INVESTIGATING AUTOPHAGY DYSFUNCTION INDUCED BY A PARKINSON'S DISEASE-CAUSING MUTATION IN VPS35

by

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DEDICATION

I would like to dedicate this dissertation to my mother, Lutfa Begum, and my father, Mujibur Rahman. I hope I have made you proud.

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ABSTRACT

Parkinson's Disease (PD) is an idiopathic disorder with no known cure. With number of cases steadily rising around the world, it is imperative to turn to the underlying cellular and molecular mechanisms of the disease manifestation and neurodegeneration to craft novel modes of therapy. VPS35 is one of the few genes that have identified and definitively linked to familial PD. The particular mutation that has been associated is known to cause dysfunction of a key cellular process known as autophagy. This process is primarily responsible for clearance of unwanted, damaged or misfolded proteins, among other things. Our study reveals an important link between VPS35, PI3K-AKT signaling pathway and autophagy dysfunction that might be a potential mechanism of PD pathogenesis. We also address possible perturbed dopaminergic neurogenesis in the substantia nigra brain region as a second plausible disease mechanism, as well as a target for therapeutic intervention.

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LIST OF ABBREVIATIONS

PD	Parkinson's Disease
SN	Substantia Nigra
SNPC	Substantia Nigra Pars Compacta
DA	Dopamine
RA	Retinoic Acid
VPS35	Vacuolar Protein Sorting Protein 35
WT	Wild-type
MUT	Mutant
CDC	Center for Disease Control and Prevention
D620N	Aspartic Acid to Asparagine mutation at amino acid position 620
CRC	Cargo Recognition Complex
CIMPR	Cation Independent Mannose-6-Phosphate Receptor
β2AR	Beta-2 adrenergic receptor
GPCR	G-protein coupled receptor
TGN	Trans-Golgi Network
APP	Amyloid Precursor Protein
SNCA	α-synuclein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
shRNA	Short Hairpin RNA
siRNA	Small interfering RNA

DRD1	Dopamine Receptor D1
WASH	Wiskott–Aldrich syndrome protein and SCAR homolog
UPR	Unfolded Protein Response
PE	Phosphotidylethanolamine
PI3K	Phosphatidylinositol 3-kinase
mTOR	Mammalian target of rapamycin
PI	Phosphatidylinositol
PI3P	Phosphatidylinositol 3-phosphate
ECM	Extracellular Matrix
HA	Hyaluronan
RNA-seq	RNA sequencing
KEGG	Kyoto Encyclopedia of Genes and Genomes
HMW	High Molecular Weight
LMW	Low Molecular Weight
Baf A1	Bafilomycin A1
HAS	Hyaluronan Synthase
ECL	Enhanced Chemiluminescence substrate
NCBI	National Center for Biotechnology Information
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
MMP	Matrix Metalloproteinases

CHAPTER ONE: A REVIEW OF THE CONTRIBUTIONS OF VPS35 MUTATIONS IN PARKINSON'S DISEASE

Parkinson's Disease (PD) is a multi-system neurodegenerative disease where approximately 90% of cases are idiopathic. The remaining 10% of the cases can be traced to a genetic origin and research has largely focused on these associated genes to gain a better understanding of the molecular and cellular pathogenesis for PD. The gene encoding vacuolar protein sorting protein 35 (VPS35) has been definitively linked to late onset familial PD following the identification of a point mutation (D620N) as the causal agent in a Swiss family. Since its discovery, numerous studies have been undertaken to characterize the role of VPS35 in cellular processes and efforts have been directed toward understanding the perturbations caused by the D620N mutation. In this chapter, we examine what is currently known about VPS35, which has pleiotropic effects, as well as proposed mechanisms of pathogenesis by the D620N mutation. A brief survey of other VPS35 polymorphisms is also provided. Lastly, model systems that are being utilized for these investigations and possible directions for future research are discussed.

Introduction

Parkinson's disease (PD) is a complex neurological disorder involving both motor and non-motor symptoms. Affecting 1% of the population over 60, it is the second most common neurodegenerative disorder (Lau & Breteler, 2006; Williams-Gray et al., 2013). The prevalence of the disease rises with age. According to a meta-analysis report by Pringsheim, Jette, Frolkis, & Steeves, (2014), the prevalence is 41 per 100000 between

ages 40 and 49, rising up to 428 between ages 60 and 69 and 1087 between 70 and 79. Another report states that there is a greater incidence in men compared to women, at a ratio of approximately 1.5:1 (Elbaz et al., 2002; K. S. M. Taylor, Cook, & Counsell, 2007; Wooten, Currie, Bovbjerg, Lee, & Patrie, 2004). The case burden in the US alone is estimated to rise to more than one million by 2030 (Marras et al., 2018).The loss of motor function occurs primarily due to a loss of dopamine signaling in the basal ganglia. This loss of dopamine is, in turn, due to the progressive loss of dopaminergic neurons in a region of the brain known as the substantia nigra. However, it is not only the basal ganglia that is affected in PD. Research suggests that it involves multiple areas of the brain and results from a complex interplay between genetic and environmental factors. Clinically described motor symptoms include resting tremors, bradykinesia, rigidity of limbs and gait defects. Moreover, there are numerous non-motor symptoms such as cognitive defects and dementia, mood disorders and sleep disorders that add to the suffering of the patients (Ascherio & Schwarzschild, 2016; Pringsheim et al., 2014; Tysnes & Storstein, 2017). According to the centers for disease control and prevention (CDC), medical complications due to PD rank as the 14th leading cause of death in the United States (Beard et al., 2017). With no known cure for this disease, research has been focused on understanding the cellular and molecular processes that may be responsible for the neurodegeneration.

In terms of neuropathology, some of the hallmarks of PD are the loss of dopamine producing neurons in the substantia nigra and the formation of toxic protein aggregates in surviving neurons. Neurofibrillary tangles composed of α-synuclein aggregates formed structures called Lewy bodies in these neurons. This is also accompanied by

mitochondrial dysfunction (Goswami, Joshi, & Singh, 2017). The pathology of the α-synuclein aggregates resembles features of prion disease. This has led to the hypothesis that α-synuclein has prion properties (Brundin & Melki, 2017; Surmeier, Obeso, & Halliday, 2017). Some of the genes associated with PD that have been described include SNCA, LRRK2, VPS35, CHCHD2, GBA, Parkin, PINK1, DJ-1, ATP13A2, FBXO7 and PLA2G6. These genes have also been reported to disrupt mitochondrial function and biogenesis (Helley, Pinnell, Sportelli, & Tieu, 2017). Protein homeostasis, particularly synucleinopathy, and mitochondrial health have therefore become major areas of focus for research into characterizing PD pathogenesis and developing therapeutics.

Identified VPS35 Mutations

The VPS35 gene was identified by two independent studies in 2011 as linked to familial PD (Vilariño-Güell et al., 2011; Zimprich et al., 2011). These studies were conducted using exome sequencing technology on DNA obtained from families that exhibited evidence of hereditary PD. Subsequent studies have largely concluded that the D620N mutation is relatively rare, with a calculated prevalence of about 0.115%, from 15 reported case studies involving 21824 PD patients worldwide (Ando et al., 2012; Blanckenberg, Ntsapi, Carr, & Bardien, 2014; Chen et al., 2013; H Deng et al., 2012; Hao Deng, Gao, & Jankovic, 2013; Gagliardi, Annesi, Tarantino, Nicoletti, & Quattrone, 2014; Gambardella et al., 2016; Guella et al., 2012; Guo et al., 2012; Gustavsson et al., 2015; Koschmidder, Mollenhauer, Kasten, Klein, & Lohmann, 2014; Kumar et al., 2012; Lesage et al., 2012; Shannon et al., 2014; Sharma et al., 2012; Sheerin et al., 2012; Sudhaman et al., 2013; Y. Zhang et al., 2012). Other polymorphisms in the VPS35 gene that were identified include L774M, P316S, R524W, I241M, M57I, G51S, R32S, I560T,

H599R, M607V. However, all of these sequence variations, with the exception of D620N, have yet to be definitively linked to PD.

In 2005, a study identified VPS35 levels to be reduced in affected brain regions in Alzheimer's disease (Small et al., 2005). Another study then reported increased Amyloid β depositions in mice heterozygous for VPS35 knockout ($Vps35^{+/m}$) (Wen et al., 2011). Similarly, other studies have linked abnormal microglial activity and abnormal hippocampal development, as observed in Alzheimer's Disease, to VPS35 depletion, but no polymorphisms in the VPS35 gene have been associated(Appel et al., 2018; C.-L. Wang et al., 2012).

VPS35 as Part of the Retromer Complex and Related Functions

VPS35 was originally identified in yeast as part of a protein complex called the retromer complex. This complex is involved in cellular trafficking (M. N. Seaman, McCaffery, & Emr, 1998). Especially, its role in endosome to Golgi retrograde transport has been very well characterized (Follett et al., 2014b; Matthew N J Seaman, 2012; Trousdale & Kim, 2015). Since its identification, retromer dysfunction has been implicated in Alzheimer's disease, Parkinson's disease and several other neurodegenerative diseases, along with various developmental processes, including wing and eye development in *Drosophila*, and bone development (Belenkaya et al., 2008; Chan et al., 2016; P. Zhang, Wu, Belenkaya, & Lin, 2011). In this context, the interaction of the retromer complex and the Wnt signaling pathway has been extensively studied (Belenkaya et al., 2008; Chan et al., 2016; Small & Petsko, 2015; S. Wang & Bellen, 2015; P. Zhang et al., 2011; Q. Zhang et al., 2018). Structurally, VPS35 forms a trimer with VPS26 and VPS29, to form what is known as the cargo recognition complex (CRC).

