resources and expertise that has been required for many advances in research methodologies.

The proliferation and increasing accessibility research techniques like iPSCs, genetic engineering using CRISPR-Cas9 genome editing, and RNA sequencing now allow us to overcome many of these barriers. The goal of this thesis is to use these techniques to develop novel *in vitro* human neuronal models of LND, and to use these models to examine how HPRT deficiency impacts distinct neuronal subtypes.

3. Purine and Mitochondrial Changes in a Knockdown Model of LND

3.1. Introduction

Many of the current challenges in LND research stem from the lack of understanding of the downstream effects of HPRT disruption in neurons. Although HPRT is considered a housekeeping gene and expressed fairly consistently across cell types, it is more highly expressed in the brain than other tissues (Lo and Palmour 1979) and more expressed in the midbrain than other brain regions (Stout et al. 1985). This suggests that HPRT function is particularly important in these regions and may be connected to the particular dysfunction of midbrain dopaminergic neurons in LND patients. The importance of HPRT in the brain may also explain the prevalence of neurological symptoms, and the relative lack of symptoms related to the dysfunction of other organs.

Still, the relationship of HPRT function to neurodevelopment and behaviour is unclear. Effectively studying this connection is difficult because HPRT deficient mouse models show functional brain deficiencies but no behavioural problems, muddying the relationship between findings of studies in mice and human disease. At the same time, the rarity of the disease makes large-scale studies on patients or human brain tissue are exceptionally challenging to organize. The largest post-mortem study in LND to date has used just five patient and six control brains (Gottle et al. 2014). In order to unravel the relationship of HPRT function to neurodevelopment and brain function, new models will need to be developed. Ideally, these models will use human cells, be based on well-characterized disease modelling approaches, reflect the genetics of LND,

and be easy to work with and to distribute in the research community (Bell, Kolobova, et al. 2016).

Short hairpin RNAs (shRNAs) are a simple and effective approach to reduce a gene of interest in a target cell line based on of gene regulation by double-stranded RNA, which has been identified in plants and animals (Lau et al. 2001; Jorgensen 1990). When a lentivirus containing a shRNA is transfected into a cell, it can insert the shRNA into the host genome, resulting in stable expression of the shRNA and substantially reducing the expression of the target gene over multiple cell divisions (Rubinson et al. 2003). This stability makes this approach particularly well suited to rapidly developing cell lines that do not express a target gene, and that can easily be expanded and shared with other researchers and collaborators. shRNAs have been used both *in vitro* and *in vivo* to examine the impacts of many genes associated with intellectual disability and neurodevelopment, including AUS2 (Hori et al. 2014), FMRP (Alpatov et al. 2014; Khalfallah et al. 2017), CYFIP1 (Yoon et al. 2014), and MeCP2 (Marchetto et al. 2010). This technique is particularly powerful when combined with high throughput analyses like RNA sequencing (RNAseq).

shRNAs have previously been used in the study of LND by reducing HPRT mRNA in human embryonic stem (ES) cells, achieving an almost complete reduction in HPRT protein but these cell lines were not differentiated into any terminal cell type and were only used to examine alterations in the purinergic receptor P2Y1 receptor and signalling (Mastrangelo et al. 2012). Another study used shRNA to knock down HPRT signalling in mouse embryonic stem cells differentiated to stages of neural differentiation and used microarrays and RNAseq to examine transcriptome level effects (Kang et al. 2013). The authors of this study found significant alterations in pathways usually associated with neurodegenerative disease, among other

pathways. The decision to perform this study in mouse ES cells was puzzling since the lab had previously published work with undifferentiated human ES and iPSCs, and these models would have had substantially more disease relevance. Further, the data is presented only presented as GO terms, and no targets were validated with other techniques.

Advances in RNA sequencing technology have facilitated the examination of global changes in gene expression with a higher level of confidence and statistical significance. These approaches allow the identification of disrupted pathways using an unbiased, non-hypothesis-driven approach.

In order to improve our understanding of the dysregulation that occurs in HPRT deficient human neuronal cells, we used a shRNA-mediated knockdown of HPRT1 in the RenCell VM neural progenitor cell (NPC) line. By combining this neuronal cell HPRT1 knockdown with RNAseq, we have identified novel phenotypes of HPRT deficiency and validated those findings using targeted approaches at the RNA and protein level.

3.2. Methods

3.2.1. Cell culture

We grew the RenCell VM neural progenitors (EMD Millipore) on Poly-L Ornithine/laminin (Sigma-Aldrich) coated plates in 70% DMEM (Life Technologies) 30% F12 media (Mediatech Herndon) supplemented with 1x B27, 20µg/ml bFGF (R&D Systems), 20µg/ml EGF (Sigma-Aldrich), and 20µg/ml heparin (Sigma-Aldrich) to maintain the NPC phenotype. Media was changed every 2-3 days, and cells were passaged 1:3 using trypsin upon reaching confluence. To induce differentiation, we removed bFGF, EGF, and heparin from the growth media when the

cells were 70-80% confluent and maintained the cells changing the differentiation media every 3-5 days without passaging.

3.2.2. HPRT knockdown

To generate stable HPRT deficient NPC lines, we obtained anti-HPRT and control shRNAs packaged in replication incompetent lentiviral vector PLKO.1 from The RNAi Consortium at the Broad Institute (now commercially available through Sigma-Aldrich). We screened five hairpins targeting HPRT1 mRNA (HPRT1299782, HPRT1299783, HPRT1299857, HPRT1299874, and HPRT135052), and 7 hairpins targeting the negative controls green fluorescent protein (GFP72197) red fluorescent protein (RFP72204, RFP72212, RFP72221), firefly luciferase (LUC72253, LUC72261) and the lac operon Z locus (LacZ72232, LacZ7224). As these genes are not expressed in human cells or the RenCell VM line, they serve as controls for potential off-target effects or general effects of transfection.

To create stable cell lines, we transfected the RenCells with the PLKO.1 lentivirus. Cells were maintained at 30% confluency (~400,000 cells/well) in a 6-well plate and then dosed with 20 μl viral media in 2 ml cell culture media without penicillin and streptomycin. Culture media was changed 24 and 48 hours after selection. At 48h 0.8 μl/ml puromycin (Sigma-Aldrich) was added to the cultures to remove any cells that did not contain the lentivirus, and therefore did not integrate the shRNA. Stable cell lines were selected by continuous maintenance of low-dose puromycin in culture media (0.2 μl/ml). After creation and selection of stable cell lines, the knockdown cell lines were frozen in cell culture media without puromycin plus 10% DMSO and regrown as required.

3.2.3. qPCR

We extracted RNA from cultured RenCell VM NPCs, induced pluripotent stem cells, and fibroblasts using the RNeasy Mini Kit.

rtPCR samples were run in triplicate, and replicates contributing to a CT standard deviation over 0.3 between replicates were removed before proceeding. To account for possible differences in loading or other possible causes of RNA level differences RNA expression was normalized to the housekeeping gene B-Actin, the expression of which is predicted to be consistent across samples. Expression is presented normalized to control levels. Means were compared using a T-test.

Table 3 List of rtPCR primers used

Target	**Primer (Taqman)**
ACTB	Hs99999903_m1
ADORA1	Hs00379752_m1
ATP5A	Hs01081389_g1
ATP5H	Hs01046892_gH
EIF3E	Hs01066186_m1
GAPDH	Hs02758991_g1
HPRT1	Hs02800695_m1
LDHB	Hs05001188_g1
NT5E	Hs00159686_m1
RPL9	Hs01591540_g1
SLC29A2	Hs00155426_m1

3.2.4. RNA sequencing

RNA was extracted from two independent replicates for each shRNA construct, then sent to the McGill University and Genome Quebec Innovation Center for library construction and sequencing. There, expert technicians prepared RNA sequencing libraries from high-quality RNA (RNA integrity number > 9; Agilent 2100 Bioanalyzer). Before library preparation, external RNA

controls from the External RNA Control Consortium (Life Technologies) were added to each sample to assess sequencing depth and create standard curves allowing us to determine the depth and quality of sequencing and library preparation. Three libraries were run per lane of an Illumina HiSeq 2000 flow cell (100 bp paired-end reads), which achieved an average of 69529790 million mapped reads per library. The quality of the sequencing of each sample, including Phred score distribution and primer or adapter reads, was determined using FASTQC (Andrews 2010). End sequences corresponding to TruSeq adapters or Illumina universal primers are removed using Trimmomatic (Bolger, Lohse, and Usadel 2014) and the FastX toolkit (Gordon and Hannon 2010). Whenever the average Phred score of the extremities of a read falls lower than 15, these edges were trimmed, and only reads longer than 20 bp were kept. Finally, PRINSEQ (Schmieder and Edwards 2011) was used to remove mRNA poly-A tails. Sequence reads were then aligned to the appropriate reference genome using TopHat2 splice-aware alignment (Kim et al. 2013). To maximize the amount of usable data generated from the paired-end run, orphaned reads were aligned separately from paired reads, then the alignments were combined. Finally, HTSeq (Anders, Pyl, and Huber 2014) was used to count the reads that were aligned to each gene, and generate raw read counts.

To determine differentially expressed genes in the RNAseq dataset we use R, and the bioinformatics statistical package DESeq2 (Love, Huber, and Anders 2014). DESeq2 scales the counts of each sample based on their total number of reads to provide counts not biased by inter-sample variability in RNA concentration or sequencing efficiency. Principal component analysis (PCA) was performed on the normalized read counts to determine the largest sources of variance within our dataset. We then run differential expression analysis using a general linear model and the Benjamin-Hochberg multiple testing correction.

3.2.5. Western blots

Table 4 List of Antibodies Used

Targeted antigen	Host species	Dilution	Manufacturer	Cat#
ADORA1	rabbit	1:1000	Origene	TA310176
ATP5A	Mouse	1:2000	Abcam	ab14748
ATP5H	Mouse	1:300	Abcam	ab173006
ENT2	rabbit	1:2000	Abcam	Ab48595
FOXA2	Mouse	1:100	Abcam	ab117542
HPRT1	Rabbit	1:100	Abcam	ab10479
Nanog	Rabbit	1:100	Abcam	ab109884
Nestin	Mouse	1:2000	Stemcell technologies	69001
OCT4	Rabbit	1:100	Abcam	ab109884
OCT4 (OCT3)	Mouse	1:1000	Stemcell technologies	69001
Otx2	Rabbit	1:500	Abcam	ab114138
PAX6	Rabbit	1:500	Stemcell technologies	69001
SOX1	Rabbit	1:1000	Stemcell technologies	69001
SSEA4	Mouse	1:100	Abcam	ab109884
TRA-1-60	Mouse	1:100	Abcam	ab109884
Tuj1	Mouse	1:200	Abcam	ab78078

We performed western blots to quantify protein levels of genes of interest identified in the RNAseq. NPCs containing the shHPRT and control constructs lysed in RIPA buffer with protease inhibitor cocktail (Sigma-Aldrich), and centrifuged at 15000 G for 20 minutes at 4^0C the supernatant, containing the solubilized proteins, was collected at stored at -80^0C. Protein concentrations were measured using the Peirce BCA assay (Thermo-Fischer), and equal amounts of each sample were run in duplicate on 4-15% gradient stain-free gel (Bio-Rad) for 60 min at 110v. Proteins were transferred to a nitrocellulose membrane and blocked with 3% skim milk powder for 20 min. Primary antibodies (Table 4) were incubated overnight at 4^0C in TBST 4% BSA. HRP-conjugated secondary antibodies were incubated for 1h at room temperature. Blots

were visualized using the Pierce ECL Western Blotting Substrate (Thermo-Fischer) on the Bio-Rad Chemidoc illumination system and normalized to whole cell protein. Means were compared using a T-test.

3.3. Results

3.3.1. Creating HPRT deficient human neural progenitor cells

RenCell VM (EMD Millipore) is a commercially available immortalized neural progenitor cell line, isolated from the ventral midbrain of a human fetus and immortalized through overexpression of the Myc oncogene. RenCell VM progenitors can be expanded over numerous passages maintaining a normal karyotype and can be differentiated into electrically active neurons (Donato et al. 2007), as well as astrocytes and oligodendrocytes by the withdrawal of growth factors (Hoffrogge et al. 2006). The ease of working with this cell line makes it an ideal candidate for preliminary studies of the effects genetic disruptions, particularly those thought to have neurodevelopmental effects, in neural progenitor cells.

To reduce the levels of *HPRT1* expressed in the NPCs, we used lentiviral transfection with anti-*HPRT1* short hairpin RNA (shRNA). shRNAs are processed using the same machinery as microRNAs in the cell, allowing them to target and degrade specific mRNAs based on sequence complementarity. This approach can be used to dramatically reduce the levels of a target mRNA and protein (Root et al. 2006). Stable knockdowns, in which the target gene in continuously reduced can be generated using lentivirally encoded shRNAs. In these cell lines, the shRNA sequence integrates into the host genome, creating a line that will continuously express it across many passages. This approach is rapid and efficient and allowed us to generate robust transcriptome-wide data on the impacts of HPRT removal.

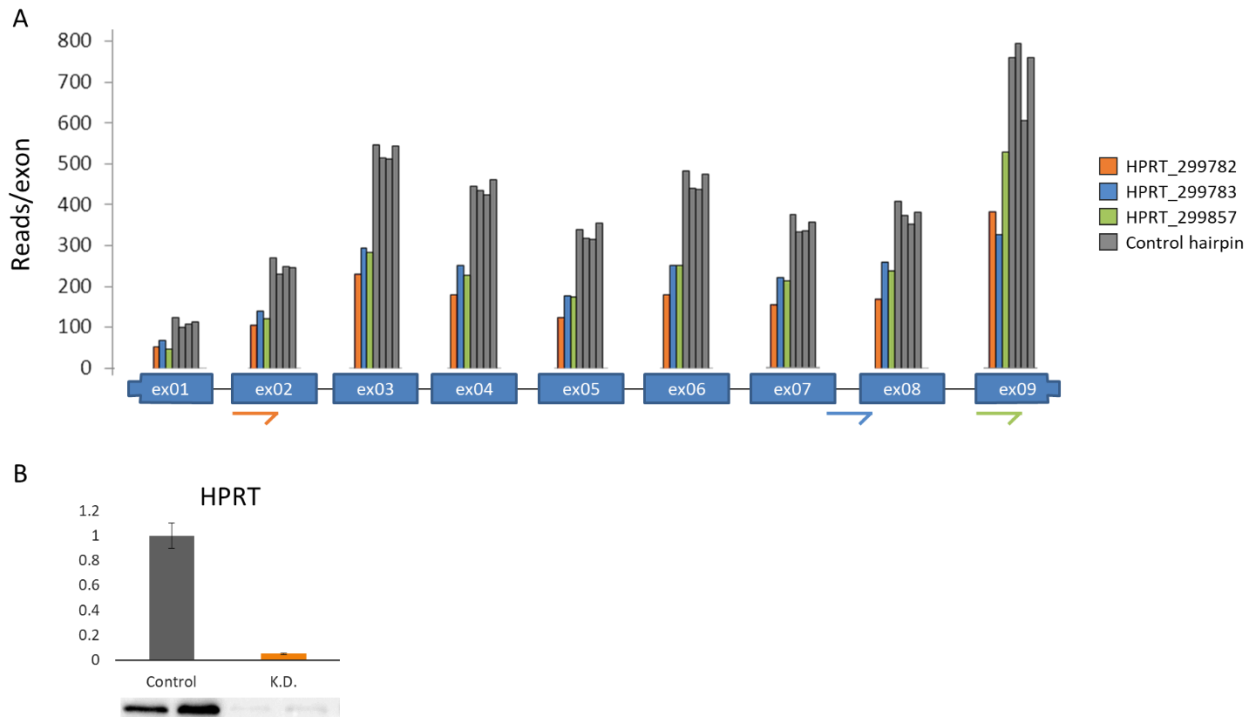

Figure 2 Knockdown of HPRT in immortalized fetal NPCs. A) *Top: Raw read count for each exon in in the shHPRT NPCs (TRCN0000299782 orange, TRCN0000299783 blue, TRCN0000299857 green) and control transfected lines (grey) from RNA sequencing. Bottom: Schematic depiction of the nine exons of HPRT1 including the targets of anti shHPRT hairpins (TRCN0000299782 orange, TRCN0000299783 blue, TRCN0000299857 green).* ***B)*** *Western blot quantification of HPRT in the HPRT299782 knockdown line reveals a depletion of HPRT protein in the shHPRT NPCs ($p < 0.001$). Data presented as mean ± standard error*

We screened five anti-HPRT hairpins and seven hairpins serving as non-target controls. The effectiveness of HPRT1 shRNAs was validated by measuring HPRT1 expression using rtPCR, revealing that hairpin knockdown efficiency reduced *HPRT1* mRNA by 35-80% (Figure 2a). While this reduction in RNA levels is not as large as the reduction in enzyme function LND, we believe that any changes occurring in these cell lines should also be occurring in fully HPRT deficient cell lines. Further, western blot analysis comparing the knockdown HPRT299782 to the

control line LacZ7224 suggested there may be a greater reduction at the protein level (Figure 2b).

RNA sequencing reveals deficits in adenyl nucleotide binding and energy metabolism

We selected the three most effective HPRT knockdowns (HPRT1_299782, HPRT1_299783, and HPRT1_299857), and three controls (LUC_7224, LUC_72261, RFP_72212) to be sent for RNA sequencing (RNAseq). To our knowledge, this is the first transcriptome-wide study of a human neuronal model of LND. Each cell line was grown in 2 independent cultures, and RNA was processed and sequenced at the Genome Quebec Innovation Center.

Unsupervised hierarchical clustering of the significantly differentially expressed genes perfectly clustered all technical replicates (separate cultures using the same hairpin) and biological replicates (separate hairpins targeting HPRT1 and controls), which also clustered tightly in a PCA analysis (A and 4B).

To better understand the processes that are differentially regulated in response to HPRT deficiency we divided the genes into those that were increased or decreased in the shHPRT lines (genome-wide q value <0.05) and used DAVID version 6.6 Gene Ontology clustering (Huang, Sherman, and Lempicki 2009) to determine the types of biological processes and molecular functions of genes that were disproportionately affected by HPRT removal. Figure 3 shows a representative term from each of the top 5 enriched ontology clusters among the increased (Figure 3 C) and decreased (Figure 3 D) gene sets. The most enriched clusters among increased genes exclusively consisted of genes related to mitochondrial function and protein translation, while several of the clusters enriched in decreased genes involved purine nucleotide binding and regulation. Surprisingly, when Cluster 3, associated with purine nucleotide binding was examined in more detail the majority of the terms related to adenyl related nucleotides (Figure 3 E). HPRT

is directly involved in guanosine and inosine metabolism but is several metabolic steps away from adenosine (Figure 1). These findings were of particular interest since genes related to energy metabolism were increased, while genes related to adenyl nucleotide binding were decreased. These two gene functions are closely related through ATP. Targeted validation of RNAseq results.

To validate these findings using a targeted, hypothesis-driven approach, we selected genes related to the enriched GO terms that were significantly differentially expressed between shHPRT1 lines and controls, had a large effect size (>30% change in expression), and have well-understood functions that could be related to disease pathology. To represent adenyl nucleotide binding genes we selected the adenosine receptor A1 (ADORA1), a Gi-coupled receptor expressed throughout the brain and the equilibrate nucleotide transporter SLC29A2, which encodes the protein Equilibrative Nucleoside Transporter 2 (ENT2) transporting adenosine and hypoxanthine into and out of neurons and astrocytes. As representative genes from the mitochondria-related clusters, we selected ATP5H, a component of the mitochondrial ATP Synthase Core complex, and Lactate Dehydrogenase B (LDHB), which catalyzes the conversion between lactate and pyruvate, contributing to the regulation of pyruvate levels for the TCA cycle. The RNA and protein used for the RNAseq, rtPCR, and western blots were all obtained from independently grown cell cultures.

Adenosine related genes ADORA1 (RNA p=0.042, protein p=0.11) and ENT2 (RNA p=0.003, protein p=0.030) were decreased across all methods of study (Figure 4). And western blotting validated the increase in the mitochondrial gene LDHB (p=0.016), while the increase in ATP5H was not statistically significant (p=0.14, Figure 5). While the changes in protein levels of ADORA1 and ATP5H did not reach the level of statistical significance, the effect size and

direction are consistent with our RNA level findings and absence of statistical significance is likely due to the small sample sizes available for this experiment.

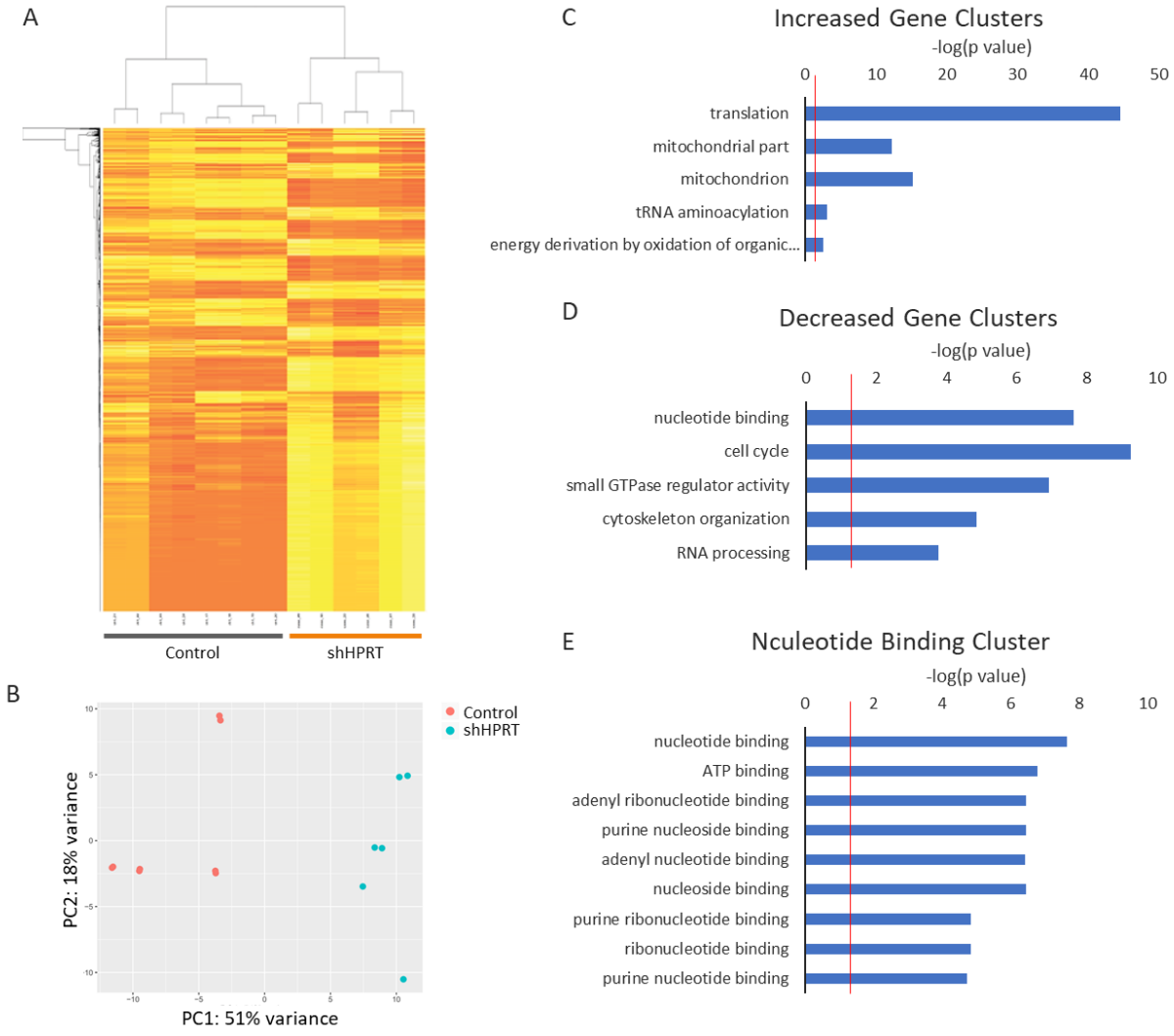

Figure 3 RNAseq reveals deficits in adenyl nucleotide binding and energy metabolism A) *Unsupervised hierarchical clustering of significantly differentially expressed genes by expression level perfectly groups shHPRT and Control RenCell VM NPCs.* ***B)*** *PCA shows segregation by group along the first principal component, accounting for 51% of the observed variance between samples, and tight clustering of technical replicates.* ***C)*** *DAVID Gene Ontology clustering of genes that are increased or* ***(D)*** *decreased in the shHPRT RenCell VM NPCs compared to control RenCell VM NPCs.* ***E)*** *Significantly enriched gene ontologies in the nucleotide binding cluster are related to adenosine binding, (multiple testing corrected $p<0.05$ indicated by red line)*

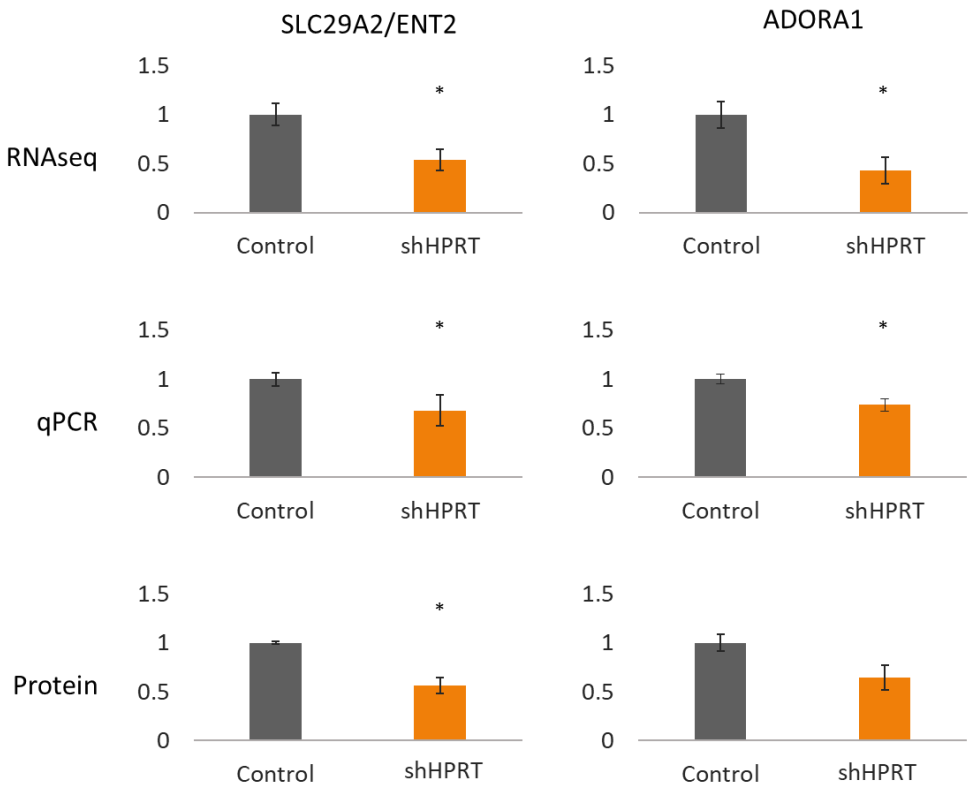

Figure 4 Adenosine related genes are decreased in shHPRT RenCell VM cells. A) *RNAseq shows significant decreases in SLC29A2 (q<0.0005) and ADORA1 (q<0.0005),* ***B)*** *rtPCR validates reduction of SLC29A2 (p=0.042) and ADORA1 (p=0.003)* ***C)*** *Western Blot validates protein level reductions in ENT2 (p-=0.030 and ADORA1 p=0.11). All data normalized to control expression and presented as mean ± standard error, asterisks denote p<0.05*

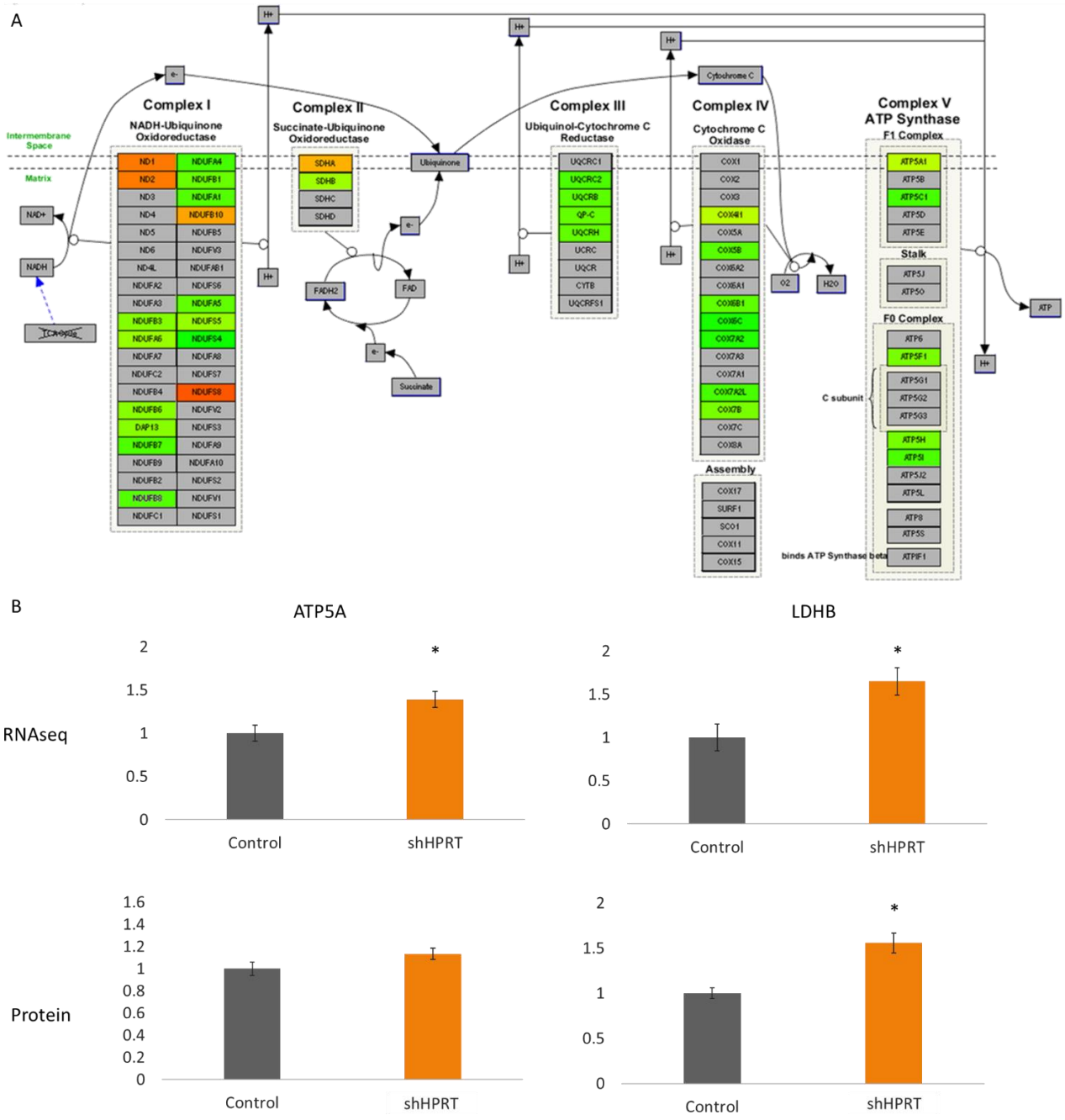

Figure 5 Mitochondrial genes are increased in shHPRT RenCell VM NPCs. A) *Schematic depiction of electron transport chain complexes from Wikipathways, colour coded to indicate significantly differentially expressed genes.Green indicates genes that are significantly increased in shHPRT cells , red and orange indicate genes significantly decreased in shHPRT cells . **B)** RNAseq shows significant increases of ATP5H (q<0.0005) and LDHB (q<0.0005) **C)** Western blots show increased ATP5H (p=0.14) and LDHB p=0.015). RNAseq and western blot data normalized to control expression and presented as mean ± standard error, asterisks denote p<0.05*

3.4. Conclusions

We have developed a unique resource of transcriptomic data from human neuronal HPRT knockdown and control cell lines. To our knowledge, this is the first report of transcriptome-wide gene expression in a human neuronal model of LND. Studying this dataset revealed that HPRT deficiency causes an increase in the expression of mitochondrial genes and a decrease among adenyl nucleotide related genes, an effect that was replicable across independent cell cultures and multiple methods of detection at the RNA and protein level. While each of these systems has been previously implicated in LND, this is the first time that they have been shown to be caused by a reduction in HPRT.

Much of the previous work on LND has focused on guanyl nucleotides, which are the direct product of HPRT metabolic activity, however, ATP has previously been shown to be reduced in patient fibroblasts (Fairbanks et al. 2002) and murine astrocytes (Pelled, Sperling, and Zoref-Shani 1999). Similarly, ATP signalling through the purinergic receptors is decreased in HPRT deficient embryonic stem cells (Mastrangelo et al. 2012) and neuroblastoma cells (Erdorf, von der Ohe, and Seifert 2011). There has not been a consensus on ATP levels in LND, as other studies in LND patient erythrocytes (Micheli et al. 1993) and mouse brain homogenate (Micheli et al. 2009) did not identify significant differences in ATP. These discrepancies are likely to be caused by cell type differences between each of the models used. For instance, one paper confirmed the reductions in ATP seen in cultured mouse astroglia but saw a smaller, not statistically significant, decrease in neurons cultured from the same mice (Brosh et al. 2000). This is a clear reminder of the importance of using the most relevant cell types possible.

LND patient cells also show a dysregulation of the broader adenosine system. Patient lymphocytes have been shown to have decreased adenosine transport, particularly through ENT2

(Torres et al. 2004) which may be caused by a buildup of hypoxanthine (Prior, Torres, and Puig 2007). Neither of these studies examined gene expression of the ENT transporters. Instead, they used measures of transporter function and hypothesize competitive inhibition of adenosine transport by hypoxanthine. Our gene expression data suggest that a gene regulatory mechanism reduces the availability of ENT2 transporters in addition to – or instead of – this inhibition. Both our data and the and that of the other group show that these effects are specific to ENT2, and do not impact ENT1.

Based on the gene expression changes seen in our data and the results of others, a possible model of LND is one in which, to compensate for the reduction GMP and IMP, an increased amount of AMP is deaminated IMP, which is in turn metabolized into GMP. While this process would at least partially maintain the GMP pool, it would reduce the AMP pool. To maintain the level of ATP required for cellular function, the cell may require more mitochondria, resulting in the increased expression of mitochondria-related genes. This is supported by the findings of Shirley et al. (2007), who reported on a human neuroblastoma line which did not show any changes in overall ATP concentration but showed a significant increase in the ratio of ATP:AMP and the adenyl energy charge, which could be the result of an increased proportion of the available AMP that is converted to ATP.

These results are also particularly interesting in the context of increased protein translation, which is indicated by our gene ontology analysis. Protein translation is among the most energy-intensive processes in the cell (Jewett et al. 2009), and increased translation may, therefore, exacerbate any other energy imbalances. Increased protein translation has also recently been shown in fragile X syndrome, (Ifrim, Williams, and Bassell 2015), tuberous sclerosis (Ehninger

et al. 2008), autism (Gkogkas et al. 2013; Kelleher and Bear 2008), and Rett Syndrome (Li et al. 2013), suggesting that this may be a common phenotype in intellectual disability.

This set of experiments contained several limitations. Firstly, the shHPRT1 knockdown reduced endogenous HPRT1 expression to 30-50% of baseline, however, patients only experience symptoms of LND with under 10% residual HPRT activity. It is unclear if this level of knockdown will capture all of the effects of the absence of HPRT1 or if some systems will respond differentially to partial depletion. Secondly, these NPCs grew for a substantial period and differentiated to their current state in the presence of HPRT (i.e., before the transfection with the shRNA). It is possible that some of the effects of the absence of HPRT occur earlier in the development and differentiation of NPCs, and these differences would not be seen in this model. Finally, RenCell VM is an immortalized cell line; this may impact the response of a variety of gene networks to HPRT depletion, particularly those related to growth and metabolism.

In summary, we have developed a novel model of LND in expandable, human-derived cells. We examined genome-wide transcription profiles in these cells using RNAseq, which is currently the gold standard technique for gene expression analysis, and are the first to have performed this level of transcriptomic analysis in a model of LND. Our results show that HPRT knockdown is sufficient to cause substantial transcriptomic changes to the energy metabolism and adenyl related gene pathways in NPCs.