This CRC then associates with a dimer of sorting nexins, belonging to the SNX-BAR family of proteins. This association is further facilitated by RAB7. Together, the retromer complex plays important roles in vesicular sorting (Bonifacino & Hurley, 2008; Lucas et al., 2016; McGough & Cullen, 2011; M. N. J. Seaman, Harbour, Tattersall, Read, & Bright, 2009; Matthew N J Seaman, 2012). Deficiency of either VPS35 or VPS29 leads to the degradation of the other two CRC components (Fuse et al., 2015). Transmembrane receptors such as the cation independent mannose 6 phosphate receptor (CIMPR) and the membrane iron transporter DMT1 are well characterized targets of retromer mediated sorting (Arighi, Hartnell, Aguilar, Haft, & Bonifacino, 2004; Lucas et al., 2016; Tabuchi, Yanatori, Kawai, & Kishi, 2010). In addition, there have been several studies on the role of the retromer in recycling of beta-2 adrenergic receptors (β2AR) in dendritic cells and trafficking of G protein coupled receptors (GPCRs) (Bahouth & Nooh, 2017; Bowman, Shiwarski, & Puthenveedu, 2016; Bunnett & Cottrell, 2010; Choy et al., 2014; Feinstein et al., 2011; McGarvey et al., 2016; Pavlos & Friedman, 2017; Sposini et al., 2017). The retromer is known to carry cargo from endosomes to the trans-Golgi network (TGN), performing retrograde transport, as well as carrying cargo from endosomes to the plasma membrane, allowing recycling of membrane bound receptors (Trousdale & Kim, 2015). Moreover, included in the list of retromer cargo, are degradative enzymes such as Cathepsin D, which are important for lysosomal function and could potentially influence autophagy. Furthermore, amyloid precursor protein (APP) and α-synuclein (SNCA) are also reported to be sorted by the retromer complex (Aufschnaiter, Kohler, & Büttner, 2017; Follett et al., 2014b; Follett, Bugarcic, Collins, & Teasdale, 2017; Gallon & Cullen,

2015; C. Li, Shah, Zhao, & Yang, 2016; Miura et al., 2014; Reitz, 2018). These findings have sparked considerable interest in the contributions of the retromer to human diseases.

Proposed Mechanisms of VPS35 in Neuronal Dysfunction

The importance of the roles played by the retromer complex in signal transduction and receptor trafficking points to the dysfunction of the retromer as the major potential mechanism of neuronal degeneration caused by the VPS35 D620N mutation (Follett et al., 2014b, 2017; Reitz, 2018; Small & Petsko, 2015; S. Wang & Bellen, 2015). Out of the three components of the core retromer complex, VPS35-VPS26-VPS29, knockdown of any one component, except VPS26, leads to the degradation of the other two components (Fuse et al., 2015). Therefore, given that any free VPS35, existing outside the retromer complex, is rapidly degraded, the role of VPS35 D620N in PD is likely to involve altered retromer function. Trafficking defects of AMPA receptors was reported in a VPS35 heterozygote mouse model as well as cultured mouse hippocampal and cortical dopaminergic neurons treated with VPS35 shRNA (Munsie et al., 2015; Tian et al., 2015). Additionally, dopamine receptor D1 (DRD1) is also reported to be recycled with the involvement of VPS35 (C. Wang et al., 2016).

Studies have reported reduced α-synuclein degradation in VPS35 deficient cells (Braschi et al., 2010; Miura et al., 2014; Sugiura, McLelland, Fon, & McBride, 2014; F.-L. Tang et al., 2015). Formation of toxic α-synuclein aggregates and fibril formation are hallmarks of PD (Patel & Witt, 2018). This accumulation has been attributed to abnormal sorting of degradative enzymes that are normally targeted to the lysosome. However, this accumulation could also be partially due to autophagy defects, which have also been reported in HeLa cells expressing the VPS35 D620N mutation, attributed to impaired

WASH (Wiskott–Aldrich syndrome protein and SCAR homolog) complex association (McGough et al., 2014; Zavodszky et al., 2014). Given that autophagy is a major process responsible for removing proteins, macromolecules and organelles, this is another potential mechanism for the neurodegeneration caused by VPS35 (Barth, Glick, & Macleod, 2010; Lynch-Day, Mao, Wang, Zhao, & Klionsky, 2012; Tofaris, 2012)

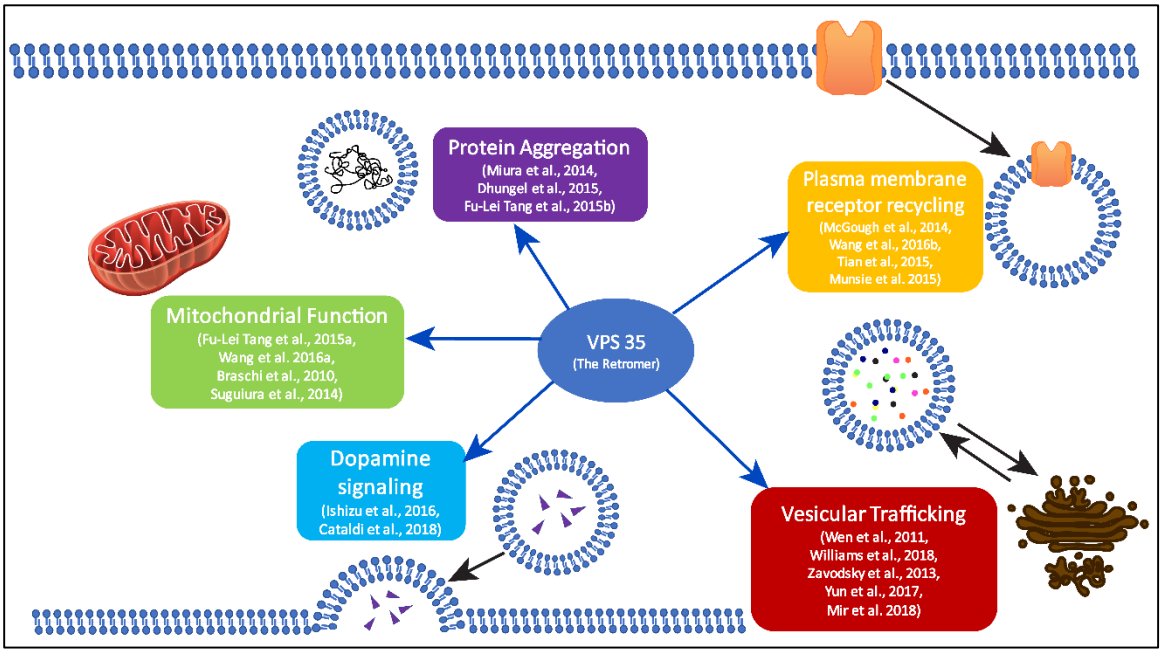

Figure 1.1 Cellular processes affected by VPS35 mutations

Furthermore, defects in mitochondrial fusion and function have also been reported in some studies (Fu-Lei Tang et al., 2015; W. Wang et al., 2016). Mitochondrial dysfunction has been shown to affect neuronal function severely and is believed to be a driving force of neurodegeneration (Haelterman et al., 2014; Hauser & Hastings, 2013; Mullin & Schapira, 2013; Subramaniam & Chesselet, 2013; Winklhofer, 2014). Also, it is noteworthy that autophagy is the means by which damaged mitochondria are turned over (Ashrafi & Schwarz, 2013). Therefore, disruption in autophagy due to VPS35

D620N mutation may also result in, if not exacerbate, any mitochondrial dysfunction that may be directed by the VPS35 D620N mutation.

Current Model Organisms and Systems for Studying VPS35

Cell Culture

A variety of tissue culture model systems are available for studying the structure and function of VPS35, and the pathogenic effects of its mutations. SH-SY5Y neuroblastoma cells can easily be induced to adopt a dopaminergic state by treatment with retinoic acid (Korecka et al., 2013; Krishna et al., 2014; Shipley, Mangold, & Szpara, 2016). This system was used by Tang et al (2015) to demonstrate mitochondrial impairment due to VPS35 deficiency as well as due to the D620N mutation (Fu-Lei Tang et al., 2015). This system was also used to characterize the role of VPS35 in lysosomal clearance of AIMP2, which is a substrate of the PD associated gene, Parkin (Yun et al., 2017). Microglial BV2 cells have been used in some studies to understand the role of VPS35 in Alzheimer's Disease, and microglial physiology (Lucin et al., 2013; Yin et al., 2016). In addition, HEK 293T cells also present a popular choice for understanding cellular pathways affected by VPS35 and retromer function due to their high plasmid transfection efficiency (Follett et al., 2014a; Williams et al., 2018; Yang et al., 2008). McGough et al (2014) used HeLa and RPE-1 cell lines to establish the interaction of VPS35 and FAM21 of the WASH complex (McGough et al., 2014). Cell lines can readily be transfected or virally transduced with an siRNA, shRNA or overexpression vectors to modulate the expression of VPS35 and other genes of interest (Nayerossadat, Maedeh, & Ali, 2012). Korolchuk et al. (2007) knocked down VPS35 in Drosophila S2 cells using an RNAi approach, in order to identify novel proteins important for

endocytosis, where candidate proteins were selected based on binding predictions to either α adaptin or clathrin heavy chain, or on having a predicted membrane bending domain (such as the BAR domain). These candidate proteins were then knocked down to screen for subsequent endocytosis defects. VPS35 was among the short list of proteins that were identified as exhibiting a significant reduction in endocytosis, where the effect due to VPS35 was the most severe (Korolchuk et al., 2007).

In addition to immortalized cell lines, primary neuronal and glial cultures have also been used to investigate VPS35 functions and mutations (F.-L. Tang et al., 2015; Tsika et al., 2014; Wen et al., 2011; Williams et al., 2018). Tang et al (2015) isolated and cultured dopaminergic neurons from brains of mice expressing microRNA directed against VPS35 (F.-L. Tang et al., 2015). These cells were found to have a reduced expression of MFN-2 or Mitofusin, and exhibited mitochondrial fragmentation, as well as impaired mitochondrial function. Similar results were obtained using SH-SY5Y and NLT neuroblastoma cultures (Fu-Lei Tang et al., 2015). In the study conducted by Tsika et al (2014), rat primary cortical cultures were used to show that overexpression of human VPS35 resulted in neuronal cell death and higher sensitivity to cellular stress like as reported in PD cases (Tsika et al., 2014). Wen et al (2011) demonstrated using mouse hippocampal slice cultures that VPS35 haploinsufficiency exacerbates long term potentiation impairment already present in mice expressing Swedish mutant form of the amyloid precursor protein (Wen et al., 2011). Williams et al (2018) discovered a novel link between the PD gene, Parkin, and VPS35, wherein Parkin mediates ubiquitination of VPS35, which does not affect VPS35 turnover, but may have a role to play in the retromer mediated endosomal sorting (Williams et al., 2018). Using primary cortical

neurons, they identified ATG9A, a WASH-dependent retromer cargo, to be missorted upon silencing of the Parkin gene. Using cell lines and primary cultures can therefore be very powerful tools for deciphering molecular mechanisms under precisely controlled conditions.