4. An iPSC Model Of LND Replicates Adenosine And Mitochondrial Alterations

4.1. Introduction

We have shown that removing HPRT from an NPC line causes substantial changes to metabolic and adenyl nucleotide binding gene networks, but this knockdown data has several shortcomings, each of which could be addressed by using an iPSC based model on LND. iPSCs are created from patient cells, and therefore have the same mutations and gene function as was present in the patient. iPSCs can also be differentiated into a wide array of cell types, closely replicating the physiology of each cell type. While the first cells differentiated from iPSCs were neurons (Takahashi et al. 2007; Takahashi and Yamanaka 2006) other groups have gone on to generate a wide array of cell types including beating cardiomyocytes (Zhang et al. 2009), hematopoietic progenitors (Raya et al. 2009; Wahlster and Daley 2016), and many neuronal subtypes including cortical neurons (Zeng et al. 2010), retinal ganglion cells (Tanaka et al. 2015), spinal motor neurons (Dimos et al. 2008), serotonergic neurons (Lu et al. 2016), and midbrain dopaminergic neurons (Kriks et al. 2011), to name just a few (Suzuki and Vanderhaeghen 2015). Developing iPSC based models that can differentiate into any cell type would be preferable since HPRT removal can have differential effects on different cell types (Song and Friedmann 2007).

iPSCs are generated by transfecting patient cells – often fibroblasts – with the reprogramming factors OCT4, KLF4, SOX2, and c-MYC. These genes are master transcription factors that trigger the expression of a cascade of genes that return the terminally differentiated cell lines to a pluripotent state (Takahashi et al. 2007). This state is self-perpetuating, so simply a transient

expression of the Yamanaka factors or transfection with a few copies of mRNA is sufficient to trigger a stable pluripotent phenotype (Warren et al. 2010).

Many rare developmental disorders have been modelled using iPSCs including Fragile X syndrome (Liu et al. 2012), Timothy Syndrome (Paşca et al. 2011), Rett syndrome (Marchetto et al. 2010), and others (Saporta, Grskovic, and Dimos 2011a). The ability of these cell lines to generate neurons with the specific mutation found in patients without requiring genome engineering makes them a revolutionary research tool, particularly in rare disease research where patient samples can be difficult to obtain (Bell, Peng, et al. 2016). The use of iPSCs in developmental disorders has lead to the discovery of new phenotypes, and the clarification of mechanisms in many developmental disorders (Avior, Sagi, and Benvenisty 2016).

While iPSC cell lines from LND patients have been used to study the basic biology of stem cell lines and as a selectable marker in genome editing studies (Mekhoubad et al. 2012), no papers have used differentiated human iPSCs to identify phenotypes in LND or to further our understanding of the impacts of HPRT deficiency on neural function. This may be because of the difficulties of working with human iPSCs which can be a difficult hurdle to overcome. This project also was also the first use of human iPSCs, or pluripotent cells of any kind in our facility. Therefore, a significant effort was required at the outset to develop our expertise in pluripotent cell culture, and to standardize and operationalize the methods that would be used by our lab for this and future projects.

With this in mind, we generated and differentiated iPSCs and iPSC-derived NPCs from LND patients and controls. We found that iPSC-derived NPCs showed similar adenosine and energy-related dysfunction to the HPRT knockdown RenCell VM lines, mutually validating each model.

4.2.Methods

4.2.1. Fibroblast culture

We obtained fibroblasts from three patients (GM20394, GM20393, GM01662) and two controls (NCRM1, BC1) through the Coriell Cell Repositories (Table 5). Fibroblast lines were maintained in DMEM supplemented with 10% FBS at 37^0C, 5% CO_2, and passaged 3:1 using trypsin upon reaching confluence (every 2-5 days depending on the cell line). Fibroblast lines were cryopreserved in DMEM supplemented with 10% FBS and 10% DMSO and stored in liquid nitrogen vapour.

4.2.2. Reprogramming iPSCs

iPSCs and iPSC-derived NPCs were derived from LND patients and controls (Table 5). Fibroblasts were reprogrammed to iPSCs following a protocol we developed to rapidly create iPSCs from fibroblasts (Bell, Peng, et al. 2016). Approximately 5.0×10^5 patient or control fibroblasts were transfected with 5 μg of episomal reprogramming vector containing Oct4, Sox2, Myc3/4, Klf4, shRNA P53, and a puromycin resistance cassette (ALSTEM) via electroporation with three 10ms pulses of 11650 V on the Neon Transfection System (Invitrogen, Burlington). Following transfection, the cells were plated on tissue culture plates coated with Matrigel (Corning) in DMEM 10% FBS. The following day, the media was replaced with fresh DMEM 10% FBS + 2 μg/mL puromycin (Sigma-Aldrich). Puromycin selection was maintained for 48 hours, to ensure that only cells containing the resistance cassette – and therefor reprogramming vectors – were maintained in the culture. Typically, between 1,900 and 2,100 cells remained following puromycin selection. After puromycin selection was complete, the induction process was started by replating the selected cells in TesR-E7 media (Stem Cell Technologies, Vancouver). The TesR-E7 media was changed every day during the induction and purification

process. Approximately 15 days after the removal of the selection media, distinct clusters of clonal cells were observed. These colonies were tracked until they formed large (~500–1,000 μm in diameter), robust, colonies with distinct edges. Typically, 18-24 such colonies formed per 2000 replated cells. The colonies were then detached from the plate using the gentle dissociation ReLeSR media (Stem Cell Technologies, Vancouver) which selectively releases colonies of iPSCs from cell culture plates while leaving non-iPSCs attached to the bottom. The floating colonies were then picked and individually plated on new Matrigel-coated plates in mTesR1 media (Stem Cell Technologies, Vancouver) supplemented with ROCK inhibitor y-27632 (Sigma-Aldrich) at a final concentration of 10 μM. This process was repeated an additional 1-3 times until pure cultures of iPSC colonies can be observed. Once formed, iPSC colonies can be maintained in culture in mTesR1 media with daily media changes, cryopreserved in fetal bovine serum with 10% DMSO (Dimethyl sulfoxide, Sigma-Aldrich), or terminally differentiated. An additional control iPSC (HiFb4) line was obtained from the Harvard Stem Cell Institute. Multiple iPSC cultures were produced from each fibroblast cell line and were verified as pluripotent by ICC staining for the pluripotency markers TRA160, SSEA4, OCT 4, and Nanog and by differentiation to an NPC Phenotype.

4.2.1. Differentiating forebrain-like NPCs

We tried several different procedures to produce forebrain-like NPCs (fNPCs), including some based on adherent cell culture with dual SMAD inhibition (Chambers et al. 2009), and some based on creating very uniform neurospheres in Aggriwell plates (Stemcell Technologies) or 96 well v-bottom plates. Taking into account the time required, price, and reprogramming efficiency, we found the most effective protocol to be based on Zeng et al. (2010), incorporating some elements of other protocols, and advances in the reagents available. This protocol has been

shown to produce NPCs that differentiate into mature neurons that express the forebrain markers BR1, MAP2, CTIP2, and VGLUT1.

Table 5 Patients fibroblasts used for iPSC and NPC generation

ID	Clinical Description	Source	fNPC	mdNPC
GM20394	Classic clinical and enzymatic phenotype; Deletion of exon 1 resulting in no mRNA.	Coriell Cell Repositories	x	
GM20393	Classic clinical and enzymatic phenotype; 140 A>G [Glu47Gly];	Coriell Cell Repositories	x	x
GM01662	Variant Phenotype, Lacks self-mutilation and mental retardation; Exon 2,3 duplication and elongated HPRT mRNA; No detectable HPRT activity;	Coriell Cell Repositories	x	
DP	Classic clinical and enzymatic phenotype; 508C>T [Pro169Stop]	Dr. Hyder Jinnah		x
MS	Classic clinical and enzymatic phenotype; 151C>T [Ala50Stop]	Dr. Hyder Jinnah		x
TS	Classic clinical and enzymatic phenotype; 371insTT [Ser123Shift]	Dr. Hyder Jinnah		x
CT	Classic clinical and enzymatic phenotype; 289insA [Thr96Shift]	Dr. Hyder Jinnah		x
SK	Control	Dr. Hyder Jinnah		x
AK	Control	Dr. Hyder Jinnah		x
JS	Control	Dr. Hyder Jinnah		x
NCRM1	Control	Coriell Cell Repositories	x	x
BC1	Control	Coriell Cell Repositories	x	x
HiFib4	Control	HSCI	x	

After optimization, our finalized neural differentiation program was as follows: iPSC colonies were dissociated using Gentle Cell Dissociation Reagent (Stem Cell technologies) and resuspended in Neural Induction (NI) media (DMEM/F12 supplemented with N2 (Invitrogen), B27 supplement (Invitrogen), 1 mg/ml BSA, 10 μM Y27632 (AdooQ® Bioscience), 10 mM SB431542 (Selleckchem), 200 ng/ml noggin (GenScript®), onto low-bind plates (Corning). Cells were plated at a density of 2-3x10^6 cells per 100mm^2 plate. Cells were cultured in suspension and monitored for the formation of Embryoid Bodies (EBs), which occurred approximately four days after suspension. Three days after the formation of EBs, cells were plated onto polyornithine and laminin-coated tissue culture plates after one week in neural progenitor (NP) media (DMEM/F12 supplemented with N2 and B27 supplements, with 20 ng/mL of the growth factors bFGF and EGF GenScript, and 1 μg/mL laminin Sigma-Aldrich). EBs were allowed to attach for 24hrs, then were dissociated and replated on fresh plates. Cells were then maintained in NP media for approximately seven more days, with the media being changed every third day until cells adopted a consistently NPC morphology and stained positive for NPC markers (Fig. 5B). fNPCs could be maintained and expanded in NP media for up to 11 passages, or differentiated into mature neurons. If mature neurons were desired, fNPCs at approximately 70% confluency were cultured in Final Differentiation (FD) media (DMEM/F12 with N2 and B27 supplements, 20 ng/mL of the neurotrophic factors BDNF and GDNF GenScript, and 1 μg/mL laminin) for one week, with media being exchanged every two days, followed by culturing in neural maturation (NM) media (DMEM/F12 with N2 and B27 supplements and 1 μg/mL laminin) for up to 120 days. Half media exchanges were performed every 3 days. Mature neuronal differentiation was performed as a validation of the neurogenic potential of the fNPCs, but all other experiments were conducted using undifferentiated progenitors.

4.2.1. Immunocytochemistry

Cells fixed with 3% paraformaldehyde (Sigma-Aldrich) on slides for fifteen minutes then permeabilized with 0.5% TX-100 (Sigma-Aldrich), and blocked in 0.5% PBS-BSA. Primary antibodies (Table 4) were incubated in 0.5% PBS-BSA for 30 minutes at room temperature. Samples were washed, then incubated with secondary antibodies (Table 4) in 0.5% PBS-BSA for thirty minutes in the dark. Samples were washed three times with 0.5% PBS-BSA, and twice with PBS. Coverslips were fixed, sealed, and visualized on an Apotome Florescent microscope (Zeiss).

4.2.2. rtPCR

To measure the level of transcription of target genes, RNA was extracted from cells using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized with M-MLV Reverse Transcriptase (Invitrogen). TaqMan primers were designed by Life Technologies (Table 3). Real-time PCR reactions were run in triplicate with the ABI 7900HT Fast Real-Time PCR System, and data were collected with Sequence Detection System (SDS) software (Life Technologies), and data were normalized to B-Actin expression. Means were compared using a T-test except for the HPRT inhibitor study where means were compared using and ANOVA.

4.2.3. Western blots

We examined the levels of our target proteins using western blots. To extract the protein, duplicate independent cell cultures were lysed in RIPA buffer with protease inhibitor cocktail (Sigma-Aldrich), centrifuged at 15000 G for 20 minutes at 4^0C, and the supernatants containing the solubilized protein were stored at -80^0C until use. Protein concentrations were measured using a BCA assay, and equal amounts of each sample were run in duplicate on 4-15% gradient stain-free gel (Bio-Rad) for 60 min at 110v. Proteins were transferred to a nitrocellulose

membrane and blocked with 4% skim milk powder for 20 min. Primary antibodies (Table 4) were incubated overnight at 4^0C in TBST 4% BSA. HRP-conjugated secondary antibodies were incubated for 1h at room temperature. Blots were visualized using the Pierce ECL Western Blotting Substrate (Thermo-Fischer) on the Bio-Rad Chemidoc illumination system and normalized to whole cell protein. Means were compared using a T-test.

4.2.4. [^{35}S]- GTPγS

[^{35}S]- GTPγS is a protocol used to measure the amount of radiolabelled GTP that is recruited to a Gi-coupled receptor following activation with an agonist (Pradhan et al. 2009). To collect the cellular membrane which contains the adenosine receptors, approximately $5x10^6$ cells were collected and resuspended in 0.8ml 0.25M Sucrose, then lysed using a polytron. The lysates were centrifuged for 10 minutes at 2500 RPM at 4^0C. The supernatants were diluted 1:10 in TMEN media and centrifuged at 30000 RPM at 4^0C for 30 min.

Pellets were resuspended in 1.5ml 0.32M sucrose. 5µl was used to quantify the protein in each sample. To analyze ADORA1 activity levels, samples were first cleared of endogenous adenosine using 1µM Adenosine Deaminase. Next 5µg per well of the cellular membrane was treated in quadruplicate with increasing doses of the ADORA1 agonist 2-Chloro-N6-cyclopentyladenosine (CCPA) and incubated for 1 h at 25°C in assay buffer containing 30 mM GDP to block nonspecific binding, and 0.1 nM [^{35}S]GTPγS. GTPγS is non-hydrolysable, and therefore remains bound to a receptor following stimulation, until saturation. By measuring radioactivity produced by the labelled GTP, we can determine the amount of agonist needed to recruit half maximal GTP (EC_{50}) and the maximal activation in response to an agonist (βmax). Bound radioactivity was quantified using a liquid scintillation counter. Binding in the presence of 10 µM GTPγS was

defined as non-specific binding and subtracted from each well. Data are expressed as % change from baseline, defined as binding in the absence of agonist.

4.3.Results

4.3.1. Generation of patient-derived fNPCs

Substantial effort was put into finding and optimizing iPSC induction and neural differentiation protocols that work consistently and efficiently in our hands. We developed the iPSC protocol described here, the full details of which can be found in Bell, Peng, et al. (2016). Using this technique we were able to consistently produce high quality iPSC colonies that could be expanded for multiple passages with minimal differentiation and stained uniformly for the pluripotency markers SSEA, OCT4, TRA160, and Nanog and could differentiate efficiently into cells with an NPC phenotype (Figure 6).

To induce neural differentiation we evaluated protocols based on both 3D differentiation (Zeng et al. 2010) and monolayer differentiation (Chambers et al. 2009) and found that, while monolayer differentiation protocols were faster and simpler, they provided a much less consistent induction. We also did not find that the use of Aggriwell plates (StemCell technologies) substantially increased the consistency of our neural inductions, and that simply allowing the neurospheres to form spontaneously from the iPSC colonies was the most time and cost-effective way to generate neurons. On the other hand, we found that including many of the factors from the dual SMAD inhibition based monolayer protocol (Chambers et al. 2009) during embryoid body formation improved the efficiency of the protocol, resulting in an approach similar to that used by Brennand et al. (2011). Once we had established a protocol that was able to consistently generate high-quality neural progenitors from iPSCs, we were able to generate neural progenitors from all

patient and control iPSCs that expressed neural progenitor markers nestin and Sox2 (Figure 6) and can differentiate into βIII-Tubulin positive neurons following growth factor removal. The ability to consistently and efficiently produce NPCs and neurons shows that the iPSCs we produced can produce the neurons for our experiments.

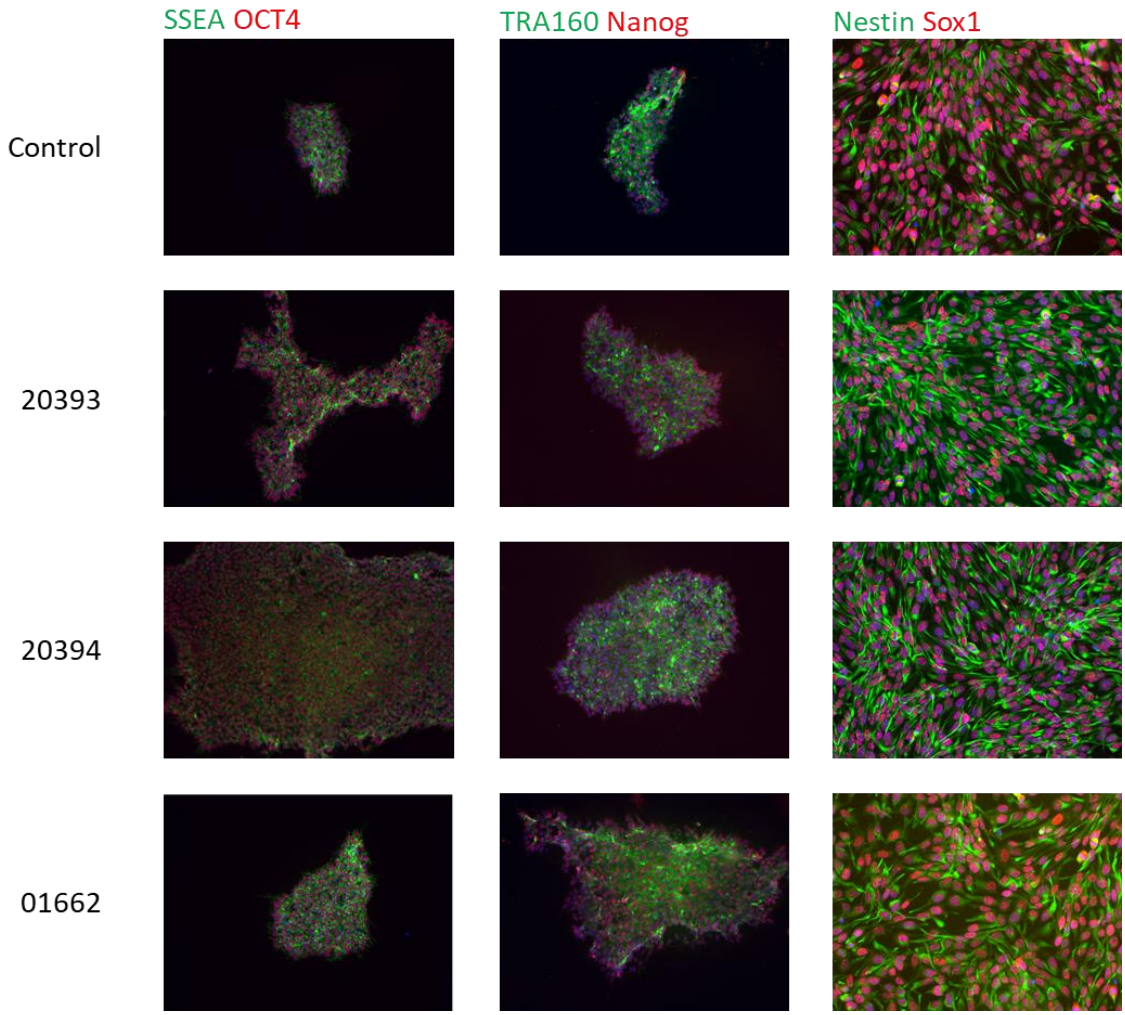

Figure 6 Generation of pluripotent iPSCs and tripotent NPCs. *Induced pluripotent stem cells derived from patients (20394, 20393, 01662) and controls (NCRM1) express the pluripotency markers SSEA4, OCT4, TRA16 and Nanog. NPCs differentiated from these iPSCs express the NPC markers Nestin (green) and SOX1 (red). Nuclei stained with Dapi (blue).*

4.3.2. Patient fNPCs replicate adenosine and mitochondrial phenotypes

To validate the differential expression of mitochondrial and adenosine related genes identified in the RenCell VM HPRT knockdown lines, we examined protein and RNA expression levels in the patient and control fNPCs. Concordant with our RenCell data, patient cells lacking HPRT show increased protein expression of key components of the ATP-synthase complex, ATP5H ($p=0.008$) was significantly increased while ATP5A did not reach the threshold of significance ($p=0.068$) it was increased by 56% (Figure 7 A-B). Adenosine binding genes were also decreased with SCL29A2 (ENT2) significantly decreased at both the RNA ($p=0.001$) and protein levels ($p=0.042$) ADORA1 mRNA was significantly decreased ($p=0.0001$), while protein did not reach statistical significance ($p=0.075$ Figure 7 C-D). Although ADORA1 and ATP5A proteins were not significant, the magnitude and direction of the differences of expression was similar to that seen in the HPRT1 knockdown cell lines, and we therefore consider them a validation of the RNA level finding that the absences of HPRT has the effect of increasing mitochondrial genes and decreasing adenosine binding related genes.