Mice

Homozygous knockout of VPS35 is embryonically lethal in mice. To overcome this challenge several mice models have been developed, including a hemizygous deletion mutant (Wen et al., 2011). This mutant was used to demonstrate that VPS35 interacts with the protease BACE-1, predominantly responsible for Amyloid beta production, and that VPS35 reduction increases BACE-1 activity in the mouse hippocampus. In addition, A VPS35 D620N knock-in strain of mice has been developed recently by Cataldi et al (2018), in collaboration with Jackson Laboratories, to further characterize dopamine release and monoamine transporters in a VPS35 D620N mutant background (Cataldi et al., 2018). These mice were generated by Cre-recombinase mediated recombination of the VPS35 exon 15, on chromosome 8. These mice exhibited no detectable movement disorders and were similar in locomotion to non-transgenic mice. However, the dopamine turnover was reported to be increased. Dopamine transporter levels were reported to have been reduced while vesicular monoamine transporter levels were increased (Cataldi et al., 2018). Mir and colleagues (2018) used these same mice to study the interaction between LRRK2 and VPS35 (Mir et al., 2018). Using this model, they were able to demonstrate that the VPS35 D620N mutation increases LRRK2 catalyzed phosphorylation of three different RAB proteins, RAB 8a, 10 and 12. They concluded that the VPS35 D620N mutation causes a gain of function with

respect to the kinase activity of LRRK2 and is an upstream regulator (Mir et al., 2018). A similar mouse model, generated using a CRISPR/Cas-9 mediated knock-in strategy, was used previously by Ishizu et al. (2016) to characterize reduced dopamine release in the striatum of heterozygous mice with one gene copy of VPS35 D620N knocked in ($VPS35^{D620N\ KI}$), while the other copy had one base pair deleted in exon 15, creating a premature stop codon, referred to as $VPS35^{Del1}$ (Ishizu et al., 2016). These $VPS35^{D620NKI}$/$VPS35^{Del1}$ heterozygous mice also displayed premature death and significant neurodegeneration throughout life, indicating that this model could be of considerable value for Parkinson's Disease research.

Rat

A transgenic rat model generated via lentivirus mediated gene transfer has been described and utilized by Tsika et al., (2014). These rats overexpressed one of two human VPS35 constructs, one of wild type and the other containing the D620N mutation. However, the researchers concluded that the human D620N construct did not alter vesicular sorting of retromer cargo proteins in primary cortical neurons from these animals. They went on to confirm this finding in yeast and human (patient donated) fibroblasts. Contrary to this finding, the human VPS35 D620N overexpression was found to induce neurodegeneration in the substantia nigra of these rats suggesting that, in this model, VPS35 D620N might be primarily causing neuronal loss without altering retromer function (Tsika et al., 2014).

Drosophila Melanogaster

MacLeod et al (2013) used an overexpression vector to demonstrate that overexpression of wild type VPS35 reduced the defects caused by LRRK2 G2019S

mutation, and those caused by RAB7L1 knockdown. This was shown in LRRK2 mutant flies and primary rat neuronal cultures (MacLeod et al., 2013). Similarly, a VPS35 overexpressing strain of flies was used by Linhart et al in 2014, to demonstrate the rescue of LRRK mutation derived eye defects, by eye specific VPS35 overexpression in Drosophila (Linhart et al., 2014), obtained from the Bloomington Drosophila Stock Center. In another study, flies with the Drosophila VPS35 (dVps35) knocked out, were shown to be embryonically lethal (Inoshita et al., 2017). This lethality could not be rescued by knock-in of human VPS35 wild type or human VPS35 D620N. However, it was rescued by knock-in of dVps35 WT and by knocking in dVps35 D647N (the Drosophila analog of the D620N mutation in humans). Using transgenic flies expressing wild type dVps35 or dVps35 D647N, in all three possible dVps35 heterozygote and homozygote backgrounds (+/+, +/-, -/-), they were then able to demonstrate that Drosophila LRRK (dLRRK) and dVps35 affect synaptic architecture and endocytosis via the same pathways (Inoshita et al., 2017).

Saccharomyces Cerevisiae and Caenorhabditis Elegans

Dhungel et al (2015) discovered that in VPS35 deficient yeast cells, EIF4G1 upregulation was highly toxic, but the two mutations by themselves were not lethal (Dhungel et al., 2015). EIF4G1 is a translation initiation factor scaffold protein, responsible for the translation initiation complex formation (Villa, Do, Hershey, & Fraser, 2013). While they could not establish a direct interaction between the two proteins, a few common pathways were identified leading to the hypothesis that they were functionally related. The authors also noted that in a VPS35 null background, overexpression of EIF4G1 resulted in fewer but larger synaptic boutons. This group then

investigated proteotoxic stress in yeast cells and activation of the unfolded protein response (UPR). It was seen that there was increased proteotoxic stress and UPR activation in VPS35 deletion mutants, only upon overexpressing EIF4G1. This led to the conclusion that the EIF4G1 upregulation combined with retromer dysfunction was causing toxic protein aggregation. In addition, they demonstrated that Sortillin functions downstream of VPS35 or in parallel and is able to suppress the EIF4G1 overexpression toxicity in VPS35 deletion mutants. Lastly, this group showed, using yeast, *C. elegans* and transgenic mice that VPS35 deletion increases alpha synuclein driven toxicity. In transgenic mice, increased toxicity was also seen after overexpression of VPS35 D620N and VPS35 P316S (Dhungel et al., 2015). Therefore, altered VPS35 function can facilitate protein aggregation, a hallmark of PD.

Future Research Directions

With the advent of CRISPR/Cas-9 mediated genome editing technology, therapeutic applications targeting the VPS35 gene can now be conceived (Calatayud, Carola, Consiglio, & Raya, 2017). This could potentially be done in inducible pluripotent stem cells, obtained from patients, genetically modified and transplanted into the affected individuals' brains. This technique has recently been described and is awaiting U.S Food and Drug Administration approval (Loring, 2018). A major obstacle in such an approach is the prion like properties of α-synuclein aggregates (Brundin & Melki, 2017). The pre-existing toxic α-synuclein aggregates in the patient's existing brain environment may induce the formation of α-synuclein fibrils in the transplanted cells, thus negating any corrections made in the genome. Similar outcome was observed in patients who received

embryonically derived grafting (Kordower, Chu, Hauser, Freeman, & Olanow, 2008; J.-Y. Li et al., 2008).

In addition, further characterization of the VPS35 D620N knock-in mice is now required to properly investigate the cellular pathways that are perturbed. With this in vivo model system, work done with in vitro settings must now be validated. Furthermore, a cell line containing the D620N mutation in the endogenous VPS35 gene would add validity to findings already reported using cells that have a VPS35 D620N construct stably overexpressed. Finally, characterization of the pathways perturbed by mutations in VPS35, is far from complete. A better understanding of the interplay of VPS35 and these pathways is required, so that therapeutic interventions can be crafted.

CHAPTER TWO: ENHANCED HYALURONAN SIGNALING AND AUTOPHAGY DYSFUNCTION BY VPS35 D620N

The motor features of Parkinson's disease result from loss of dopaminergic (DA) neurons in the substantia nigra with autophagy dysfunction being closely linked to this disease. A PD-causing mutation in VPS35 (D620N) has been reported to block autophagy. In order to identify signaling pathways responsible for this autophagy defect, we performed an unbiased screen using RNA sequencing (RNA-Seq) of wild-type or VPS35 D620N-expressing retinoic acid-differentiated SH-SY5Y cells. We report that VPS35 D620N-expressing cells exhibit transcriptome changes indicative of alterations in extracellular matrix (ECM)-receptor interaction as well as PI3K-AKT signaling, a pathway known to regulate autophagy. Hyaluronan (HA) is a major component of brain ECM and signals via the ECM receptors CD44, a top RNA-Seq hit, and RHAMM to the autophagy-regulating PI3K-AKT pathway. We find that high (>950 kDa), but not low (15 - 40 kDa), molecular weight HA itself can inhibit autophagy. In addition, VPS35 D620N facilitated enhanced HA-AKT signaling. Transcriptomic assessment and validation of protein levels identified the differential expression of CD44 and RHAMM isoforms in VPS35 D620N mutant cells. Based upon this expression profile, these data suggest that the RHAMM isoform V3 may contribute to the enhanced activation of HA-AKT signaling and autophagy inhibition by VPS35 D620N.

Introduction

PD is a Progressive Neurodegenerative Disease

In the USA, with a prevalence of 1% in people over 60 and over 4% by age 85, Parkinson's disease is the most common disease of motor system degeneration (Lau & Breteler, 2006). This disease is clinically characterized by a resting tremor, rigidity, postural instability, and bradykinesia. PD's primary motor features result from a loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SN) region of the midbrain. Specifically, the loss of dopamine produced by this neuronal subset causes dysfunction of the basal ganglia, a cluster of nuclei responsible for coordinating movement. No therapies to date deal with the underlying neuronal loss and thus only symptomatic treatments exist for PD (Jankovic & Stacy, 2007).

Autophagy Misregulation in PD

Macroautophagy (hereafter referred to as autophagy) is an orderly process of cytoplasmic engulfment by lipid membrane-bound vesicles termed autophagosomes leading to degradation of the encapsulated material (La Spada & Taylor, 2010). Autophagy proceeds in distinct steps that include induction, cargo recognition, autophagosome formation and transport, autophagosome-lysosome fusion, degradation of cargo, and the release of degraded material into the cytoplasm. Following the induction of autophagy, Atg8 (LC3) undergoes cleavage, and a Phosphatidylethanolamine(PE) moiety is conjugated (now LC3-II). LC3-II is unique to autophagosomes and is commonly used as a marker for autophagy (He & Klionsky, 2009). Autophagosomes fuse with endosomes and multivesicular bodies that in turn fuse with lysosomes containing lytic enzymes that facilitate the degradation of cargo.

Autophagy is primarily devoted to adapting to nutrient limitation, degrading misfolded proteins, and removing dysfunctional organelles (A. M. K. Choi, Ryter, & Levine, 2013). Studies of neurodegenerative disease and protein turnover pathways have indicated that the maintenance of protein quality control presents a particular challenge for neurons and other specialized cells of the nervous system (Malgaroli, Vallar, & Zimarino, 2006; J. P. Taylor, Hardy, & Fischbeck, 2002). This appears to be of particular importance to PD pathology as protein aggregates of α-synuclein are believed to be removed by autophagy, at least before fibril formation, and whose presence may signal autophagy dysfunction (Riedel, Goldbaum, Schwarz, Schmitt, & Richter-Landsberg, 2010; Tanik, Schultheiss, Volpicelli-Daley, Brunden, & Lee, 2013). Moreover, inflammation and other proteotoxic insults can facilitate α-synuclein aggregation, perhaps by overloading the autophagic machinery with damaged proteins and organelles or by inhibiting autophagy directly. A link between PD and autophagy can also be traced genetically. For example, at least seven monogenic causes of PD strongly connected with autophagy regulation exist, and some are directly involved in lysosomal biology (Dehay et al., 2010, 2013). As a result, considerable effort has been made to understand autophagy dysfunction in PD, with the goal of targeting this process for therapy.