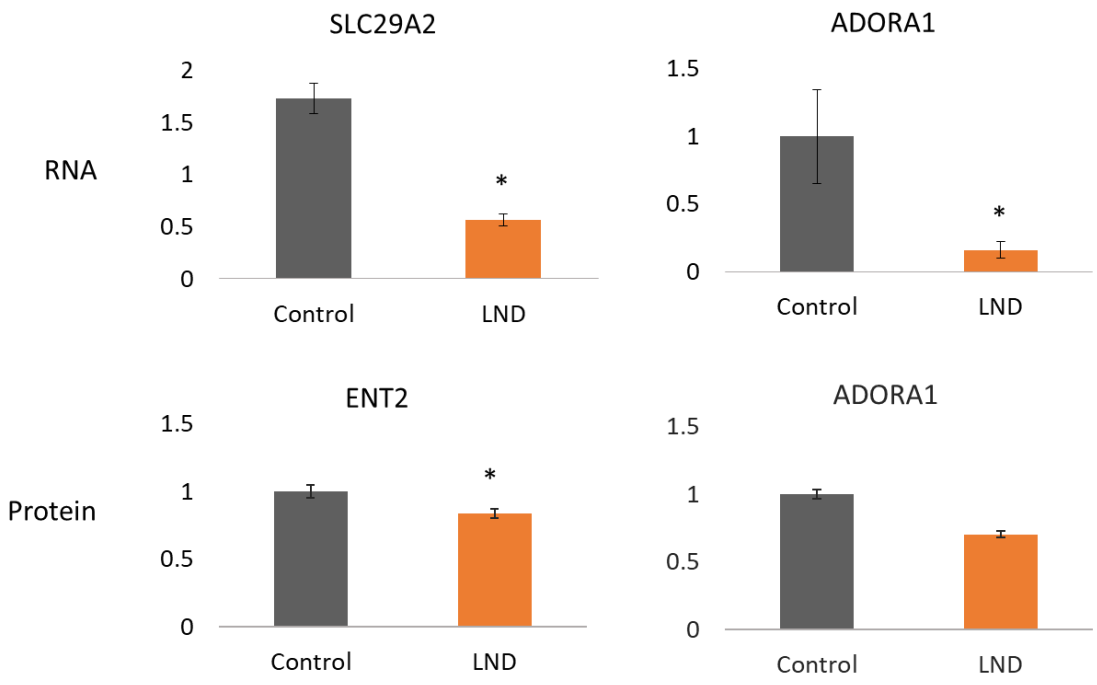

Figure 7 Adenosine related genes are decreased in LND patient-derived fNPCs. A) *rtPCR shows significant decreases in SLC29A2 (p=0.0011) and ADORA1 (p=0.0001)* ***B)*** *Western Blot validation of gene expression changes protein level reductions in ENT2 (p=0.042) and ADORA1 (p=0.075). Data presented as mean ± standard error, values normalized to controls asterisks denote p<0.05*

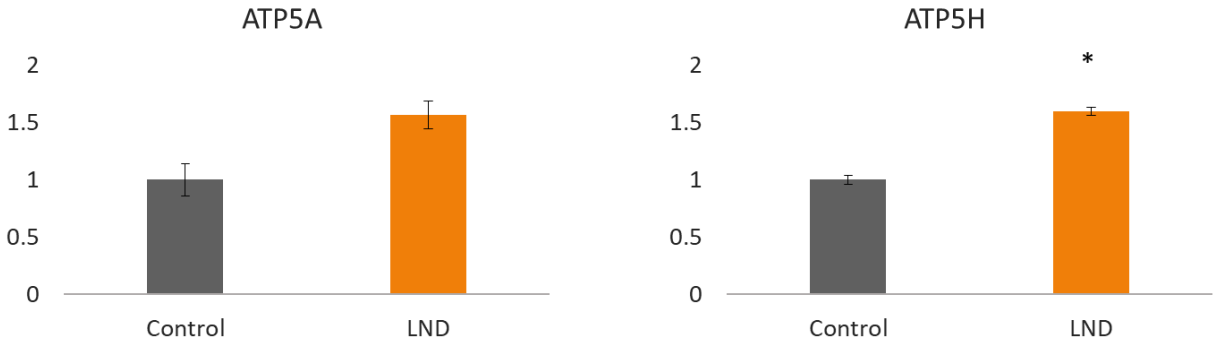

Figure 8 Mitochondrial proteins are increased in LND patient-derived fNPCs. *Patient lines show increased protein expression of ATP5A (p=0.068) and ATP5H (p=0.008) measured using western blot. Data presented as mean ± standard error, asterisks denote p<0.05*

4.3.3. Pharmacological inhibition of HPRT replicates phenotypes from patient cells

Because of our small sample size and line to line noise inherent in iPSC studies, it is possible that the changes in gene expression we saw were idiosyncratic to the cell lines, and not an effect of HPRT deficiency. To assess this possibility, we treated two of our control cell lines, NCRM1 and HiFib1, with azathioprine, an HPRT inhibitor. Duplicate cultures of each cell line were treated with 1 and 10 μg/ml azathioprine for 48 hours and 6 days; RNA was extracted, representative genes were assessed with rtPCR. 6 days of HPRT inhibition with 10 μg/ml azathioprine was sufficient to induce decreases in ADORA1 ($p=0.005$) and SLC29A2 ($p<0.001$), but did not alter ATP5H expression ($p=0.48$) (Figure 9). This confirms that the chronic removal of HPRT activity is sufficient to induce these changes in iPSC derived neurons, and therefore that the absence of HPRT is what causes these changes in the shHPRT knockdown lines and the patient-derived iPSC lines.

4.3.1. GTP-y-S shows functional reduction of ADORA1

Finally, if the absence of HPRT is reducing the amount of adenosine receptor available, it should be observable at the functional level by measuring the response of receptors to an ADORA1 agonist. In collaboration with the lab of Dr. Brigitte Kieffer, we used GTP-γ-S to measure the ability of the ADORA1 receptor to recruit GTP in response to stimulation. Following the removal of all endogenous adenosine using Adenosine Deaminase (ADA), increasing concentrations of the specific ADORA1 agonist CCPA were added to cell membrane collected from patient and control derived fNPCs. The control cells showed a maximal activation (βmax) of 293% (95% CI = 260.8 - 321.9%) of baseline while the patient lines only achieved 123% activation (95% CI = 110.6 - 135%). This approach measures the amount of GTP recruited by saturated receptors, and therefore a reduction in maximal activation can indicate a reduction in the number of available

receptors, or the ability of the receptors to become activate or recruit GTP. In conjunction with our previous data, this is strong evidence that the reduced expression of ADORA1 substantially limits the ability of the cells to respond to adenosine signalling. Importantly, the concentration of CCPA that was required to induce 50% maximal activation (Km) was not significantly different between patient and control derived lines, indicating that the receptors that are present are similarly able to bind CCPA.

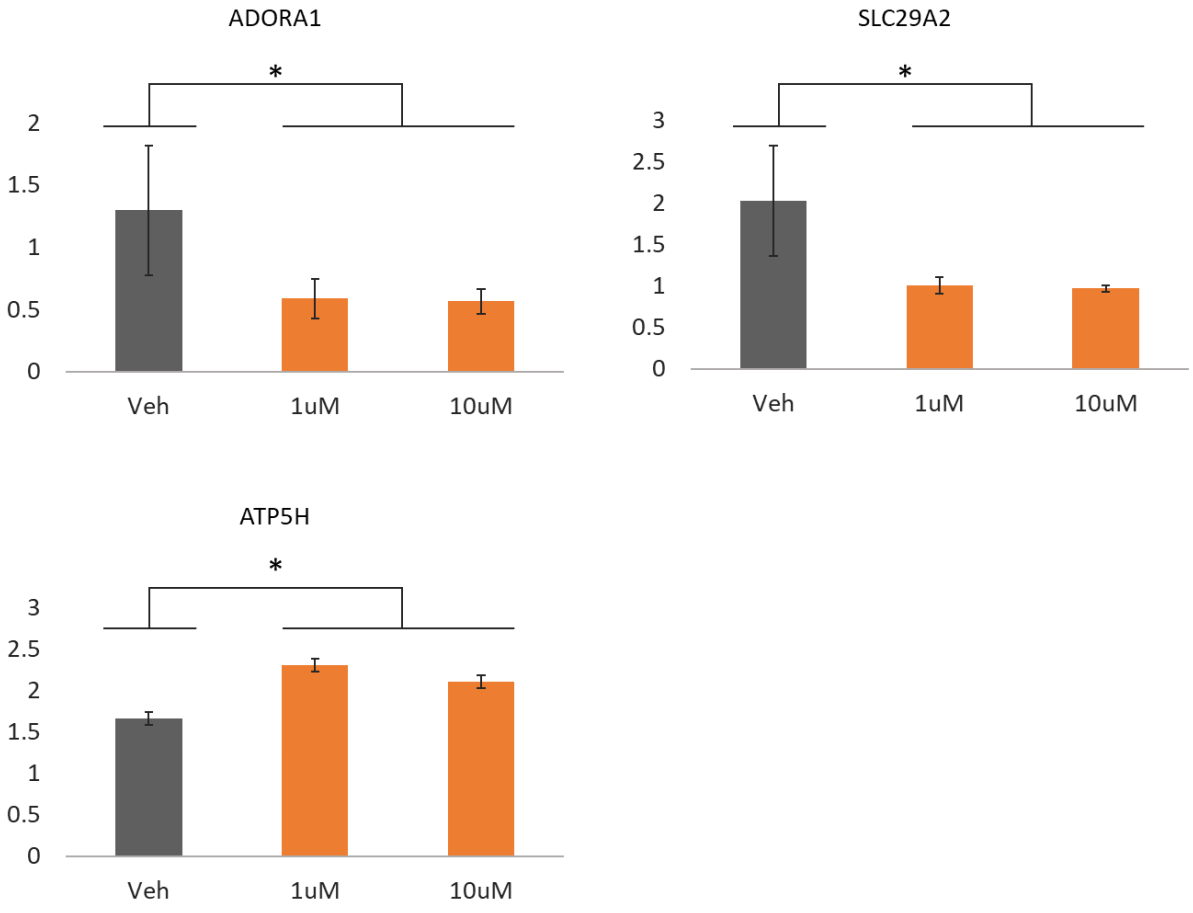

Figure 9 Pharmacological inhibition of HPRT replicates effects on gene expression. *Two control NPC lines (hifib1 and NCRM1) treated for six days with DMSO (Veh) 1 μM or10 μM of the HPRT inhibitor Azathioprine replicate the effects of phenotype of LND patient cell lines with altered expression of* ***A)*** *ADORA1 (p=0.0266),* ***B)*** *SLC29A2 (p=0.0071), and* ***C)*** *ATP5H (p=0.0004). Data presented as mean ± standard error, asterisks denote p<0.05.*

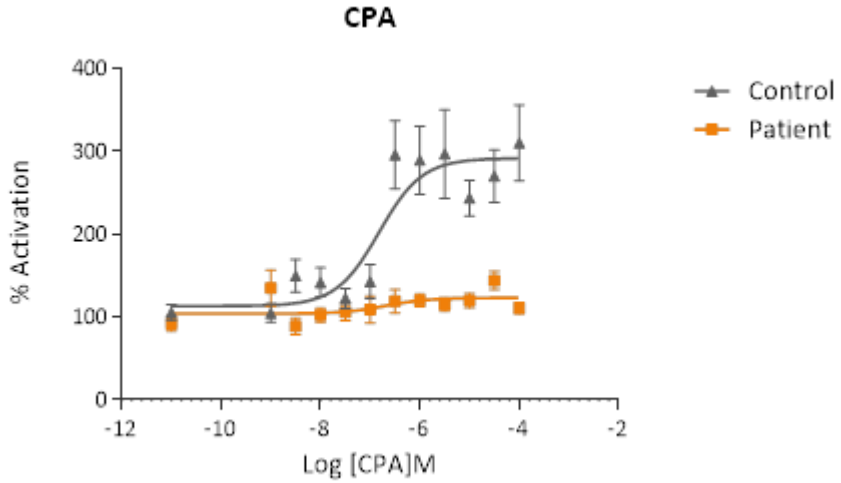

Figure 10 Patient-derived fNPCs show reduced response to an ADORA1 agonist. *Percent of baseline radiation measured from recruiting radiolabelled GTP in response to increasing concentrations of the ADORA1 agonist 2-Chloro-N6-cyclopentyladenosine (CCPA). Control fNPCs recruit 123% more GTP at maximal stimulation, while patient-derived fNPCs only increase to 291%. logEC50 CCPA in the patient and control fNPCs were 6.69±1.83 and 6.82±0.53, respectively. Data presented as mean ± SEM for each concentration.*

4.4. Conclusions

We have now shown deficiencies in adenosine receptors and increases in components of mitochondrial ATP synthase in two independent human neuronal models of genetic HPRT deficiency, and in a thorough pharmacological inhibition of HPRT. The similarity in gene expression changes between the shHPRT knockdown cell lines and iPSC derived cells confirms that the alterations to the adenosine signalling and mitochondrial pathways are effects of the absence of HPRT and not off-target effects of shHPRT, and further demonstrates the validity of both LND models.

By looking at the data from individual cell lines we can see that the cell line 01662, which was derived from a patient who does not show behavioural phenotypes despite the complete absence of detectable HPRT function, displayed intermediate protein levels of ADORA1 and ENT2,

correlating with disease severity. It is possible that this correlation is merely the result of having a small sample size and single intermediate patient. Larger studies using cells from patients with a range of disease severities will be an important next step in delineating the relationships between molecular phenotypes and specific disease characteristics like self-harm and dystonia.

These data also present some of the strongest available evidence implicating adenosine signalling in LND. As discussed in previously, studies in lymphocytes have shown that cells from LND patients have reduced purine transport (Torres et al. 2016), another study analysed metabolite concentrations in the blood of patients LND patients and found significant reductions of ATP and Succinyl-AMP (an intermediate in the conversion of IMP to AMP). Interestingly, these studies did not report significant changes in any components of guanosine metabolism (Ceballos-Picot et al. 2015). Others have reported mixed and inconsistent results when measuring nucleotide metabolites across different cell culture conditions and tissues (Brosh et al. 2000; Hershfield and Seegmiller 1977; Harkness, Mccreanor, and Greenwood 1991; Pelled, Sperling, and Zoref-Shani 1999; Zoref-Shani et al. 1993). Ours is the first data to both indicate a reduction in ADORA1 expression in a human neuronal cell line and demonstrate a functional impact of this reduction in ADORA1 binding capability and signalling.

Our data also indicate an increase in the expression of mitochondrial proteins in the context of LND. Mitochondrial funtion in LND has not been well studied, but would make sense in the broader context of changes in the adenosine system. Work from other groups have shown that increases in componants of ATP synthase, including ATP5A can increase mitochondrial fusion, which is protective against increased oxidative stress(Wang et al. 2012). These chagnes may therefore represent a protecitve or compensatory mechanism in the patient cell lines.

Although the connection between adenosine signalling and LND behaviours is not obvious, adenosine has been connected to both DA function and, to a lesser extent, aggressive behaviours. ADORA1 receptors form heteromeric complexes with dopamine D1 receptors (DRD1), wherein ADORA1 signalling negatively regulates DRD1 signalling (Ginés et al. 2000; Ferré et al. 1994) and can modulate phasic DA release (Ross and Venton 2015). ADORA2A forms heteromeric complexes with DRD2 where they again have mutually antagonistic interactions (Fuxe et al. 2005; Mayfield et al. 1996) and have become an active subject of research in Parkinson's disease and restless leg syndrome (Uchida, Kadowaki-Horita, and Kanda 2014; Shao et al. 2018; Jenner 2014). Further, aggressive behaviours have been characterized in mice lacking the ADORA1 and ADORA2A receptors (Giménez-Llort et al. 2002; Ledent et al. 1997), and adenosine reduces aggressive behaviours caused by clonidine administration (Ushijima, Katsuragi, and Furukawa 1984), and allopurinol treatment, which increases the overall availability of adenosine, has reduced aggression in some patients without metabolic disorders (Lara, Belmonte-de-Abreu, and Souza 2000).

These data suggest that the relationship between HPRT genotype, adenosine phenotype, and neurological phenotype is fertile grounds for further research.

5. iPSC Derived Midbrain-Like NPCs do not Replicate Adenosine and Mitochondrial Alterations

5.1. Introduction

Although reductions in striatal dopamine are the most consistently documented phenotype of LND, studies using different models and methodologies show substantially different effect sizes. For instance, an early study in 3 post-mortem LND patient brains suggested a loss of 60-90% of dopamine terminals in patient brains based on decreases in DA levels, DA metabolite homovanillic acid (HVA), the activities of two enzymes responsible for producing DA, DOPA-decarboxylase (DDC) and tyrosine hydroxylase (TH). These effects were seen in the caudate, putamen, and nucleus accumbens, but no significant differences were seen in the SN (Lloyd et al. 1981). A more recent analysis in postmortem brains from 5 patients did not measure DA concentration directly but showed a reduction in TH levels across the brains of 5 LND patients showed that dopamine projections were morphologically normal, and not reduced in number, only in the intensity of TH staining (Göttle et al. 2014). This reduction in TH was observed in all the studied tissues, including the substantia nigra (SN). TH staining in HPRT knockout rats, on the other hand, shows both normal dendritic branching and normal levels of TH intensity (Isotani et al. 2016). Interestingly, the authors of each paper used these data to support the same conclusion; that the amount of dopamine produced by each neuron was decreased. This conclusion is also supported by PET imaging which has shown a 57% reduction in presynaptic dopamine storage in the SN and Ventral tegmental area (VTA), which is more than the caudate (39%), putamen (31%), or cortex (44%) (Ernst et al. 1996).

HPRT knockout mice present a similar picture. Dopamine concentrations are reduced across multiple studies (Jinnah et al. 1994; Finger et al. 1988; Williamson et al. 1991; Dunnett et al. 1989; Jinnah, Langlais, and Friedmann 1992), and reductions are consistently greater in the striatum than the SN or VTA. However, the level of dopamine deficiency reported in these studies varies widely, from 20-60%. While some of this variability can be explained by strain differences and age, these factors cannot account for the whole range of findings, and some of the discrepancies remain unexplained (Jinnah et al. 1999).

In vitro models have also been used to examine dopaminergic dysfunction in LND. The two most common models, the murine MN9D and human SH-SY5Y cell lines both suggested an impact of HPRT dysfunction on dopaminergic differentiation and development. Ten independent HPRT deficient MN9D subclones each showed an increase of the dopaminergic markers EN1 and EN2, and lmx1a, each of which is important in directing the differentiation of midbrain dopaminergic neurons (Ceballos-Picot et al. 2009). HPRT deficient SH-SY5Y have shown changes in the same genes (Guibinga et al. 2012), but the actual production of dopamine in these cell lines has not been investigated. This may be a least in part because the dopaminergicity of these lines had been questioned and their relationship to actual midbrain neurons is unclear (Balasooriya and Wimalasena 2007; Rick et al. 2006).

We have generated induced pluripotent stem cells and midbrain-like neural progenitors (mdNPCs) from an expanded sample of LND patients and controls to assess gene expression and dopamine production in the cell type most affected in LND.

5.2. Methods

5.2.1. Reprogramming iPSCs

Fibroblasts from 10 LND patients and controls were provided by Dr. Hyder Jinnah and maintained as previously described. Due to the time and cost requirements of reprogramming large numbers of iPSCs five patients (CT, DP, MS, TS, and GM20393), and four controls (JS, SK, MK, and NCRM1) were selected for reprogramming. Of these, CT, DP, MS, TS, JS, SK, and MK fibroblasts were provided by the Jinnah Lab, while GM20393 and NCRM1 were also used in the generation of fNPCs, allowing more direct comparisons between the datasets.

Fibroblasts were reprogrammed using episomal reprogramming vectors containing Oct4, Sox2, Myc3/4, Klf4, shRNA P53, and a puromycin resistance cassette (ALSTEM). Episomal vectors were chosen because of their high reprogramming efficiency, ease of transfection, and reduced risk of genomic instability compared to lentivirus and Sendai virus reprogramming vectors. $5.0x10^5$ cells were transfected with 1ug of reprogramming vector using electroporation (11650 Volts, 10 ms, three pulses). Following transfection, cells were plated on tissue culture plates coated with Matrigel (Corning) in TesR-E7 media (Stem Cell Technologies). The following day, the media was exchanged for fresh TesRE7 media supplemented with 2 μg/ml puromycin (Sigma-Aldrich) to select for cells with the puromycin resistance gene provided by the reprogramming vectors. Puromycin selection was maintained for 48hrs, after which the media was exchanged with fresh TesR-E7 media. During the induction process, TesR-E7 media was changed every day. Colony formation occurred 10-12 days following puromycin selection. Colonies were tracked until they formed robust, distinct cell populations 500-1000μm in diameter, then individually plated on matrigel coated plates in mTesR1 media (Stem Cell Technologies) supplemented with 10 μM ROCK inhibitor y-27632 (Sigma-Aldrich). iPSC

colonies were passaged manually until pure colonies form consistently, then cryopreserved in FBS with 10% DMSO (Sigma-Aldrich) or differentiated into mdNPCs.

5.2.2. CRISPR knockout of HPRT1

One of the biggest limitations in iPSC research is that it the differences of genetic background from patient to patient add substantial noise to the system. To help reduce this noise genome editing can be used to either knock the gene of interest out of a control cell line or to repair the mutation in a patient line through facilitated homologous recombination. Recently, with the invention of CRISPR genome editing, these genome editing has become substantially easier and more affordable. Our research group recently developed a simplified protocol that simultaneously reprograms a somatic cell to an iPSC and performs CRISPR genome editing (Bell, Peng, et al. 2016) which we used to create an HPRT knockout in the control cell line NCRM1.

10^6 NCRM1 fibroblasts were seeded in a 10cm culture dish. Once cells were more than 75% confluent (1 day), they were lifted using 0.05% Trypsin/EDTA and centrifuged at 1,000 rpm for 5 minutes at room temperature, then resuspended in DBPS, split in half, and centrifuged again. The cell pellets were resuspended in Solution R (citric acid) and transfected with 3μg of HPRT1 CRISPR9 in an RFP construct from DNA2.0 and 3μl of reprogramming vector using a NEON Transfection system (Thermo-Fischer). Immediately after electroporation, the cell suspension solution was added to warm Fibroblast Medium in one 10 cm culture dish pre-coated with gelatin and incubated overnight at 37 °C, 5% CO2. The following day cells were detached using accutase A and successfully transfected (RFP+) colonies were selected using fluorescence-activated cell sorting (FACS) and replated at low density in TesR-E7 media (Stem Cell Technologies). The next day, the media was exchanged for fresh TesR E7 media supplemented with 2 μg/ml puromycin (Sigma-Aldrich). Cells containing the CRISPR construct were selected

by RFP sorting then plated in media containing puromycin which selects for cells containing the reprogramming vectors. As with unedited cells, robust colonies began to form 10-12 days after plating, and cell culture then proceeds identically.

Because of the low-density plating after FACS, each colony can be assumed to form a single cell and therefore contains only one genetic mutation. As the colonies were expanded, DNA was extracted using the Qiagen miniprep kit, and knockouts were confirmed by PCR and electrophoresis, with a change in band size indicating a successful deletion or insertion, and by Sanger sequencing performed at Genome Quebec.

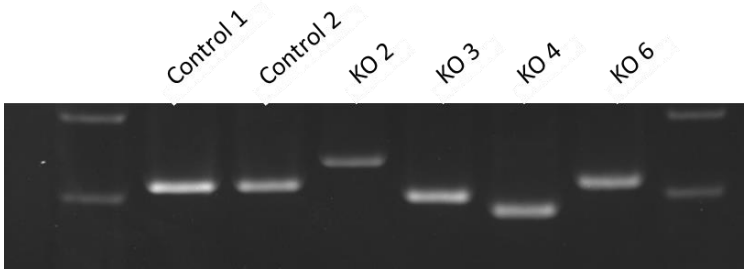

Figure 11 Confirmation of HPRT CRISPR knockout *(Control 1, Control 2), Three confirmed knockouts (KO2, KO3, KO4) and one unconfirmed knockout (KO6) as indicated by shifts in band size. All three knockouts were also confirmed by Sanger sequencing.*

5.2.3. Differentiating dopaminergic neurons from iPSCs

iPSCs from five patients (GM20393, CT, DP, MS, and TS), four controls (NCRM1, JS, SK, and MK), and one CRISPR knockout line (HPRT-KO1) were used to generate dopaminergic neurons (Table 3). To generate dopaminergic neurons, we used a protocol based on floor plate differentiation into ventral midbrain neurons in vivo (Kriks et al. 2011). iPSC colonies were dissociated with gentle dissociation buffer and 10^6 cells seeded into 10 cm uncoated petri dish with neural induction medium supplemented with 200ng/ml of the SMAD inhibitor noggin and

200 ng/ml C24II sonic hedgehog (SHH), forming floating spherical Embryoid bodies (EBs). After five days EBs were plated onto poly-lysine and laminin-coated culture dish in Neural Induction medium 1. The EBs adhered to the dish and after three days, 3μM of the GSK3 inhibitor CHIR-99021 was added to the media. After another three days, SHH and CHIR-99021 were removed from the media and cultures were maintained until rosettes form (7-14 days). Rosettes were carefully selected, released using gentle dissociation reagent and replated onto poly-D-lysine/laminin-coated plates in Neural Progenitor medium (DMEM/F12 supplemented with 2 mM L-glutamine, 1x B27, 1x N2 supplement, 200 ng/ml SHH, 100ng/ml FGF8, and 1μg/ml laminin. Cells were then expanded as neural progenitor cells or frozen in FBS 10% DMSO.

To generate mature neurons, neural progenitor cells were detached by gentle dissociation medium and replated onto poly-D-lysine/laminin-coated plates in final differentiation medium (N2/B27 Medium with 20 ng/ml BDNF, 20 ng/ml GDNF, 0.5 mM dCAMP, 200 μM ascorbic acid, and 1 μg/ml laminin) then grown to the desired age with media changes every 3-5 days.

5.2.4. Immunocytochemistry

Cells were fixed with 3% paraformaldehyde (Sigma-Aldrich) on slides for fifteen minutes then permeabilized with 0.5% TX-100 (Sigma-Aldrich), and blocked in 0.5% PBS-BSA. Primary antibodies (Table 4) were incubated in 0.5% PBS-BSA for 30 minutes at room temperature. Samples were washed then incubated with secondary antibodies (Table 4) in 0.5% PBS-BSA for thirty minutes in the dark. Samples were washed three times with 0.5% PBS-BSA, and twice with PBS. Coverslips were fixed, sealed, and visualized on an Apotome Fluorescent microscope (Zeiss).

5.2.5. HPLC

5.2.5.1. Nucleotides

To measure the levels of adenosine nucleotides present in the cells, we followed high-performance liquid chromatography (HPLC) the protocol of Bhatt et al. (2012) in collaboration with Dr. Thad Rosenberger. To extract nucleotides from the cells and prevent the breakdown of ATP to ADP, mdNPCs were flash frozen by floating the cell culture dish on liquid nitrogen. Frozen cells were then collected from 2 wells of a 6 well plate by scraping the cells with the culture dish on dry ice and transferred to a micro-centrifuge tube chilled in liquid nitrogen, and lysed in 0.6N perchloric acid using a Polytron® PT1200E homogenizer (Kinematica Inc, Bohemia, NY). The homogenate was centrifuged at 13,000 x g for 2 min at 4^{o} C. The supernatant was collected and neutralized with 1 mL ice cold freon/trioctylamine (4:1, by Vol.). The pellet was saved for protein determination and normalization. The neutralized nucleotide extract was vortexed for 30 sec then centrifuged at 13,000 x g for 2 min to induce phase separation. The upper aqueous layer was collected and stored at 4° C until derivatization. The protein pellet was washed once with acetone, dried using a nitrogen evaporator and then re-suspended in 1N sodium hydroxide for 24 hr. The dissolved pellets were boiled for 5 min, sonicated, then the protein content was measured using a Bradford assay (BioRad)

Nucleotide standards (adenosine, ATP, ADP, AMP), sodium acetate (99%),trichlorofluromethane (freon),chloroacetaldehyde (50%), trioctylamine, glucose, hexokinase, and, myokinase were purchased from Sigma-Aldrich (St. Louis, MO), tetrabutylammonium phosphate (TBAP) was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ), and HPLC grade acetonitrile, mono-basic potassium phosphate (KH_2PO_4), perchloric acid, acetone, sodium hydroxide, and bovine serum albumin were obtained from EMD Chemicals Inc. (Gibbstown, NJ). All standard stock

solutions were prepared by dissolving 10-25 mg of a pure standard nucleotide in 1-1.5 mL of deionized water, and stored at -20° C until use. The standard solutions were diluted 1000-fold and the absorbance was recorded at 259 nm. The molar concentration of the stock standards was calculated using the molar extinction coefficients. All calibration procedures were performed using freshly made stock standards while the working standards used for method development, analyte stability, and other aspects described herein were stored at 4° C for no more than one month before use.

To convert the nucleotides into their fluorescent, N^6-etheno-derivatives, 50 µL aliquots of nucleotide extract were mixed with 150 µL freshly prepared mix of chloroacetaldehyde (7.8 M) to 1 M acetate buffer (pH 4.5), vortexed, centrifuged at 450 x g for 2 min at 22° C, then heated to 60°C for 60 min. After the reaction the tubes were immediately placed on ice to stop the reaction, centrifuged at 450 x g for 2 min at 22° C, then diluted with water to 1:6 for analysis. A100 µL aliquot of this solution was placed in microvial and 50 µL of the derivatized sample was used for analysis.

The HPLC analysis of etheno-adenine nucleotides was performed on System Gold® 125 Solvent Module (Beckman Coulter, Inc., Fullerton, CA) equipped with a System Gold® 508 auto-sampler and an in-line Jasco FP-2020 fluorescence detector (Jasco Corporation, Tokyo, Japan). Separation was performed on a Waters Sunfire™ ODS column (5µm, 250 x 4.6 mm, Milford, MA) equipped with a C18 SecurityGuard cartridge (Phenomenex, Torrance, CA) at 22° C. Pump control and peak integration was achieved using the 32 Karat™ software (Ver. 7.0, build 1048, Beckman Coulter, Inc., Fullerton, CA). The mobile phase consisted of buffer A, 30mM KH_2PO_4 + 0.8mM TBAP, pH 5.45; and buffer B, acetonitrile/30mM KH_2PO_4 (1:1, by Vol.) + 0.8mM TBAP, pH 7.0. The pH of buffer B was adjusted to 7.0 before the addition of acetonitrile. All

buffers were filtered using a 0.45-µm Supor-450 membrane filter (Pall Corporation, Ann Arbor, MI) prior to the addition of the ion-pairing reagent TBAP.

The etheno-adenine nucleotides were eluted off the column with the following gradient. The initial buffer B concentration was maintained at 10 % for 0.5 min and then increased to 25% over a period of 2.5 min then held constant for 4 min. At 7 min the concentration of solvent B was increased to 50% over a 4 min period then held constant for 10 min. At 21 min the proportion of buffer B was decreased back to the starting concentration of 10% over a 4 min period then held constant for 5 min until the end of the run. The re-equilibration time between samples and the flow rate was held constant at 20 min and 1 ml/min, respectively. The quantification of the etheno-adenine nucleotides was performed using an excitation wavelength of 280 nm with an output emission wavelength set at 410 nm. The identity of etheno-adenine nucleotides was determined by comparing retention times to known nucleotide standards and were further confirmed by enzymatic peak shift analysis. Means were compared using a two way ANOVA.

5.2.5.2. Dopamine

Dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and HVA were determined using high-pressure liquid chromatography and electrochemical detection (HPLC-EC) as previously described (Domenger et al. 2012). 5-10 million cells per line were lysed in 20µL 0.25 M perchloric acid and centrifuged at 4 °C for 15 min at 10 000 RPM. The supernatant was transferred to new tubes and pellets were reconstituted in 0.1 N sodium hydroxide for protein quantification using a Pierce® BCA protein assay kit (Thermo-Fischer). 20 µl of the supernatant was loaded into a refrigerated (10 °C) autosampler (ESA, model # 542) and injected onto a Luna C18(2) 100 Å 75 × 4.6 mm 3 µm analytical column at a flow rate of 1.5 ml/min with the potential of the electrochemical detector (ESA CoulArray, model # 5600A) set at − 250 mV and + 300

mV. The mobile phase was prepared from 6.0% methanol, 0.341 mM 1-octanesulfonic acid sodium salt, 168.2 mM sodium acetate, 66.6 mM citric acid monohydrate, 0.025 mM ethylenediaminetetraacetic acid disodium and 0.71 mM trimethylamine, and adjusted to pH 4.0–4.1 with acetic acid. The position of the peak for each analyte was compared to an external standard solution which contained 25 ng/mL each of DA, DOPAC, and HVA in 50 mM acetic acid. Chromatographic peak analysis was accomplished by identification of unknown peaks in a sample matched according to retention times from known standards using ESA's CoulArray software. Means were compared using a two way ANOVA.

5.2.1. Seahorse

Seahorse experiments were conducted at the McGill metabolomics core facility using an XF96 (Agilent). Patient and control cells were plated in six replicates at 50,000 cells/well in NPC growth medium on a Seahorse XF96 Spheroid Microplate in one day before analysis. Immediately preceding analysis the cells are washed two times with Seahorse XF Base Medium supplemented with Glucose (10mM) Sodium Pyruvate (1mM) and L-Glutamine (2mM) pH 7.4, then incubated in a CO2 free incubator for 1 hour.

The Seahorse system measures baseline oxygen consumption rate (OCR), then sequentially injects compounds to measure the effect on oxygen consumption, which correspond to different mitochondrial functions. Oligomycin (1 μM) inhibits ATP synthase (complex V), and the decrease in OCR following oligomycin treatment correlates to ATP production. Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP, (0.5 μM)) is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential, this allows free passage of electrons through the electron transport chain, resulting in maximal oxygen consumption by complex IV. Spare capacity is the difference between basal and maximal

respiration and relates the cells' ability in increase respiration in times of increased demand. Finally, a mixture of rotenone (0.5 μM) and antimycin A (0.5 μM) are injected, inhibit complex I and complex III respectively, entirely shutting down mitochondrial respiration and allowing for calculation of non-mitochondrial oxygen consumption. The resulting OCR measurements are analyzed using the Seahorse XF Report Generator.

5.2.2. Mouse brain collection

HPRT Knockout brains were provided by Dr. Jasper Visser. Brains were collected from HPRT knockout and control C57Bl/6j mice (Hooper et al. 1987) at 265-331 days. Brains were flash frozen, then sectioned. 500μm x 0.5mm punches of VTA and 1mm punches of striatum were collected from six KO and six control mice, RNA was extracted using the RNeasy Lipid Tissue kit (Qiagen) and quantified using the Agilent Tapestation.