The PD-Causing VPS35 D620N Mutation Affects Retromer Function and Blocks Autophagy

In 2011, two independent groups reported the mutation of VPS35 (D620N) as being causal for a familial form of PD (Vilariño-Güell et al., 2011; Zimprich et al., 2011). VPS35 has a well-established and critical role in retromer function. The retromer is a highly conserved complex of proteins and an essential element of the endosomal sorting

machinery that directs the recycling of plasma membrane receptors. The retromer is comprised of a cargo recognition trimer containing VPS35-VPS29-VPS26 and a dimer made of an assortment of SNX1, SNX2, SNX5, and SNX6 (McGough & Cullen, 2011). This complex associates with endosomes and facilitates the retrograde transport of transmembrane cargo to the trans-Golgi network, thereby rescuing cargo from degradation by the lysosome. However, the mechanism for VPS35 D620N-mediated neurodegeneration remains unknown.

Interest into the role of VPS35 in mediating autophagy was initially hinted at in work published by Dengjel et al where VPS35 was identified in an autophagosome-interactor screen (Dengjel et al., 2012). Subsequently, Zavodszky et al., presented direct evidence of this by reporting that the PD-causing VPS35 D620N mutation impairs autophagy in HeLa cells through defective WASH complex recruitment to endosomes (Zavodszky et al., 2014). However, the study by Zavodszky et al. focused on members of the WASH complex—namely, canonical interactors of VPS35 (Gomez & Billadeau, 2009; Hao et al., 2013). Interestingly, we did not detect an association with the principal WASH complex member WASH1 in retinoic acid (RA)-differentiated SH-SY5Y cells, a widely used cellular model of PD (Korecka et al., 2013; Krishna et al., 2014), suggesting the existence of distinct mechanism **(Fig. S1)**. Therefore, we performed an unbiased transcriptome screen of RA-differentiated SH-SY5Y cells that we engineered to stably express VPS35 or VPS35 D620N using RNA sequencing. Our analysis has uncovered altered signaling in PI3K-AKT and extracellular matrix (ECM)-receptor interaction pathways.

Autophagy Regulation by Phosphatidylinositol-AKT Signaling

Autophagy is regulated by two distinct kinase complexes: an autophagy-specific phosphatidylinositol 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) (Janku, McConkey, Hong, & Kurzrock, 2011). Members of the PI3K family phosphorylate phosphatidylinositol (PI) generating phosphatidylinositol 3-phosphate (PI3P) which serves as a docking lipid that can facilitate the complex formation and activation of proteins that include AKT. AKT then propagates this signal through a series of kinase events that result in suppression of autophagy. Our transcriptome screen revealed this pathway as a top hit affected by VPS35 D620N, suggesting it could be a major effector for autophagy blockade.

HA-AKT Signaling

The major structural component of brain extracellular matrix (ECM) is hyaluronan (HA), which not only serves as the main anchoring point for brain cells but is also an important facilitator of intracellular signaling. CD44 and RHAMM are primary receptors for HA that signal to AKT upon binding (Lin, Chang, & Ying, 2007; Onodera, Teramura, Takehara, & Fukuda, 2015; Trapasso & Allegra, 2012, p. 44). Our RNA-Seq data indicates that VPS35 D620N causes altered extracellular matrix receptor interaction and suggests dysregulated PI3K-AKT signaling. Interestingly, while studies have been reported showing HA, CD44 and RHAMM activation of the autophagy modulators AKT and mTOR there are no reports directly addressing HA regulation of autophagy. We then hypothesized that perturbed HA-AKT signaling may contribute to VPS35 D620N autophagy dysfunction.

Results

VPS35 D620N Inhibits Autophagy

To determine whether VPS35 D620N represses autophagy in a PD model system, we first engineered a human neuroblastoma cell line (SH-SY5Y) to stably express WT or D620N VPS35 (V5–tagged). This was done by obtaining a VPS35-expressing lentivirus construct from Lynda Chin (Scott et al., 2009) and generating the D620N mutation by site-directed mutagenesis. We next infected low passage SH-SY5Y cells (ATCC) and selected transgene-positive cells using blasticidin. Before conducting experiments, we differentiated SH-SY5Y cultures into post mitotic dopaminergic neuron-like cells using 10uM retinoic acid for 7 days. Western blot revealed that WT and D620N transgenic proteins are expressed equally and at a similar level to endogenous VPS35 **(Fig. 2.1A)**. In stable cell lines, no endogenous VPS35—only transgenic (shifted) VPS35—was detected, which is likely due to transgenic VPS35 titrating out endogenous VPS35 from the retromer complex. The knockdown of VPS35 results in the knockdown of the other core retromer component members (VPS29) and vice versa, suggesting that free VPS35 as well as VPS29 are rapidly degraded by the cell **(Fig. 2.2C)** (Fuse et al., 2015). Therefore, serendipitously, these cell lines express the transgene at endogenous protein levels, thereby avoiding overexpression artifacts. Upon examination of autophagy in these cells, we found autophagy to be robustly inhibited by D620N, as evidenced by the reduced LC3-II induction following Bafilomycin A1 treatment **(Figs. 2.1B and 2.1C)**. We further tested autophagy dysfunction by VPS35 D620N through assessing the clearance of intracellular alpha-synuclein aggregates **(Fig. S2)** (Outeiro et al., 2008). We report that mutant-expressing cells accumulated significantly higher levels of alpha-

synuclein aggregates which supports the notion that VPS35 D620N is a potent inhibitor of autophagy.

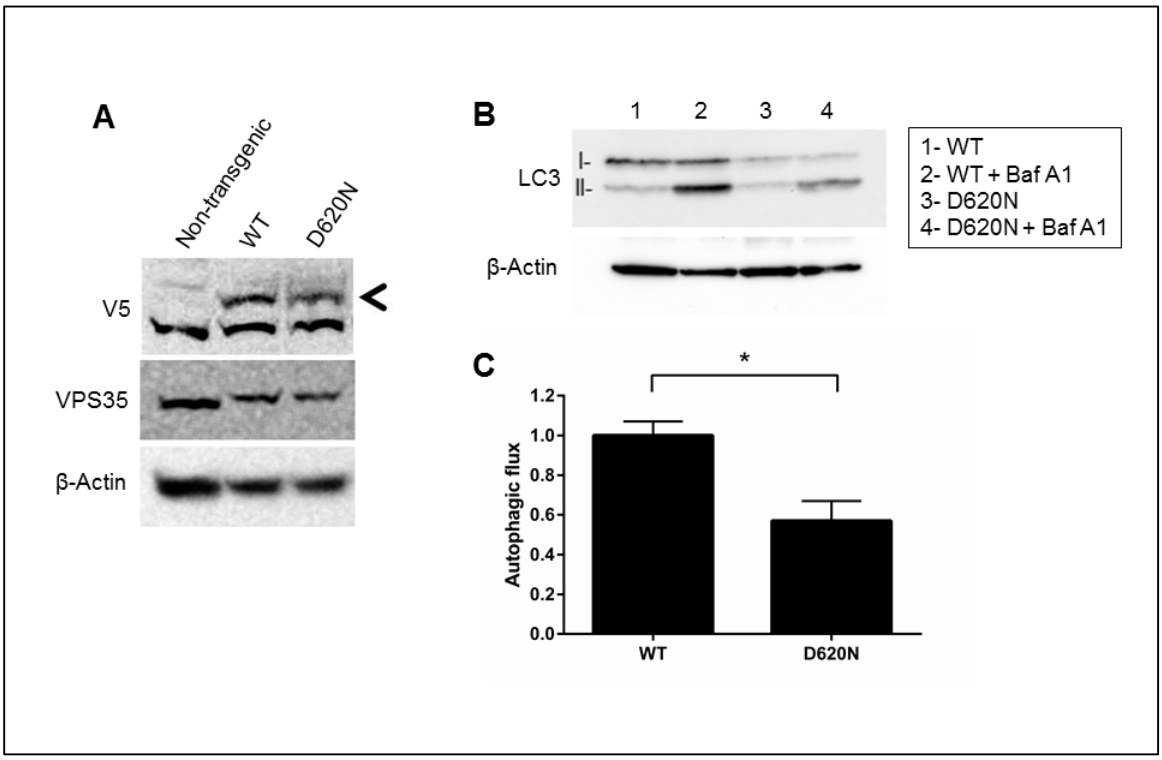

Figure 2.1 VPS35 D620N inhibits autophagy.

Transgenic and endogenous VPS35 were evaluated using western blot (A). Differentiated SH-SY5Y cells stably expressing transgenic VPS35 (WT or D620N) were treated with Baf A1 to block autophagosome/lysosome fusion. Western blot of LC3-II was performed (B) and autophagic flux quantified (C). Results from three independent experiments are displayed (*P<0.05 compared to indicated group, student's t-test; error bars = SEM).

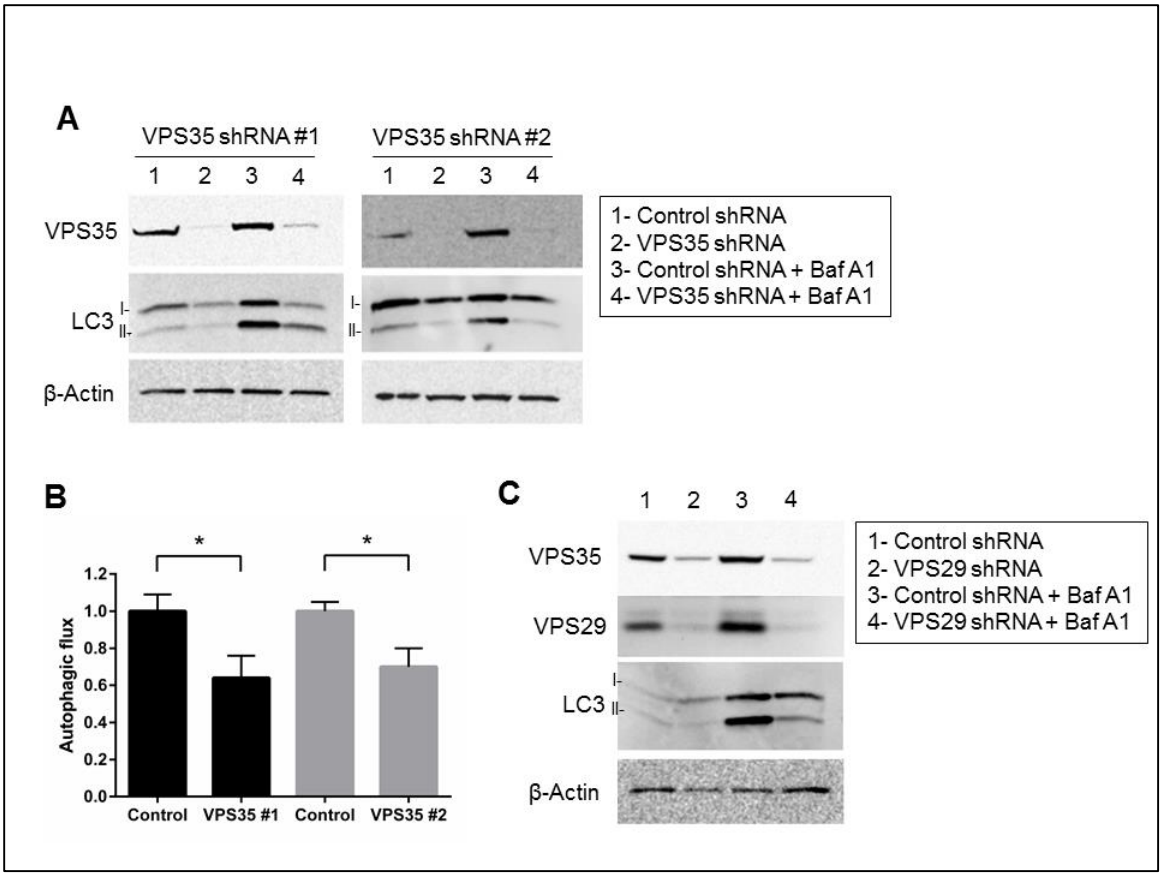

Figure 2.2 VPS35 knockdown inhibits autophagy.