5.2.3. RNAseq

RNA sequencing (RNA-seq) was performed as previously described in. Briefly, libraries were prepared at the McGill University and Genome Quebec Innovation Center. Five controls, four patient cell lines, and one CRISPR knockout cell line were sequenced on. Separately, RNA extracted from the SN and caudate of 6 wildtype and 6 $HPRT^{y/-}$ mice were sequences on an Illumina HiSeq 2500 flow cell (125bp paired-end reads), and were processed identically.

The quality of the sequencing of each sample, including Phred score distribution and primer or adapter reads, was determined using FASTQC (Andrews 2010). End sequences corresponding to TruSeq adapters or Illumina universal primers were removed using Trimmomatic (Bolger, Lohse, and Usadel 2014) and the FastX toolkit (Gordon and Hannon 2010). Whenever the average Phred score of the extremities of a read falls lower than 15, these edges were trimmed, and only reads

longer than 20 bp were kept. Finally, PRINSEQ (Schmieder and Edwards 2011) was used to remove mRNA poly-A tails and aligned to the appropriate reference genome using tophat2 splice-aware alignment (Kim et al. 2013). In order to maximize useable data generated from each paired-end run, orphaned reads were aligned separately from paired reads, then the alignments were combined. Finally, HTSeq (Anders, Pyl, and Huber 2014) was used to count the reads aligned to each gene, and generate raw read counts. Differential expression was calculated as previously described.

5.3.Results

5.3.1. Creating midbrain like neural progenitors and neurons

Fibroblasts from patients and controls were obtained through a collaboration with Dr. Hyder Jinnah, a clinician-scientist focused on LND and other movement disorders at Emory University. Based on our experience with the fNPCs we used an increased number of patients to generate mdNPCs. We reprogrammed fibroblasts from four patients and three controls into iPSCs following the protocol of Bell, Peng, et al. (2016). In addition to these lines, we included one patient (GM20393) and one control (NCRM1) iPSC line that had been used to generate fNPCs in the midbrain differentiation protocol (Table 5) and a CRISPR induced knockout of HPRT in the NCRM1 background.

mdNPCs generated through our in-house protocol uniformly expressed markers of neural progenitors (Nestin, SOX1; Figure 12A) and markers of midbrain fate (FOXA2, OTX2; Figure 12B). These markers demonstrate a successful creation of dopaminergic progenitor cells and a very low level on non-midbrain like progenitors. After four weeks of growth in the dopamine

progenitor differentiation media, cells expressed markers of mature neuronal cells (Tuj1) and of dopaminergic neurons (TH, DRD1; Figure 12A).

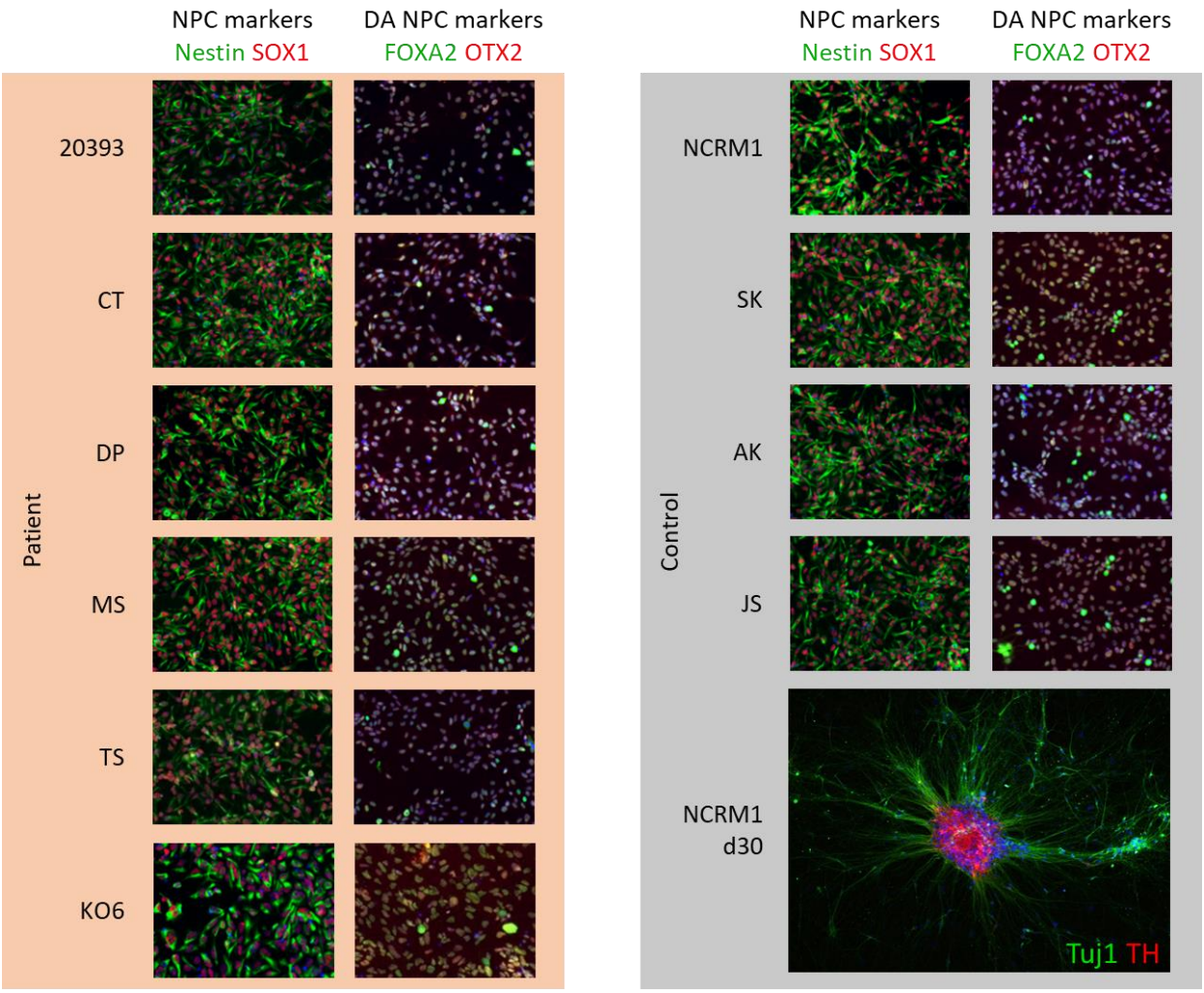

Figure 12 mdNPCs express dopamine projenitor markers and differntiate to dopaminergic neurons *A) Patient and B) Control mdNPCs derived from patient and control iPSCs stain positive for neural progenitor markers Nestin (green, left) and SOX 1 (red, left) and the midbrain lineage markers FOXA2 (green, right) and OTX2 1 (red, right). C) After 30 days of maturation, neurons express the mature neuronal marker TUJ1, and the dopaminergic markers Tyrosine hydroxylase (TH) and Dopamine Receptor D1 marker Tuj1 (green, left) and the dopaminergic markers TH (red). Nuclei stained with dapi (blue).*

5.3.2. RNAseq reveals minimal overlap with previous models

To get a clearer picture of the systems that were dysregulated in the mdNPCs and examine all genes related to energy metabolism and adenosine signalling, we sent RNA extracted from each patient and control cell line for RNA sequencing and processed them using our in-house pipeline. PCA indicated that the control sample JS was contributing a large portion of the variance and therefore we excluded JS expression data from subsequent analyses. DAVID gene ontology (Huang, Sherman, and Lempicki 2009) indicated several gene clusters that were significantly enriched in both directions, but none of these clusters overlapped with the enriched clusters from the fNPCs. Surprisingly, gene ontology also did not reveal any enrichment of gene clusters linked to dopaminergic differentiation or function (Figure 13B). Based on this unexpected result, we compared the lists of differentially expressed genes from our two RNA seq experiments (5655 in the RenCell VM line, 1263 in the mdNPCs) and determined that there were 360 genes differentially expressed in the same direction. The overlap was not significant (p=0.59).

5.3.1. Dopaminergic neurons do not show alterations in mitochondrial or adenosine related genes

We next wanted to examine the adenosine and mitochondrial systems that we had shown to be differentially expressed in the fNPCs and shHPRT1 NPCs. RNA was extracted, and rtPCR performed as previously described. As above, sample JS was excluded from the data, and the CRISPR and NCRM1 controls were pooled with the patient and control groups.

Surprisingly, we saw that the mitochondrial genes *ATP5H* and *LDHB*, and the adenosine related genes *ADORA1* and *SLC29A2* were not differentially expressed in the mdNPCs and, in fact, levels of expression were almost identical (Figure 14). We then examined other top hits from the RNAseq in shHPRT1 NPCs related to protein translation initiation (*eIF3E* and *RPL9*), another of

the top differentially regulated gene clusters. These genes were also not significantly differentially expressed in the mdNPCs.

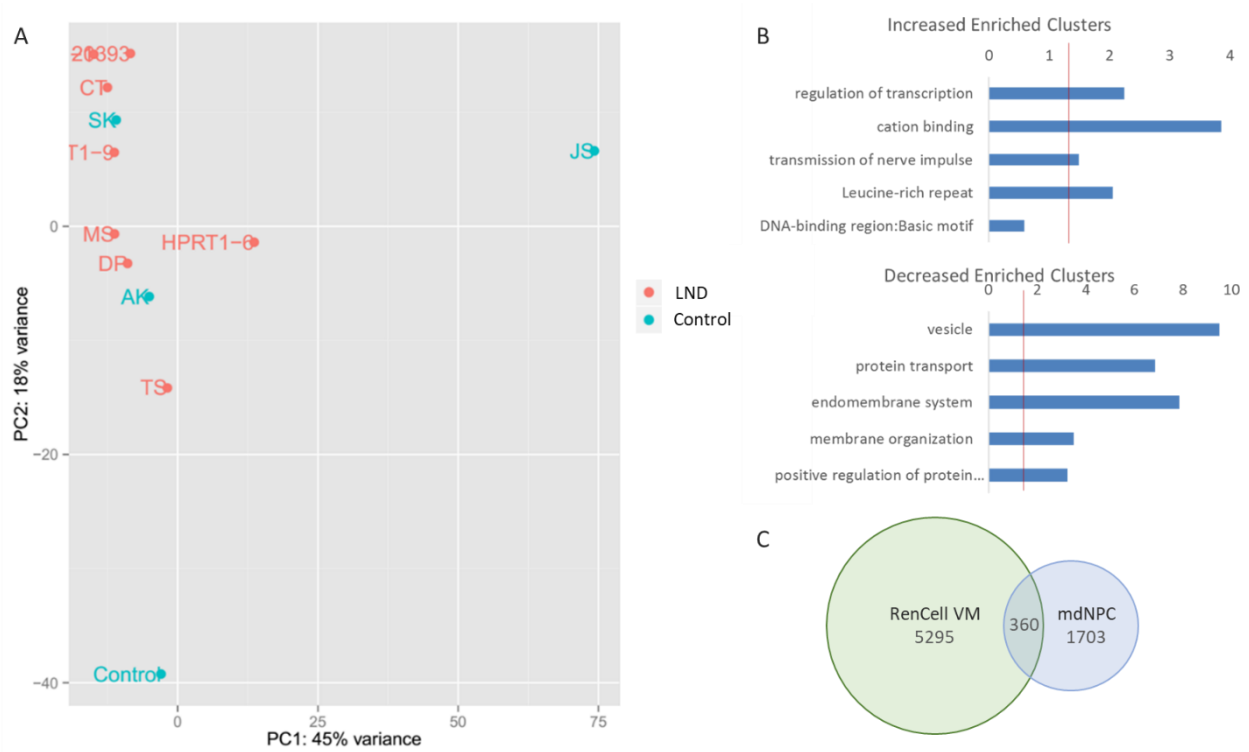

Figure 13 RNAseq in mdNPCs . A) *PCA of RNAseq results shows sample JS contributing to large sample variance.* ***B)*** *Top five enriched Biological function and molecular pathway GO clusters among increased and decreased genes, as determined by DAVID. Red line denotes multiple testing corrected p=0.05* ***C)*** *Overlap of differentially expressed genes in the RenCell VM and mdNPC experiments.*

5.3.1. HPLC shows reduction of adenosyl compounds in mdNPCs from LND patients

Based on the gene expression profiles we had observed in the fNPCs, which indicating a reduction of genes that bind to adenosyl compounds and an increase in genes required to produce ATP, we suspected that HPRT deficiency may be driving a reduction of adenosyl compounds in the fNPCs. However, these genes were not differentially expressed in HPRT deficient mdNPCS,

suggesting that these cells either have normal adenosyl levels or use an alternate pathway to respond to a deficiency of adenosyl compounds.

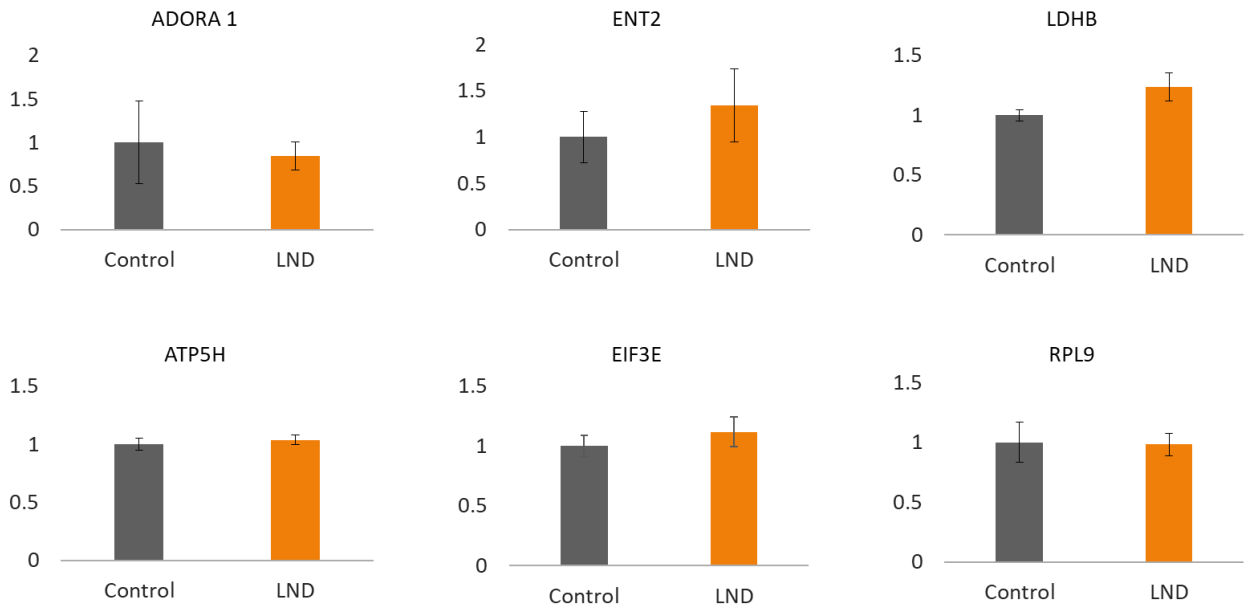

Figure 14 mdNPCs do not differentially express adenosine related or mitochondrial genes. *Patient and CRISPR modified lines do not differentially express the markers of adenosine signalling (ADORA1 p=0.71, SLC29A2 p=0.56), Mitochondria (LDHB p=0.18, ATP5H, p=0.61) or translation (RPL9 p=0.92, EIF3E p=0.52) that were detected in the cortical neurons. Data shown as mean ± SEM and normalized to control values.*

To delineate between these possibilities, we measured the adenosyl content of the cell lines using HPLC. These experiments were conducted in collaboration with Dr. Thad Rosenberger based at the University of North Dakota. We grew cultures of cortical-like and midbrain-like progenitors that had been previously created from iPSCs. NPCs were not available from the sample patient populations for each of the two cell types, but in both cases the controls BC1, NCRM1, and HIFIB4, the patient GM20393, and the CRISPR knockout NCRM1 KO were used. Additionally, GM20394 and GM01662 were used in the fNPCs, and DP, MS, TS, AK, SK, and CT were used

to create mdNPCs. Nucleotides were isolated from approximately $12x10^6$ cells and analyzed as previously published (Bhatt et al. 2012).

HPLC analysis revealed decreases in each of Adenosine, AMP, ADP, and ATP across the samples, (Figure 15). With significant main effects of genotype for AMP ($p=0.016$) and ADP ($p=0.011$). Adenosine ($p=0.051$) and ATP ($p=0.053$) had p values only slightly above the threshold for statistical significance. There was a significant effect of cell type on levels of ADP ($p=0.042$) and ATP ($p=0.006$), and near significant for adenosine ($p=0.059$). The interaction term was only significant ADP ($p=0.049$). This indicates that the cortical-like and mdNPCs have similar reductions in adenosyl compounds, although they do not demonstrate the same gene expression patterns in response to those reductions.

Evidently, the absence of HPRT is causing a reduction in adenosine nucleotides across the cell types. It is possible that this adenosine is being used to replenish the guanosine pool, or that the reduction in adenosine reflects a global depletion of purine nucleotides. In either case, the mdNPCs are not responding through the same molecular pathways as the forebrain or same mechanisms are governing the reductions in the adenosyl nucleotide pool – namely that some of the adenosine is being recruited to increase the levels of guanosine – but that mdNPCs are using other mechanisms to compensate for the change.

To further evaluate mitochondrial function and ATP production we used the Seahorse Mito Stress Test kit. This test measures the oxygen consumed by cells at a basal state and following treatment with various compounds that isolate individual elements of mitochondrial performance, however, this did not reveal any significant deficiencies in mitochondrial function (Figure 16).