VPS35 expression was knocked down in differentiated SH-SY5Y cells neurons by two distinct lentivirus-delivered shRNA vectors. Following selection with puromycin, cells were treated with Baf A1 to block autophagosome/lysosome fusion. Western blot of LC3-II was performed (A) and autophagic flux quantified (B). Results from three independent experiments are shown (*P<0.05 versus indicated group, student's t-test; Error bars = SEM). Knockdown of VPS29 by shRNA using a lentiviral vector was also performed (C).

VPS35 Knockdown Inhibits Autophagy, Phenocopying the VPS35 D620N Mutation.

To determine if the D620N mutation elicited autophagy blockade through a loss of function in differentiated SH-SY5Y cells, we knocked down VPS35 expression using two distinct shRNA constructs delivered by lentivirus and assessed autophagy **(Fig. 2.2A and B)**. We found that VPS35 knockdown resulted in repression of autophagy consistent with VPS35 D620N being a loss-of-function mutation. As reported by other groups, we

found that knockdown of other core retromer components such as VPS29 by shRNA resulted in concomitant knockdown of VPS35 **(Fig. 2.2C)** (Fuse et al., 2015). Therefore, it was not surprising to observe that VPS29 knockdown also suppressed autophagy. Collectively, these data suggested that VPS35 D620N is a loss-of-function mutation, which ultimately suppresses autophagic activity.

VPS35 D620N Misregulates ECM-Receptor Interaction and Pi3k-AKT Pathway Gene Expression

Having established autophagy dysfunction by VPS35 D620N, likely through a loss-of-function, we next sought to determine a precise molecular mechanism for this phenomenon. To this end, we performed RNA sequencing of the transcriptomes of transgenic WT and D620N VPS35-expressing SH-SY5Y cells. Gene ontology was performed (1.5 fold change cutoff) using iPathways (Advaita) and KEGG annotated pathways were assigned to gene expression changes (Luo, Friedman, Shedden, Hankenson, & Woolf, 2009; Mootha et al., 2003; Subramanian et al., 2005). Top pathway hits were ECM-receptor interaction and PI3K-AKT signaling which suggests that ECM signaling is perturbed by VPS35 D620N **(Table 1)**. Examination of specific transcripts altered for ECM-receptor interaction and PI3K-AKT KEGG annotated pathways reveals that CD44 mRNA expression is misregulated in VPS35 D620N cells **(Fig. 2.3)**.

Table 2.1. ECM-receptor interaction and AKT pathway perturbation by VPS35 D620N.

RNA-Seq was performed on WT and VPS35 D620N-expressing SH-SY5Y cells. The top 5 pathways affected by VPS35 D620N expression and relevant significance values are listed.

Pathway name	**Pathway ID**	**p-value**	**p-value (FDR)**	**p-value (Bonferroni)**
Pathways in cancer	05200	9.878e-8	2.683e-5	2.914e-5
Systemic lupus erythematosus	05322	1.819e-7	2.683e-5	5.367e-5
ECM-receptor interaction	04512	2.294e-6	2.256e-4	6.768e-4
PI3K-AKT signaling pathway	04151	3.124e-6	2.304e-4	9.215e-4
Focal adhesion	04510	9.627e-6	5.680e-4	0.003

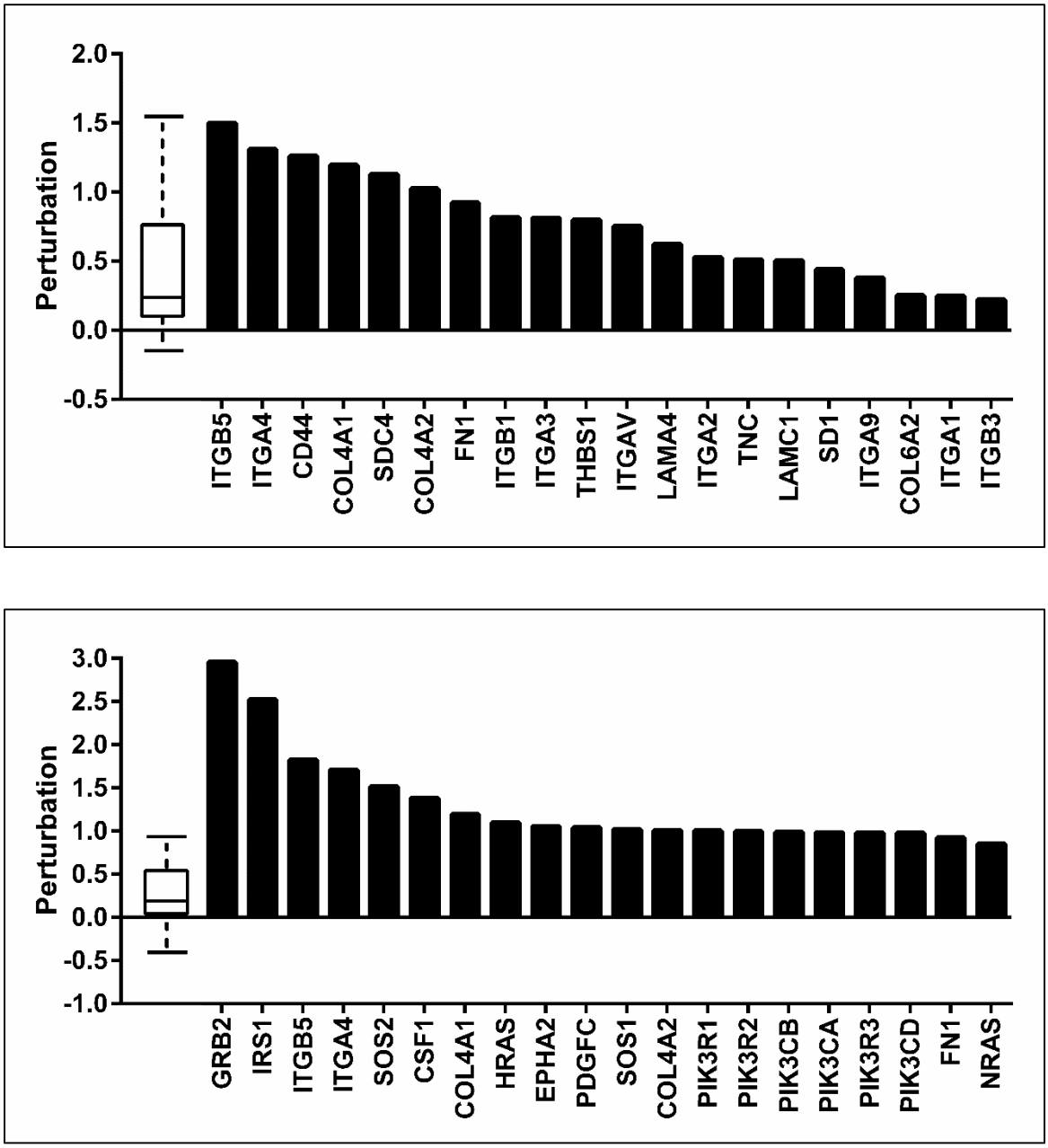

Figure 2.3. Transcriptome alteration of ECM-receptor and PI3K-AKT pathways by VPS35 D620N.

Specific transcript expression changes (Log-Fold-Change) for ECM-receptor interaction (Top) and PI3K-AKT signaling (Bottom) KEGG pathways from RNA-Seq data are shown. The distribution of all gene perturbations in the pathway are represented in the box plot where the first, median and third quartile are indicated.

Hyaluronan Inhibits Autophagy in Differentiated SH-SY5Y Cells

Our findings from RNA-Seq data indicate that HA-AKT signaling is perturbed in the VPS35 mutant cell line. These results pose the novel question of whether the HA-AKT signaling axis controls autophagic response. To test this directly, we added HA at concentrations reported to elicit biological effects in human monocytes and fibroblasts to differentiated SH-SY5Y cells (Maharjan, Pilling, & Gomer, 2011). We chose to treat cells with either high (HMW) or low molecular weight (LMW) HA since the polymer size can direct divergent bioactivities (Maharjan et al., 2011). We observed a dose-dependent repression of autophagy following treatment with HMW but not LMW HA **(Fig. 2.4)**. This is the first report of HA modulating autophagy and may indicate that the composition of ECM is an important factor in the regulation of this key process.

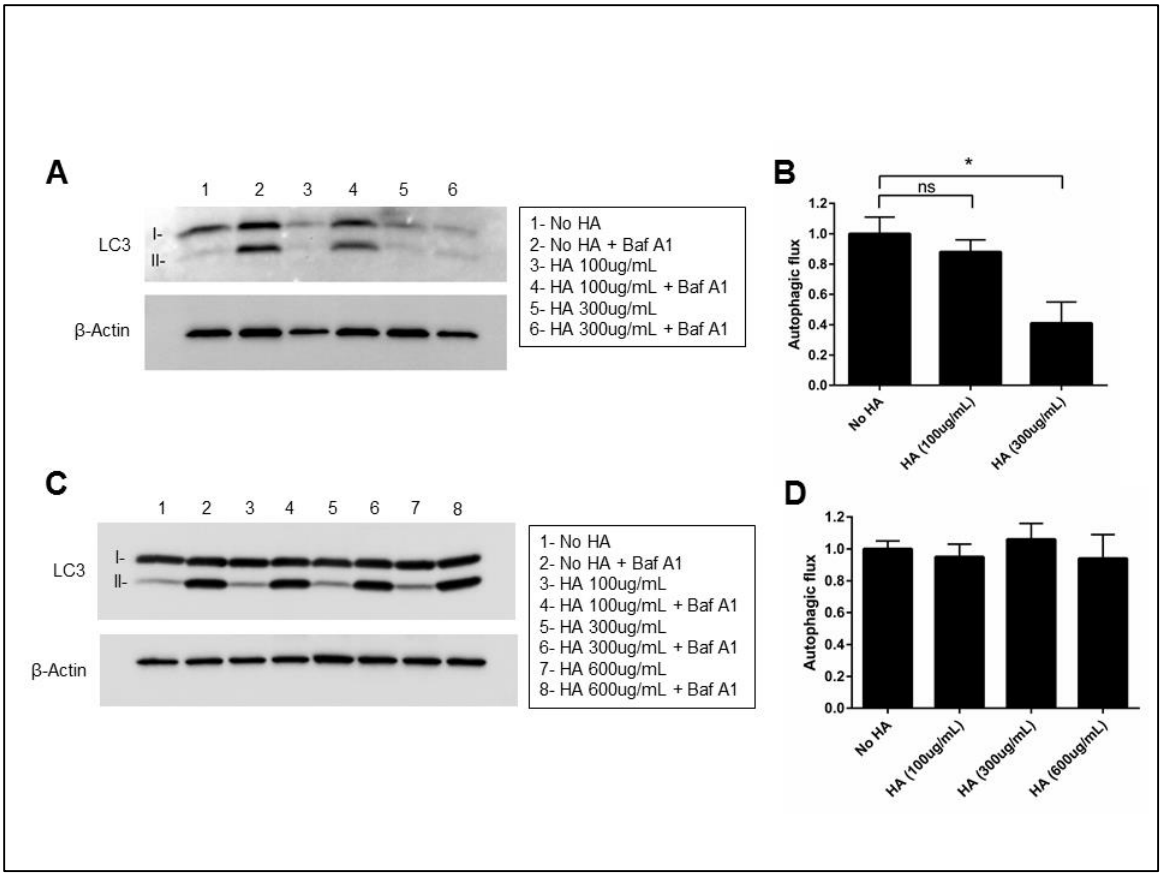

Figure 2.4. HA inhibits autophagy.

RA-differentiated SH-SY5Y cells were treated with high molecular weight (>950 kDa) HA in serum-free media for 24 hours. The next day, cells were treated with or without Baf A1 and autophagic flux assessed by LC3-II/β-Actin western blot **(A)** and quantified from three experiments **(B)**. Similarly, low molecular weight (15 - 40 kDa) HA treatment was performed and autophagic response evaluated by LC3-II/β-Actin western blot **(C)** and represented graphically **(D)** (*P<0.05 versus indicated group, student's t-test; Error bars = SEM).

Altered Hyaluronan-AKT Signaling by VPS35 D620N

We next examined whether VPS35 D620N-expressing cells exhibited heightened sensitivity to HA. Addition of HMW HA resulted in elevated AKT activation in mutant cells **(Fig. 2.5A)**. Conversely, low molecular weight HA failed to induce AKT activation in either WT or mutant cells **(Fig. 2.5B)**. This suggests that VPS35 D620N facilitates enhanced HMW HA signaling. These results are also consistent with our findings that HMW and not LMW HA repress autophagy via canonical HA-AKT signaling.

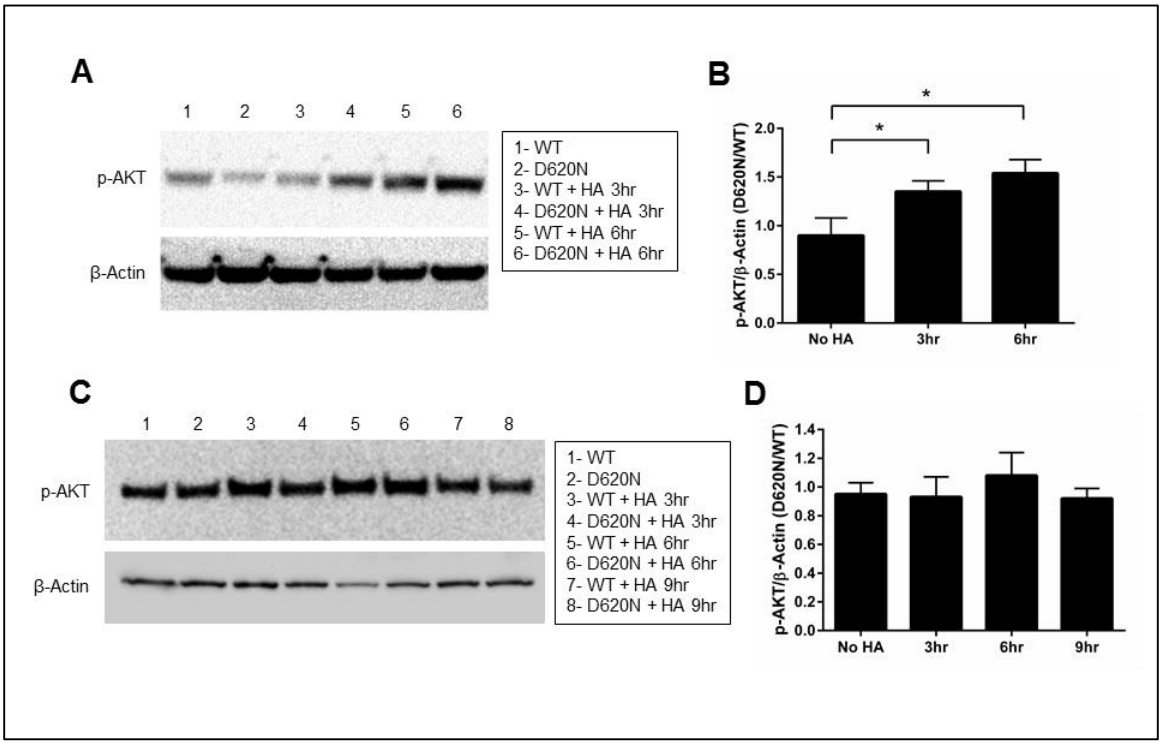

Figure 2.5. Elevated HA signaling by VPS35 D620N.

Differentiated SH-SY5Y cells expressing wild-type (WT) or D620N VPS35 were serum-deprived overnight. The following day, high molecular weight (>950 kDa) HA (300 ug/mL) (A) or low molecular weight (15 - 40 kDa) HA (300 ug/mL) (C) was added for the indicated times and western blotting was then performed (p-AKT = Ser473). Results are presented as a ratio of D620N/WT for p-AKT/β-Actin by western blot band densitometry for each time point shown (B, D) (*P<0.05 versus indicated group, student's t-test; Error bars = SEM).

AKT Pathway Activation by VPS35 D620N is Independent of Hyaluronan Production

To determine whether HA production contributes to heightened AKT signaling in VPS35-D620N cells, we measured HA produced during a 48h period **(Fig. 2.6)**, but found no detectable difference in wild-type and mutant cells. We also assessed the protein levels for the hyaluronan synthase (HAS) enzymes responsible for HA synthesis. HAS3 was not differentially expressed while we were unable to detect HAS1 and HAS2 in SH-SY5Y cells **(Fig. 2.7A)**. Taken together, these findings suggest that enhanced HA production is not responsible for the observed change in AKT signaling for VPS35 D620N-expressing cells.

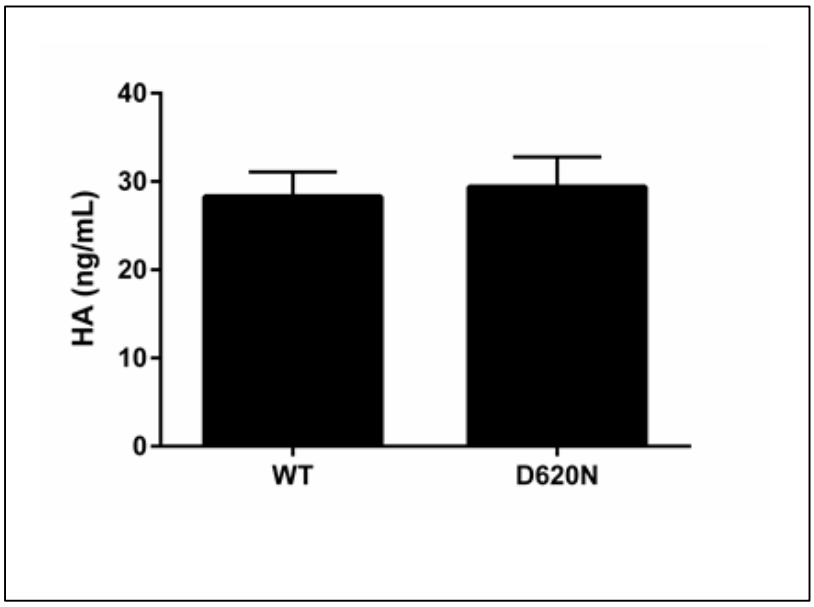

Figure 2.6. VPS35 D620N does not affect HA production.

ELISA detection of HA (all forms, >35kDa) production in cell culture supernatant (over 48h in 0.5% FBS-containing media) from differentiated wild-type and mutant SH-SY5Y cells (results from three independent experiments; student's *t*-test; P>0.05; Error bars = SEM).

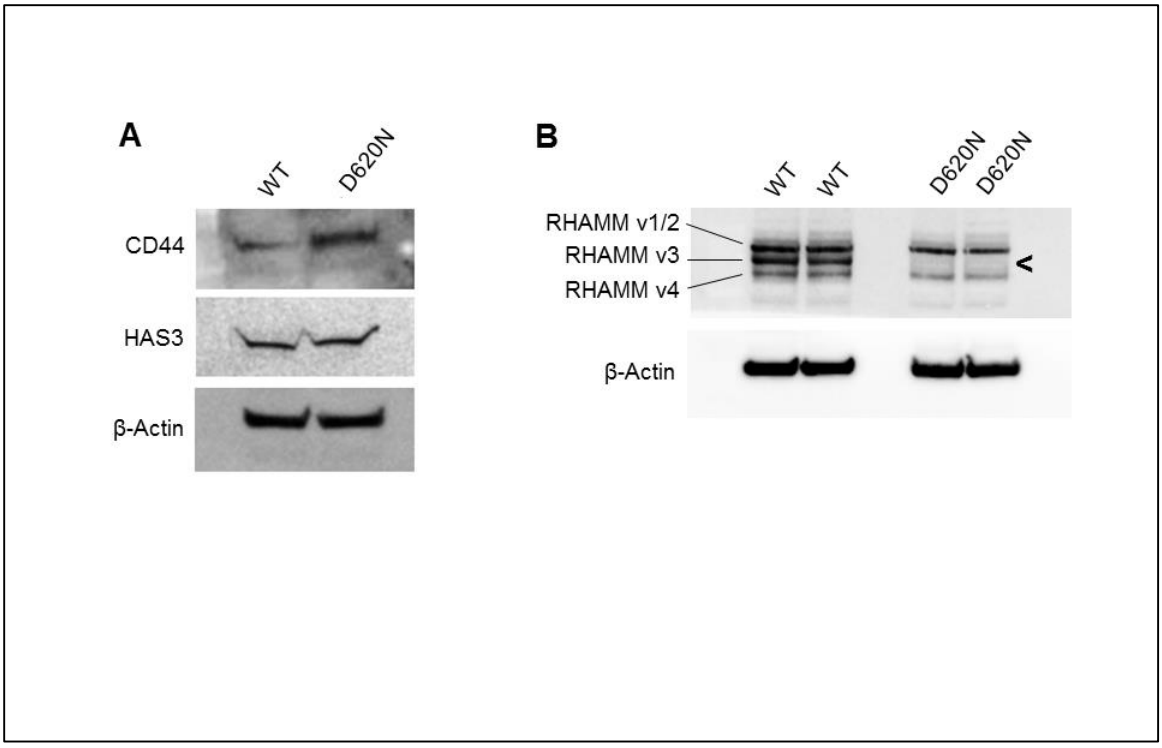

Figure 2.7. VPS35 D620N alters CD44 and RHAMM protein expression.