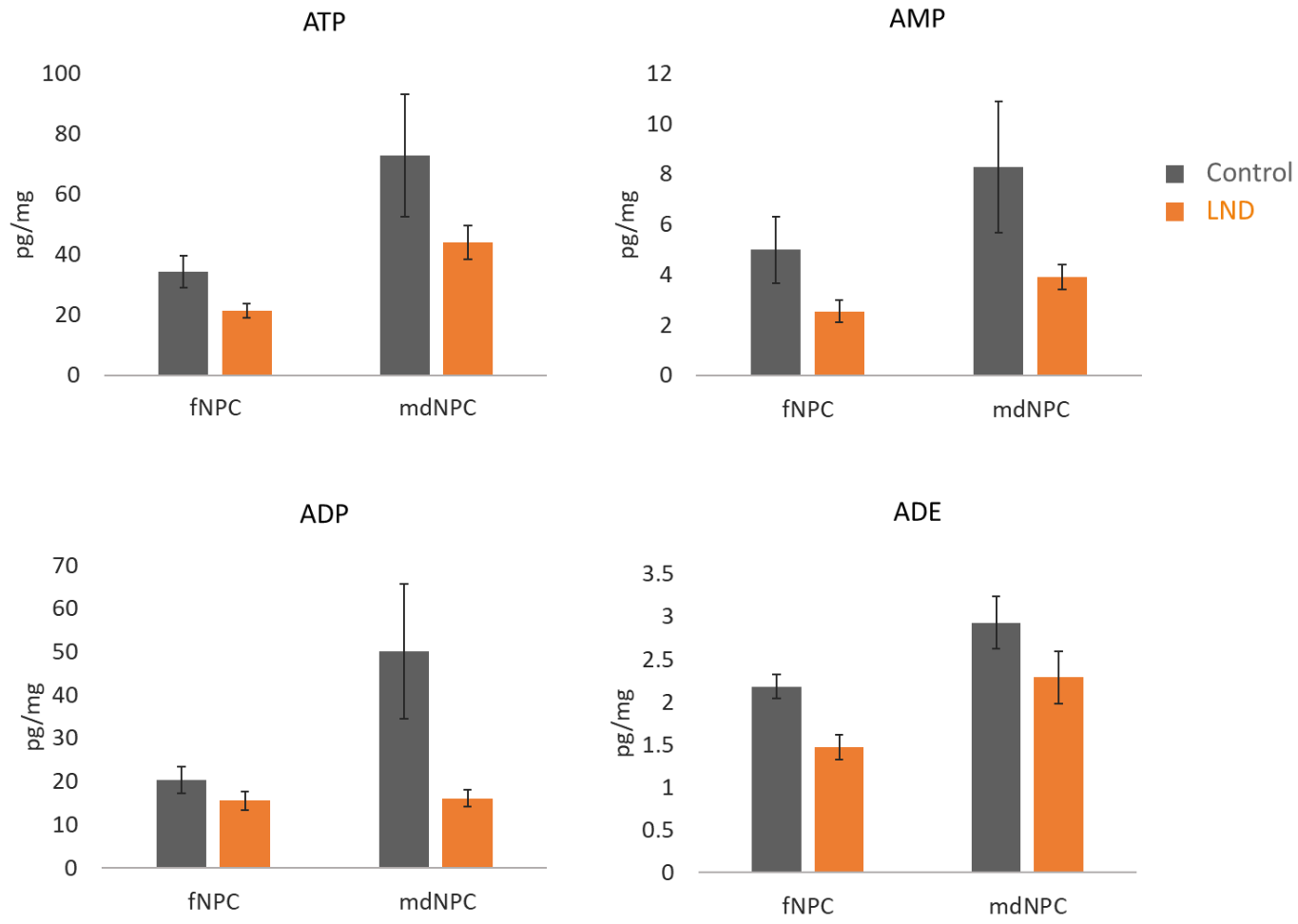

Figure 15 HPLC quantification of adenosyl compounds in fNPCs and mdNPCs (nmol/mg protein). A) *ATP quantification (nmol/mg protein) (main effect of genotype p=0.053, main effect of cell type p=0.006, interaction effect p=0.450).* ***B)*** *AMP quantification (nmol/mg protein) (main effect of genotype p=0.016, main effect of cell type p=0.096, interaction effect p=0.478).* ***C)*** *ADP quantification (nmol/mg protein) (main effect of genotype p=0.011, main effect of cell type p=0.042, interaction effect p=0.049).* ***D)*** *Adenosine quantification (nmol/mg protein) (main effect of genotype p=0.051, main effect of cell type p=0.059, interaction effect p=0.580) data shown as mean ± SEM*

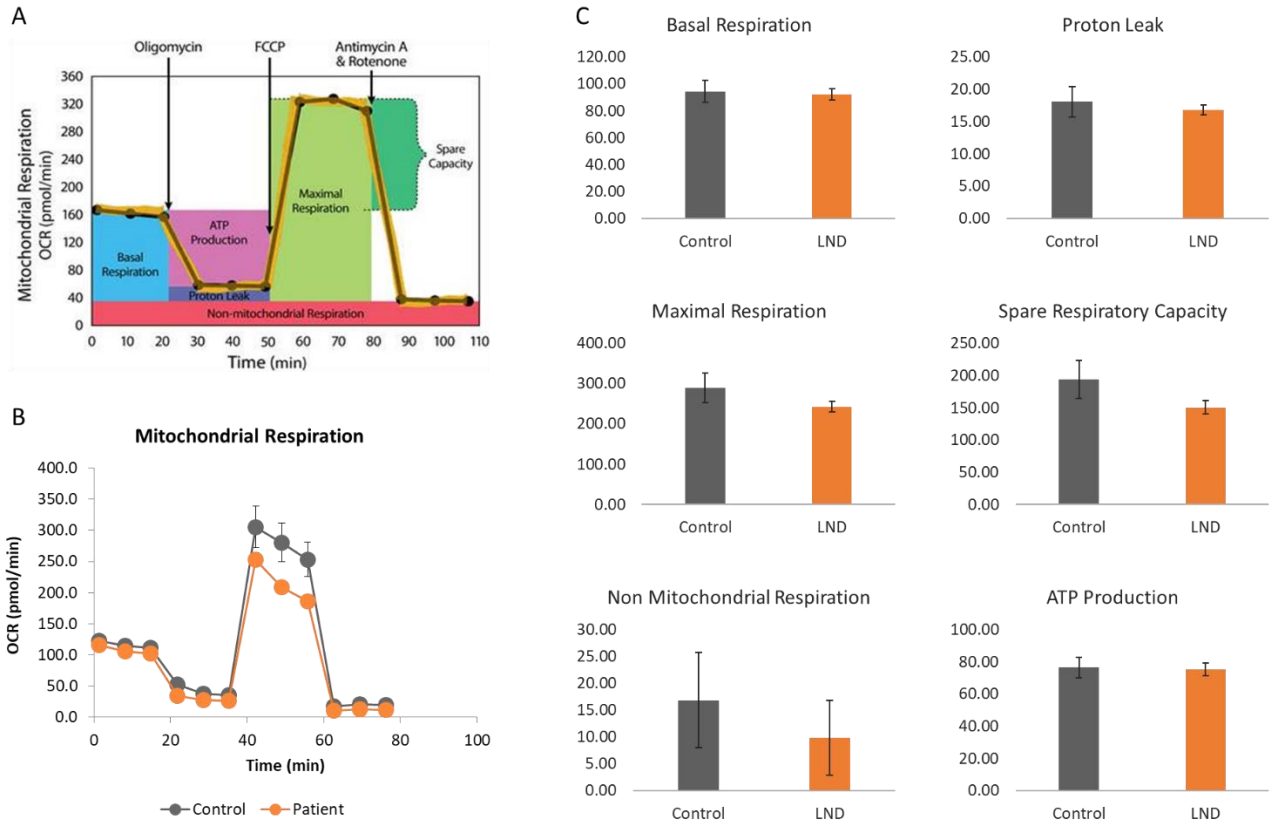

Figure 16 mdNPCs from LND patients do not show deficiencies in mitochondrial function. ***A)*** *schematic depicting the steps and measures used in the Seahorse Mito Stress Test.* ***B)*** *Control and patient oxygen consumption data following the Seahorse time course.* ***C)*** *Bar graph comparison of the oxygen consumption used in basal respiration (p=0.1867), proton leak (p=0.4051), maximal respiration (p=0.1284), spare respiratory capacity y(p=0.1226) non-mitochondrial respiration (p=0.3456), and ATP production (p=0.3079). Data presented is representative of triplicate experiments and displayed as mean ± SEM.*

5.3.2. HPLC suggests gradual DA maturation

To ensure that our mdNPCs could successfully differentiate into dopaminergic neurons, we also used HPLC to examine the levels of DA and its primary metabolites HVA and DOPAC in control and patient mdNPCs. Monoamines were extracted from the midbrain-like neurons 2 and 4 weeks after the start of differentiation. To increase the power and sample size, the CRISPR knockout and control line NCRM1 were pooled with the patient and control groups, respectively, and not considered for separate analyses.

Dopamine and the metabolite DOPAC were only present in relatively low levels in the cell lines at both time points, however very few studies producing dopaminergic neurons in vitro have successfully measured dopamine by HPLC and it is, therefore, difficult to know what levels would be considered normal at any stage of maturation. Woodard et al. (2014) followed a differentiation protocol quite similar to ours and recorded between 1 and 8 pmol of dopamine per mg of intracellular protein after 39-52 days of differentiation, which is in the range our or measurements, while (Hartfield et al. 2014) recorded 48 pmol/mg after 35 days of differentiation. However, measurements up to 6 ng/mg have been recorded in neurons isolated from adult mouse midbrain (Heyer et al. 2012).

There was not a significant difference in dopamine production between patient and control cell lines ($p= 0.43$). Although our findings are above the threshold of detection for HPLC, it is difficult to interpret changes in dopamine production in between patient and control cell lines at this low level of production. There is a significant increase in dopamine production from three weeks to four weeks in the cell lines ($p= 0.042$), suggesting that with a longer differentiation period the effects may become clearer. The same trend can be seen for the dopamine metabolite DOPAC, which is only present at low levels, and not significantly different between patients and controls ($p= 0.289$) but is significantly increased after the longer differentiation time ($p= 0.011$). On the other hand, HVA, the second primary metabolite of DA, showed a significant reduction in patient and CRISPR cells (main effect of genotype $p=0.023$, Figure 17), but did not change between timepoints 9$p= 0.057$). There was not a significant interaction of time and genotype for any of the metabolites.

The low levels of DA and DA metabolites do not necessarily mean that our protocol is unable to generate dopaminergic cells. The cells express TH, and other markers of dopaminergic neurons

(Figure 12), but may indicate that the cells still represent an early point in development before dopamine is produced at high levels. Despite this low production of dopamine, the small sample size, and high variability, our data still suggest that dopamine is reduced in HPRT deficient cells, as has been demonstrated in each other model of LND.

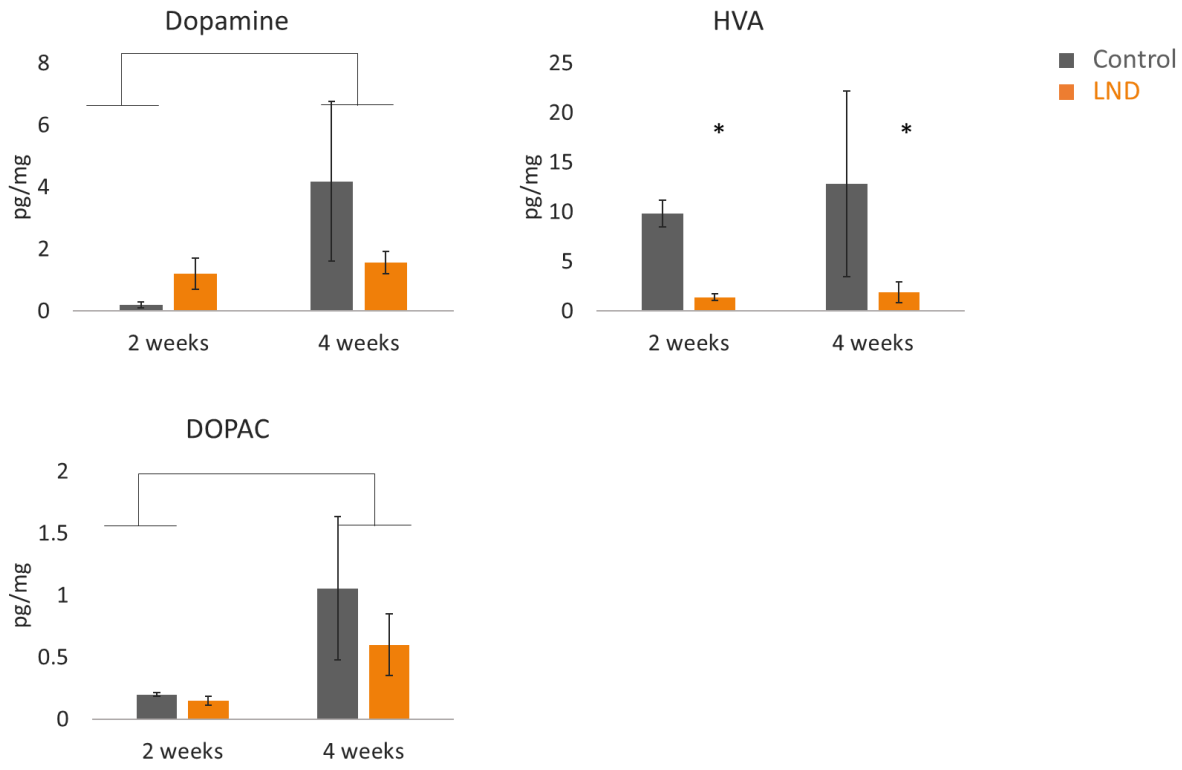

Figure 17 mdNPCs increase the expression of dopamine and metabolites on differentiation. *HPLC quantification of dopamine Homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) in patient and control derived dopaminergic neurons after 2 weeks and 4 weeks of maturation. DA Main effect of time p=0.042, main effect of genotype p=0.428, interaction p=0.085. HVA main effect of time p=0.570, main effect of genotype p=0.004, interaction p=0.683. DOPAC main effect of time p=0.011, main effect of genotype p=0.289, interaction p=0.395. data presented as mean ± SEM*

5.3.3. RNAseq in mouse brain shows regional differences

To further examine cell type differences in the HPRT deficient brain, we turned to the HPRT knockout mouse. Although the HPRT KO mouse does not display any behavioural alterations

associated with LND, it does display substantial reductions in striatal dopamine, similar to LND patients (Jinnah et al. 1994). Using the HPRT knockout mouse also allows us to access brain regions that we know have developed under physiological conditions, and accurately reflect the regional diversity of the brain. The disadvantage to working with mouse brain is that it is almost impossible to guarantee that the extracted brain sample exclusively contains the region of interest, and this is particularly true in small regions like the VTA. Even when a region is accurately extracted there is a mixture of cell types including a variety of neuronal subtypes, astrocytes, microglia and blood vessels, which can all add noise to the system and make results more difficult to interpret.

We extracted the brains from three wild six and six HPRT knockout mice, sliced them, and extracted RNA from the SN and striatum. The RNA was sent to Genome Quebec for library preparation and RNA sequencing on an Illumina Hiseq2500. Analysing the sequencing results using PCA showed that the samples segregated into two groups along principal component one, which explains 92% of the variance in the data, and that these groups correspond with brain region. There was little segregation by genotype (Figure 18 A). A similar phenomenon could be seen using hierarchical clustering, where the samples segregated by brain region but not genotype (Figure 18 B). This trend is also evident in a differential gene expression analysis, in the striatum only 4 genes were differentially expressed with genome-wide significance $p<0.05$ Hprt, Asxl3, a polycomb group protein associated with Bainbridge-Ropers Syndrome, mental retardation, and macrocephaly, Plekhf1, a lysosomal protein involved in the initiation of apoptosis, Arl14, a GTPase that controls the movements of MHC II vesicles in dendritic cells. Neither Arl14 nor Plekhf1 has been associated with disease according to the Genetic Association Database. In the

SN only Hprt was genome-wide significant, and no genes had a genome-wide significant genotype x region interaction.

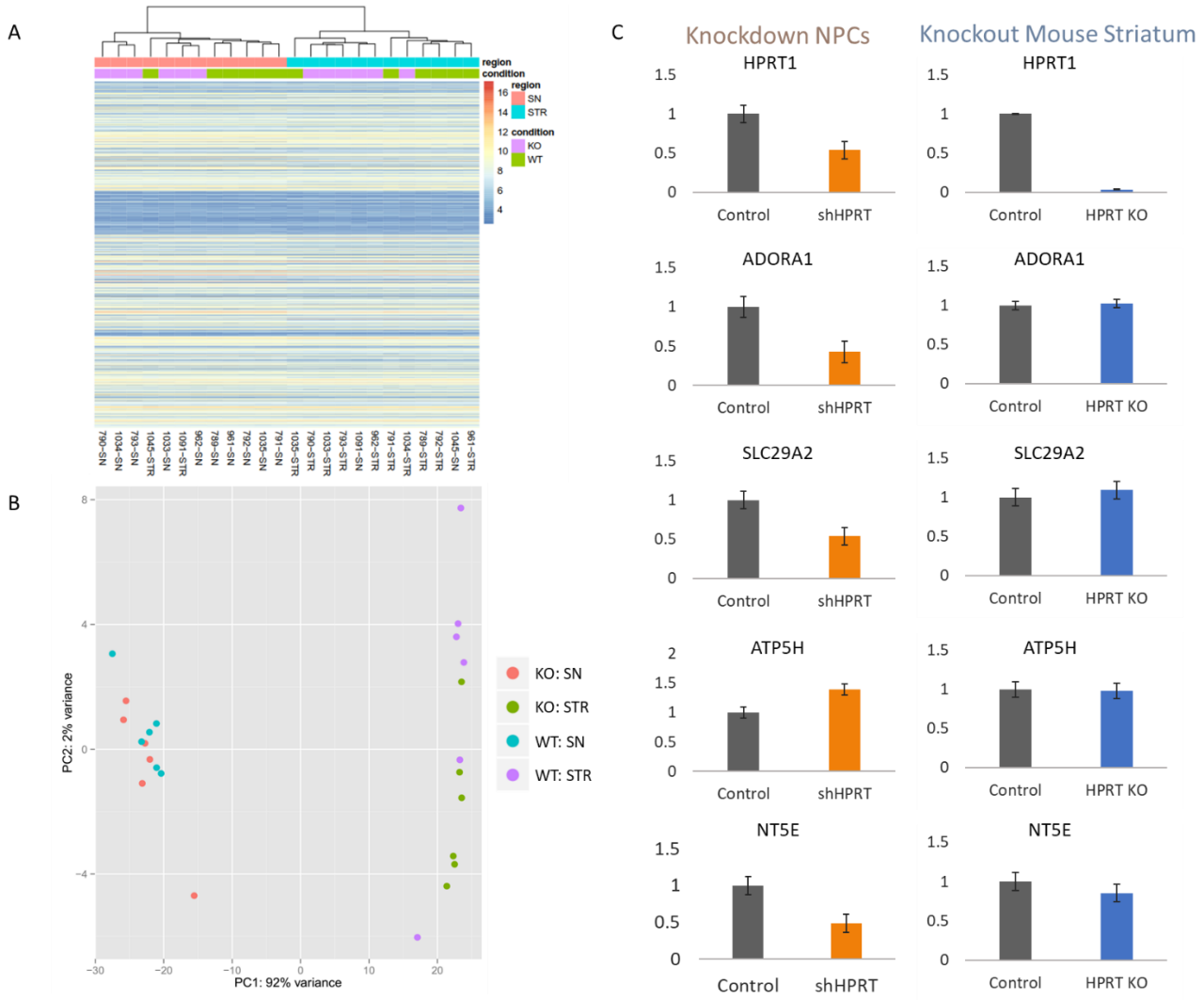

Figure 18 RNAseq reveals regional but not genotype differences in gene expression patterns in mouse brain A) *Hierarchical clustering of by the 1000 most differentially expressed genes in RNAseq data from the SN and STR of WT and HPRT KO mice correctly segregates brain region but not genotype* ***B)*** *PCA of RNAseq data generated from the caudoputamen (STR) and substantia nigra (SN) of HPRT$^{-/y}$ (KO) and control (WT) mice. Samples segregate cleanly by brain region along principal component 1, which explains 92% of the variance in the dataset.* ***C)*** *Comparison of the expression of differentially expressed genes in shHPRT RenCell VM to HPRT knockout mouse striatum. All genes are significantly differentially expressed in RenCell VM, only HPRT is differentially expressed in mouse.*

5.4. Conclusions

We have generated mdNPCs from a cohort of patients and controls. The expression of PAX6 and FOXA2 in the progenitor cells indicates a dopaminergic phenotype similar to the A9 neurons of the brain. In combination with the expression of TH after growth factor withdrawal, this suggests that these neurons are, or can become, dopaminergic. While HPLC did not identify substantial levels dopamine production in these cell lines, they are still likely to resemble human dopaminergic progenitors more closely than the adrenergic SH-SY5Y cell line that is more commonly used. Further, there were increased levels of dopamine and DOPAC over time. Differentiating functional dopaminergic neurons *in vitro* is a relatively new field and while we believe that differentiation towards a dopaminergic phenotype would continue with longer differentiation periods, these experiments require larger cell populations and more time than was available.