Western blot of differentiated SH-SY5Y cells expressing wild-type (WT) or D620N VPS35. Visualization of CD44 protein required 60-80ug total lysate and ECL Plus reagent **(A)**. RHAMM protein detection was done using 10ug of total lysate and standard ECL reagent **(B)**.

Misregulated CD44 and RHAMM Expression by VPS35 D620N

Our RNA Seq analysis revealed that ECM-receptor interaction pathways were a top hit for VPS35 D620N-expressing cells **(Table 2.1)**. From this dataset we also found that CD44 transcript expression increased significantly in VPS35 D620N-expressing cells. Consistent with CD44 pathway misregulation, assessment of protein expression revealed heightened CD44 expression in the mutant cells **(Fig. 2.7A)**. Interestingly, only a single truncated isoform of CD44 protein was observed that correlated to CD44v4. Identity of CD44v4 was confirmed by further analysis of the RNA Seq data. What's more, CD44v4 was very lowly expressed at both the protein level, requiring 60-80ug of

total lysate and a sensitive ECL reagent (WesternSure) to detect, and RNA level, nearing the limit of accurate detection in our RNA-Seq dataset **(Table 2.2)**. These factors led us to speculate that CD44 likely provides only a minor contribution to HA signaling in SH-SY5Y cells. We then examined the protein levels of RHAMM, another primary receptor for HA, and found robust expression of multiple isoforms. However, RHAMM isoform 3 (RHAMMv3) protein was strikingly absent from VPS35 D620N-expressing cells **(Fig. 2.7B)**. The identities of all four NCBI annotated isoforms were confirmed at the transcript level by RNA-Seq data. Collectively, these data suggest that the RHAMMv3 may contribute to the enhanced activation of HA-AKT signaling and autophagy inhibition by VPS35 D620N mutation, with minor contributions from CD44 misregulation.

Table 2.2. VPS35 D620N affects CD44 and RHAMM RNA splice variant expression.

SpliceR program analysis was performed on the WT and VPS35 D620N RNA-Seq dataset. Relative expression profiles are presented for all NCBI annotated splice variants as a function of FPKM for CD44 **(A)** and RHAMM **(B)**. Significance for differential expression by WT versus D620N is shown.

(A)

Isoform	**Ref. Seq.**	**Rel. Exp. WT**	**Rel. Exp. D620N**	**WT/D620N Ratio**	**p-value**
V1	NM_000610	0.0000	0.0000	INF	1
V2	NM_001001389	0.0000	0.0142	0	1
V3	NM_001001390	0.0000	0.0000	INF	1
V4	**NM_001001391**	**0.3387**	**1.0256**	**0.3303**	**0.0452**
V5	NM_001001392	0.1853	0.0000	INF	1
V6	NM_001202555	0.0000	0.0256	0	1
V7	NM_001202556	0.0000	0.0004	0.0954	1
V8	NM_001202557	0.0444	0.0000	INF	1

(B)

Isoform	**Ref. Seq.**	**Rel. Exp. WT**	**Rel. Exp. D620N**	**WT/D620N Ratio**	**p-value**
V1	NM_001142556.1	5.1525	5.5884	0.9220	0.6159
V2	NM_012484.2	0.9834	0.3711	2.6500	0.3170
V3	**NM_012485.2**	**13.5848**	**13.0535**	**1.0407**	**0.6639**
V4	NM_001142557.1	1.4806	0.6353	2.3306	0.2983

Discussion

The study of monogenic causes of PD has yielded valuable insights into the pathogenesis of this disease. The identification of VPS35 D620N as a causal familial factor has allowed for the molecular dissection of signaling pathways that could be important therapeutic points for this and other forms of PD. It is well-established that VPS35 D620N inhibits autophagy and that this mutation also hinders retromer function (Follett et al., 2014b; McGough et al., 2014; Zavodszky et al., 2014). Accordingly, research focus in the field has been placed upon canonical retromer interactors and pathways. This approach might be limiting as our group's inability to confirm WASH complex-retromer interaction in SH-SY5Y cells suggests. Therefore, in an effort to assay all potentially relevant signaling pathways we have created isogenic transgenic SH-SY5Y cell lines expressing VPS35 or VPS35 D620N and performed RNA Seq transcriptome analysis.

Identification of perturbed HA-AKT signaling as a contributor to VPS35 D620N-mediated autophagy dysfunction may offer a therapeutic target for PD as well as broaden the understanding of autophagy regulation. We report for the first time to the authors' best knowledge that HMW HA serves as a negative regulator of autophagy. Given the high concentration of HMW HA in the mammalian brain, the physiological consequences of this finding could be far-reaching. In the scope of neurological disease, defective autophagy is linked to a wide variety of maladies (Levine & Kroemer, 2008). If HMW HA and autophagic activity are coupled, then one could imagine that the concentration and quality of HMW HA might guide the path of neurodegenerative disease particularly as we age and are less adept at maintaining ECM. In the context of PD, recognition of

HA-AKT pathway involvement in the misregulation of autophagy in catecholaminergic cells could provide a novel target for treatment.

Our discovery that VPS35 D620N-expressing cells have heightened response to HMW but not LMW HA indicates that HA-AKT signaling is not initiated by a traditional ligand-receptor mechanism. This finding would imply that the responsible HA receptor(s) require scaffolding to a large ECM structure for activation. Previous work has indeed shown that cytoarchitecture is important for HA signaling (Neame, Uff, Sheikh, Wheatley, & Isacke, 1995). Maharjan and colleagues have specifically explored the signaling divergences between HMW and LMW HA (Maharjan et al., 2011). During remodeling, such as following injury, HMW HA is broken down to LMW by matrix metalloproteinases (MMP). This group reported that environments can direct monocyte differentiation and activation based upon HA polymer size. It is therefore not inconceivable that brain HA composition could serve a similarly important function.

CD44 and RHAMM are the most researched HA receptors. These proteins have largely been studied in the context of cancer metastasis due to being refined sensors for detecting the state of ECM for an invading cancer cell (Savani et al., 2001, p. 44). Upon identifying defective HA-AKT signaling we examined CD44 and RHAMM expression. We report that SH-SY5Y cells express only a single isoform (V4) of CD44 (CD44v4) protein, albeit at very low levels, and that this isoform is found in higher amounts in mutant versus control cells. We also found that CD44v4 protein and mRNA levels correlate closely. However, the extremely low level of CD44 expression coupled with the observation that the CD44 protein expression is likely controlled largely by transcriptional mechanisms suggest that CD44 plays a minor role in VPS35 D620N

dysfunction. In contrast, RHAMM protein is abundantly expressed yet isoform V3 (RHAMMv3) is strikingly absent from VPS35 D620N-expressing cells. Interestingly, RHAMMv3 transcript levels are indistinguishable for the wild-type and mutant cells indicating post-splicing regulation of expression and potentially a direct role for VPS35. Taken together, these data suggest that the RHAMMv3 may contribute to the enhanced activation of HA-AKT signaling and autophagy inhibition by VPS35 D620N.

The receptor for hyaluronan-mediated motility, RHAMM, is expressed on the cell surface as well as intracellularly. RHAMM was shown to associate with cytoskeletal components (Assmann, Jenkinson, Marshall, & Hart, 1999) in addition to the mitotic spindle (Christopher A. Maxwell et al., 2003) and direct numerous processes including mitosis and tumorigenesis (Christopher Alan Maxwell, McCarthy, & Turley, 2008; Mohapatra, Yang, Wright, Turley, & Greenberg, 1996). Thus, the study of this protein has been of particular interest in the context of cancer. Interestingly, Sohr et. al. demonstrated that overexpression of a tumor suppressor p53 mutant that lacks DNA-binding ability results in a specific loss of the RHAMMv3 isoform in DLD-1 cells (Sohr & Engeland, 2008). This group and others have also shown that p53 regulates transcription of the *RHAMM* gene (Schütze et al., 2016; Sohr & Engeland, 2008). Specific functionality of RHAMMv3 is also suggested from clinical data indicating that the RHAMMv3:RHAMMv1/v2 ratio is associated with a poor prognosis for multiple myeloma (Christopher A. Maxwell et al., 2004) and non-small cell lung carcinoma (D. Wang et al., 2016). It is important to note that RA-differentiated SH-SY5Y cells are post-mitotic so aberrant p53 activity would result from genomic instability or inappropriate signaling that is not a direct result of the cell cycle. Our RNA Seq data reveals cancer

signaling through focal adhesion or growth factor receptors as the top overall hit for altered signaling by VPS35 D620N. It is therefore not unreasonable to hypothesize that this perturbed signaling mediated by VPS35 D620N affects p53 activity which might in-turn lead to repression of RHAMMv3 expression. Further investigation into a HA-RHAMM-p53-VPS35 signaling axis could provide valuable insights into PD and cancer.

In summary, we report that VPS35 D620N suppresses autophagy in RA-differentiated SH-SY5Y cells in a manner consistent with a loss of function mutation. Using an unbiased RNA Seq screen, we identified misregulated ECM-receptor and PI3K-AKT signaling pathways in the mutant cells. Subsequent investigation revealed enhanced HMW HA-AKT signaling with an expression defect in a single isoform of RHAMM (RHAMMv3) being a likely contributing factor. These results provide a compelling rationale to further investigate ECM dynamics in PD and other neurological disorders.