Reductions in dopamine production have been a consistently observed phenotype in LND research, but our cells did not replicate this finding. It is likely that the mdNPCs are in too early of a developmental stage to express the dopaminergic machinery and that this immaturity is why the RNAseq analysis did not identify changes in the dopamine production or signalling pathway. While some work has shown changes in dopaminergic patterning genes including EN1 and Lmx1a in SH-SY5Y (Guibinga et al. 2012; Kang et al. 2011), this was also not visible in our data. It is possible that the directed differentiation protocol we followed negated these effects, or that this phenotype is cell type specific.

After the clear consistency of the adenosine and mitochondrial gene expression phenotype in the knockdown NPCs, the fNPCs, and using an HPRT inhibitor, we were initially surprised not to replicate the results in this cell population. Clearly, these cells are responding differently to

HPRT deficiency than the previous cell types examined suggesting that different neuronal progenitor cell types have different responses to the absence of HPRT1. However, a difference in the impact of HPRT deficiency between brain regions is consistent with the literature (Song and Friedmann 2007; Saito et al. 1999; Saito and Takashima 2000), and with the dopamine specific phenotypes of LND (Visser, Bär, and Jinnah 2000). Dopaminergic neurons have much higher energy requirements than other neuronal subtypes partly due to their size and complex arborization (Wellstead and Cloutier 2011; Attwell and Laughlin 2001) which are not reduced in LND (Göttle et al. 2014). Given the lack of change in mitochondrial genes, it is possible that the dopaminergic neurons are using alternate pathways to attempt to maintain energy levels in the absence of HPRT to the detriment of dopamine production and release. This would also be consistent with the reductions in ATP, ADP and AMP that were observed, and with the absence of change in mitochondrial function.

While gene ontology analysis of differentially expressed genes in the HPRT deficient cells did not provide any clear indication of which pathways may be providing this compensation, we did observe that several genes related to glutamate transport were increased. This may indicate either an altered response to excitation through glutamate neurotransmission, an increase in the amount of glucose that is being metabolised to produce ATP, or both. The use of alternate sources of energy production should be an active area of research in LND moving forward.

The mdNPCs we have generated and the data we have produced from them will be a valuable asset to the LND research community. For the first time, researchers will have access to a complete set of transactional data from patient-derived neurons, which can be used to identify LND phenotypes or to validate data previously generated in other models.

The results of our in vitro gene expression analyses, documented in the previous chapters, showed that patient-derived fNPCs, but not mdNPCs have significant changes to their adenosine and mitochondrial systems. This finding ran counter to our initial hypothesis that changes in both types of neurons would be similar in at least direction, and that mdNPCs would have if anything larger effect sizes in alignment with the effects of LND on dopamine signalling.

We initially expected that any results we found in the fNPCs would be replicated in the mdNPCs, if anything showing a greater effect size. However, our data show the opposite of that trend. Our findings reaffirm cell type differences in LND and show dopaminergic and cortical neurons respond differently to the absence of HPRT, perhaps using different pathways to compensate for the loss of purine reuptake. It is known that dopamine neurons have a higher sensitivity to changes in energy metabolism than other cell types, owing in part to their size and complexity (Pacelli et al. 2015). The difference here is that there is not a clear relationship between HPRT requirements and the size or shape of a neuron. This is perhaps partly explained by the role of HPRT in maintaining the nucleotide pool, which is used to maintain the energy charge of ATP, GTP, NAD (Deutsch et al. 2005).

While alterations in dopaminergic neurons have been the focus of several research groups, other neuronal cell types have been less well studied and may be equally important to the development of behavioural problems in LND. Regardless of the cause, it has become clear that the loss of HPRT function has cell type and brain region-specific effects, and that it will be important to consider them independently.

6. Discussion

6.1. Creating Neuronal models of LND

6.1.1. Modelling LND with RenCell VM

Many of our greatest insights into LND and HPRT function have come from *in vitro* models. This is because of both the simplicity of modifying and monitoring *in vitro* models and the unusually limited utility of *in vivo* models of LND. An unfortunate limitation of these studies has been their ability to examine the effects of HPRT deficiency in neurons and, more specifically, midbrain neurons. Most of the well-validated models of dopaminergic neuronal differentiation are in rodent dopaminergic progenitor cell lines but, because of the phenotypic differences between HPRT deficient rodents and patients with LND, these have not been used widely in LND research. The cell lines that are most commonly used to examine dopaminergic phenotypes in LND are MN9D cells and SH SY5Y cells, but each of these lines can be problematic as they do not fully recapitulate dopaminergic differentiation or dopaminergic phenotypes (Rick et al. 2006; Balasooriya and Wimalasena 2007). The RenCell VM cell line provides an alternative to these cell lines. This line was isolated from a fetal ventral midbrain sample, immortalized using the v-Myc oncogene, and, depending on the differentiation protocol used, can effectively differentiate into neurons or astrocytes (Donato et al. 2007).

When used in conjunction with genetic engineering techniques like shRNA transfection or CRISPR-Cas9 mediated genetic engineering, these cells represent an easily maintainable and expandable cell line for the study of a variety of diseases. The cell lines we have produced, which stably express anti HPRT hairpins, are now available to researchers, as are several other RenCell

VM cultures expressing shRNAs targeting other genes that have been linked to neurodevelopment (Gigek et al. 2015).

In the context of LND the use of shRNA to knock down HPRT is not an optimal model because it does not completely remove the HPRT transcript, however in the context of many other disorders that result from haploinsufficiency of the responsible gene this in fact presents an advantage as it may more closely resemble the physiological situation in which the gene is reduced but not completely absent. Further, the rapid ascent of CRISPR technologies means that HPRT knockout RenCell VM lines could now be produced with relative ease. The rapid rise of CRISPR could not have been predicted when these experiments were started, but they now serve as an important proof of concept both for the examination of neurodevelopmental disorders and for the use of RNAseq to identify substantial gene expression changes caused by HPRT deficiency.

6.1.2. Modelling LND in iPSCs

This project has further demonstrated the value of using iPSCs to model rare neurodevelopmental disorders including LND. By using cells that could be differentiated to different neuronal subtypes we were able to identify disease phenotypes that could not be seen in other cell culture models or animal models and to identify phenotypes specific to different types of neurons. The absence of HPRT does not appear to have any effect on the ability of iPSCs to differentiate into NPCs or neurons and, while this was not rigorously evaluated, there did not appear to be any gross morphological differences or differences in growth rate. This stands in contrast to iPSC based models of Rett syndrome (Li et al. 2013) and other genetically linked causes of autism (Deshpande et al. 2017) which show larger changes in soma size and RNA transcription. Although our studies were not designed to examine global transcription levels, nothing in our RNAseq data suggested global transcriptional differences between patient and control cells. The

only other study to have used iPSCs from LND patients also did not see any changes in soma size, although they did report reduced neurite growth which was not apparent in our work (Mekhoubad et al. 2012).

Another advantage of using neurons derived from NPCs is that they provide the opportunity to perform direct functional studies on the neurons. In this study, we performed HPLC to evaluate the levels of nucleotides and neurotransmitters in HPRT deficient human neuronal cells for the first time. While the results of our study are limited by sample size and neuronal maturity, they provide an important proof of concept for the viability of these types of studies. Very few others have used HPLC to detect neurotransmitters in iPSC-derived cells, but this has the potential to be an important way forward for studying neurological disorders. While we did not delve into these aspects of functional assessment, iPSC derived neurons also provide a platform for assessment of electrophysiological phenotypes either through patch clamping or using higher throughput multielectrode arrays eg, (Hempel et al. 2017; Wainger et al. 2014) and for screening for compounds that reverse identified phenotypes, provided a suitable assay readout can be developed. (Rana et al. 2017; Egawa et al. 2012).

This approach to studying NDDs is applicable beyond LND. iPSC based studies have lead to increased understanding of Fragile X syndrome (Liu et al. 2012) Timothy syndrome (Paşca et al. 2011) Rett syndrome (Li et al. 2013) and many others (Saporta, Grskovic, and Dimos 2011b). However the majority of studies in the field have focused on validating phenotypes that had been observed in other models of the diseases. While this is an important step in validating both the phenotype and disease model, more insights may be found by differentiating iPSCs into neuronal types that are not canonical parts of the disease pathway, or by using the known phenotypes to inform hypothesis generation and data analysis. For instance, Li et al. (2013) used a thorough

examination of iPSC based models of Rett syndrome to identify global transcriptional and translational repression that would have been exceptionally difficult to uncover in any other system.

The iPSC based models developed here will be a valuable resource for the LND research community and will facilitate these kind analyses without needing first to overcome the substantial barriers to entry in iPSC research for groups that do not regularly engage in it.

6.2. Gene expression changes in neuronal models of LND

6.2.1. Adenosine system

We have demonstrated reduced levels of the adenosine receptor ADORA1, the purine transporter ENT2, and the adenyl nucleotides Adenosine, AMP, ADP and ATP in various HPRT deficient cell lines. One possible explanation for these reductions would be that the adenosine pool gets used as a reserve to help resupply the guanine pool which is reduced because the cells cannot reuptake hypoxanthine or guanine. The inhibition and activation of enzymes in the purine pathway by their substrates and products can cause this kind of redistribution and balance between purines, even in healthy cells, and transiently depleting guanosine increases the amount of adenosine that is used to produce guanosine. This indicates that the system can respond to depletion of a nucleotide pool by shunting purines from adenosine, though inosine, to guanosine in states of acute G depletion (Zoref-Shani et al. 2001), but the function of this system has not been well studied under chronic G depletion.

The Adenosine system is an attractive candidate for pharmacological treatment of LND. It is well known to modulate the activity of DA, and several pharmacological modulators of the adenosine system have already been developed and used in clinical trials for Parkinson's disease (Burnstock

2017; Shao et al. 2018). These agents were not further developed into treatments for these disease because of a lack of efficacy in early studies, but several have met the requirements for safety (Chen, Eltzschig, and Fredholm 2013) simplifying the development of these treatments for LND or other disorders.

While adenosine is known to modulate dopamine signalling, its role in the differentiation of dopaminergic neurons is largely unknown. Dopamine is a very important neurotransmitter in other NDDs including schizophrenia, and neurodegenerative disorders like Parkinson's disease, and Adenosine has been proposed to play an important role in each of these disorders (Jenner 2014; Boison et al. 2012). Understanding the role of adenosine/dopamine interactions in regulating dopamine development and signalling in LND may help to clarify the involvement of adenosine in these disorders as well.

6.2.2. Transcriptomics in LND

There has been a striking lack of 'omics level data in LND research, despite the widespread availability and decreasing costs of surveying the entire genomes, epigenomes, transcriptomes, and proteomes (Joyce and Palsson 2006), and the power that these approaches have in identifying potential molecular phenotypes of disease (Gehlenborg et al. 2010), large-scale studies like this have been almost absent in LND research. Microarrays have been used to study the microRNAs that are differentially expressed in SH-SY5Y cells (Guibinga et al. 2012) Mouse embryonic stem cells (Kang et al. 2013) and in knockdown SH-SY5Y cells (Kang et al. 2011), RNA seq has been used in neuronal cells derived from mouse ES cells (Kang et al. 2013). The proteome has been examined in HPRT $^{-/y}$ mouse striatum, and compared to a Parkinson's disease model (Guo, Friedmann, and King 2007), however this study used 2D electrophoresis and only published individual information for one protein, AKT1. The associated supplementary data is not found

with the article online, and cannot be found in the database indicated in the article. The most informative proteomic study of LND was done in an immortalized rat dopaminergic cell line with a stable HPRT knockdown and used SILAC to quantify differences in protein expression in undifferentiated and differentiated cells (Dammer et al. 2015). The choice of model is curious in this case since at the time nothing was known about how the absence of HPRT affects rats *in vivo* there was no way to cross validate the results. Subsequent studies have shown that rats do not have any behavioural symptoms but, like the mouse model, they do have decreased dopamine production (Isotani et al. 2016; Meek et al. 2016), metabolites and monoamines were studied by Meek et al. (2016) but neither paper examined protein levels. While the protein level results of (Dammer et al. 2015) did not correlate with our RNA level data in human-derived cells, they did identify significant GO several similar GO clusters including Protein translation, purine ribonucleotide binding, and mitochondrial proteins. Interestingly, the expression of purine binding proteins in their model returned to normal following differentiation, while mitochondrial proteins did not.

6.3. Impacts of LND on different cell types

6.3.1. fNPCs and mdNPCs

LND research has thus far been highly focused on studying dopaminergic neurons. This makes sense because of dramatic reductions in dopamine that are seen in both human and animal brains, and because this is one of the few phenotypic changes that can be studied in the HPRT KO mouse, however fully understanding the pathobiology of LND, and the causes of the behavioural phenotypes requires understanding the systems that are dysregulated in the disease. In fact, it is easy to imagine the primary involvement of other neural circuits. For instance, the presence of compulsive behaviours suggests a possible involvement of glutamatergic connections from the

orbitofrontal cortex (OFC) to the ventromedial striatum (Ahmari et al. 2013; Evans, Lewis, and Iobst 2004). Alternately, the aggression of LND patients could be interpreted as implicating the limbic or serotonergic systems (Waltes, Chiocchetti, and Freitag 2016). HPRT is expressed throughout the brain, and it is therefore unlikely that the dopaminergic system is solely responsible for the behaviours. Beyond changes in the midbrain, LND is associated with substantial decreases in white matter volume, and perhaps have greater reductions in white matter than grey matter (Schretlen et al. 2015) The development of effective treatments for LND will likely then require understanding the impacts of HPRT deficiency in a variety of cell types, and how these work together in a system to generate such complex behaviours.

We were surprised to see that our findings in the RenCell VM model aligned more closely with the results from fNPCs than the mdNPCs, given that these cells are supposed to be predisposed to a dopaminergic fate. However, some other work has suggested that RenCell NPCs preferentially differentiate into astroglia under some circumstances. For instance, 100 % of RenCell NPCs xenoplanted into rat brain differentiated into astrocytes (Hovakimyan et al. 2012). The maintenance protocol we used for the RenCells does not contain many of the growth factors that are present in our later established dopaminergic NPC media. So it is possible that the cells we were studying had lost some of the predisposition towards dopaminergic neurons, and therefore more in common with the fNPCs, which were maintained in a more similar minimal media. This is also reflected in that the RenCell VM cells expressed high levels of glial markers after differentiation. This difference does not impact the validity of our results but does limit the generalizability, as it has become less clear precisely which cells are the closest comparison for the RenCell VM line used in our experiments.

6.3.2. Mouse brain regions

We confirmed the differential impacts of HPRT knockouts on gene expression by brain region in mice. In a principal component analysis of gene expression across $HPRT^{-/y}$ mice the first component explained 92% of the variation in gene across samples and brain region segregated almost perfectly along this component while genotype did not. The second component explained only 2% of the variation, and genotype partially segregated along this dimension.

Based on our results in cell culture and the work of other groups, we expected to see large differences in gene expression that were easy to resolve, even with our small sample size. Unfortunately, this was not the case, and we ultimately did not have sufficient power to detect any statically significant changes by genotype. This stands in contrast to targeted studies from other groups reporting, for instance, a doubling of ADORA1 expression in whole brain homogenate (Bertelli et al. 2006), or a 50% decrease in TH immunoreactivity in mouse striatum but not midbrain (Gottle et al. 2014). It is important to note that these studies also used very small sample sizes, less reliable methodologies, and were targeted studies.

Our mouse study is the first transcriptomic analysis of HPRT knockout mice and appears to support the notion that the impacts of HPRT knockout are milder in mouse brain than in human patients. In order to examine the phenotypes with more granularity larger sample sizes will be needed, but even ignoring issues of statistical significance, the magnitude of the effects on genes of interest in our study were relatively small.

6.4. Closing remarks

Lesch-Nyhan Disease is an exciting problem to work on because of the astonishing degree of separation between the cause and the phenotypes of the disease. How is it possible that the

disruption of a single gene – a universally expressed metabolic gene – can cause such a change in behaviour as complex as self-harm? Since these behaviours can be caused by a single gene, why are they not seen in other conditions that disrupt similar pathways? What does it say about aggression or self-harm in other disorders, or even suicide, that self-injury can be caused by something seemingly so basic?

These are not questions that can be answered by any disease model alone, cell culture models least of all. NPCs do not self-harm. In stead, we hope that by developing tools and taking advantage of technological and methodological advances, we can clarify the effects of the absence of HPRT at a cellular level and that this will inspire the use of still more tools and methodologies to develop novel approaches to studying LND and developing treatments.

The identification of adenosine and energy changes in patient-derived cells raises some other questions. To what extent are these changes seen in patient brains? Are they important in the development of disease phenotypes and behaviours? Can they be targeted by therapeutics to change the progression of the disease?

While these and other questions remain open LND research will continue to be an active field requiring all the imagination and creativity of those that choose to study it.

7. References

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