Methods

Tissue Culture

SH-SY5Y human neuroblastoma cells (ATCC) were cultured in T175 tissue culture flasks and maintained at 37°C with 5% CO_2 environment. Cells were cultured in DMEM/F12 50/50 media (Corning) with L-glutamine, 15% Fetal Bovine Serum (Atlanta Biologicals), 1% non-essential amino acids, 1% penicillin/streptomycin solution (Hyclone, 10000 units/mL Penicillin/10000µg/mL Streptomycin). Cells were passaged when flasks reached ~80% confluency using 0.25% Trypsin, with 2.2mM EDTA and sodium bicarbonate (Corning). Cells at passage numbers 8 thru 15 were used for experiments. For this, cells were plated on multi-well tissue culture plates at the necessary cell concentrations (e.g., 4 million cells per well on a 6-well plate). Prior to

experimentation, the cells were treated with retinoic acid for 7 days, to differentiate them into post-mitotic catecholaminergic neuron-like cells. Transgenic cells were also treated 72h prior to experimentation with 2mM sodium butyrate to reactivate transgene expression (K. H. Choi, Basma, Singh, & Cheng, 2005). 293TA cells were grown in DMEM media with 1.0 g/L Glucose, with L-glutamine and with Sodium Pyruvate (VWR), supplemented with 10% Fetal Bovine Serum (Atlanta Biologicals), 1% non-essential amino acids (Corning Cellgro) and 1% penicillin/streptomycin solution (Hyclone, 10000 units/mL Penicillin/10000µg/mL Streptomycin).

Western Blotting

Life Technologies Bolt gels and reagents were used for western blotting. Cells were lysed using RIPA lysis buffer containing a protease inhibitor cocktail and then sonicated. This was then followed by centrifugation at 15000g for 10 minutes and collecting supernatant. Total protein levels were measured and normalized across samples. Polyacrylamide gel electrophoresis was performed on the protein extracts followed by transfer onto a PVDF membrane. Transfers to PVDF membranes were performed using the iBlot2 Dry Transfer device (Life Technologies). Primary antibodies were used to detect LC3 (Novus Biologicals, NB100-2220), CD44 (Origene, TA506726), Phospho-AKT (Ser 473) (Cell Signaling, 4060), RHAMM (Genetex, GTX54121) and β-actin (Thermo Fisher, MA1-91399). Secondary antibodies (anti-mouse, Cell Signaling, 7076; anti-rabbit, Cell Signaling, 7074) were used for probing PVDF membranes after primary antibody incubation. ECL substrate (Pierce, 32106) or, for more sensitive detection, WesternSure chemiluminescent substrate (LI-COR, 926-95000) was then used

to visualize protein bands and images obtained with a ChemiDoc Touch Gel Imager (BioRad). Protein expression was quantified by densitometry using Image J (NIH).

Lentivirus Production and Transduction

Life Technologies Virapower lentiviral production kit was used to generate lentivirus. 293TA cells (GeneCopoeia) were transfected with plasmids using Lipofectamine 2000 (Life Technologies). Media was replaced the next day. The supernatant media containing active, fully assembled lentiviruses, was collected 48 hours after transfection and filtered through 400 nm pore filters, aliquoted and stored at -80°C until use. For transduction of SH-SY5Y cells, virus containing supernatant was added directly to the culture media. This media was replaced the next day and antibiotic selection (blasticidin or puromycin) for transduced cells was initiated 72 hours following viral supernatant addition.

Autophagic Flux Assay

Bafilomycin A1 (100nM) was used to block autophagy in experimental cells, four hours prior to lysis. This was then followed by comparison of LC3-II levels normalized to β-Actin between bafilomycin A1 treated and untreated cells obtained from western blot imaging as previously described (Klionsky et al., 2012).

Hyaluronan Treatment

Cells were plated at a density of 4 million cells per well on 6 well plates. Cells were then differentiated with RA for 7 days. On the day prior to lysis, cells were treated with high or low molecular weight HA in serum-free media and then lysed using RIPA lysis buffer, as described above. For autophagic flux assays, cells were treated with or

without HA in the presence or absence of Bafilomycin A1 (100nM) for 4 hours immediately prior to lysis and then autophagic flux assessed as described above.

Hyaluronan Quantification

ELISA measurement of Hyaluronan was performed using a Quantikine ELISA kit (R&D Systems, DHYAL0) according to the manufacturer's instructions. Briefly, cells were plated on 96 well plates at 50,000 cells/well. The cells were then treated with retinoic acid media for seven days. After differentiation, the media was replaced with growth media containing 0.5% FBS and RA. Supernatant was then collected after 48 hours for ELISA analysis. It should be noted that this ELISA system detects all forms of HA (>35kDa).

Differential Gene Expression, Splicing and Gene Set Enrichment Analysis

Transgenic WT and VPS35 D620N-expressing cells grown in 100mm culture plates were RA-differentiated, RNA was extracted using an RNeasy kit (Qiagen) with DNase treatment, rRNA depleted, random-primed cDNA made, and sequencing subsequently performed using Illumina with HiSeq2000/2500 (>40 million reads/sample). Sequencing and analysis were done by Idaho State University's Molecular Research Core Facility. Alignment of mRNA-Seq was conducted with a standard protocol as previously described (Trapnell et al., 2012). Briefly, raw reads were initially trimmed with FASTX-toolkit to remove adapter sequences, and aligned to a reference genome (human hg19) with TopHat (v2.0.11). To take into account novel splice isoforms, the de novo transcript assembly from the mapped reads was performed using cufflinks (v2.2.1) and the assembled transcripts were then merged into a single reference transcripts using cuffmerge. The resulting full length, mapped transcripts were further

analyzed using a Bioconductor package, spliceR (Vitting-Seerup, Porse, Sandelin, & Waage, 2014), and investigated the alternative splicing events of individual genes.

For differential gene expression analysis, the mapped reads were quantified using cuffdiff and calculate the FPKM (Fragments Per Kilobase of transcript per Million mapped reads). Genes were discarded from downstream analysis when the calculated mRNA abundance was lower than FPKM of 0.5, treating them as background noise. The quantified results were further visualized with CummeRbund (Trapnell et al., 2012) or custom scripts in R. To investigate whether the expression level of pre-defined sets of genes correlated to the observed phenotype, the Impact Analysis method was conducted using iPathwayGuide (Advaita)(Draghici et al., 2003; Khatri, Draghici, Tarca, Hassan, & Romero, 2007; Tarca et al., 2009). The number of gene-set permutations was set at 1000, and the most enriched KEGG gene sets (Kanehisa et al., 2014) were selected based on Normalized Enrichment Scores (NES).

Gaussia Luciferase Protein-Fragment Complementation Assay

Plasmids containing transgenic fusion constructs of syn-hGluc (SynLuc), syn-hGLuc1 (S1) and syn-hGLuc2 (S2) were transfected (40ng DNA/well) into transgenic (WT or VPS35 D620N) SH-SY5Y cells in 96 well plates using FuGENE 6 (Promega, E2691) according to manufacturer's instructions as previously described (Outeiro et al., 2008). Media (conditioned media) was transferred to a new 96 well plate 48 hours later for analysis. The cells were then washed with PBS and new media (without phenol red or FBS) added. Aggregation of alpha-synuclein was determined by assessing complementation of luciferase activity. Luciferase activity was measured within the conditioned media and living cells by automated injection of coelenterazine (40 µM,

P.J.K., 55779-48-1) into each well and detected using a plate reader (Victor X3 microplate reader, Perkin-Elmer) with a 1 second signal integration time.

Statistical Analysis and Data Management

Statistical analysis was performed using Prism (version 6.0; GraphPad Software, San Diego, CA). Data between two variables was based on the student's *t*-test. Data was considered significantly different at $p < 0.05$. The datasets generated during and/or analyzed during the current study are available from the corresponding author upon request.

Supplementary Figures

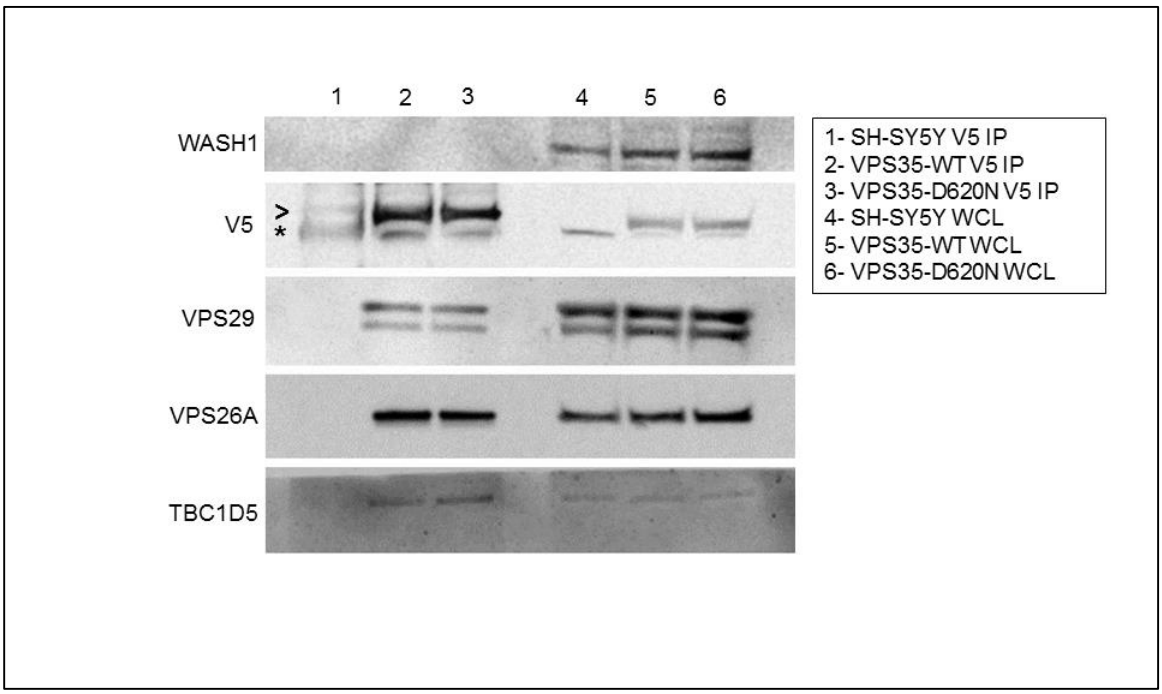

Figure S1. WASH1-VPS35 interaction is not detected in SH-SY5Y cells.

Non-transgenic (SH-SY5Y), WT-VPS35, and VPS35-D620N-expressing SH-SY5Y cells were RA-differentiated and then WASH1-VPS35 interaction assessed with co-immunoprecipitation (IP) by pulling transgenic VPS35 down with a V5 antibody. The blot was then subjected to western blotting with the indicated antibodies (> denotes the VPS35 band; * signifies a non-specific band found in all samples). Whole cell lysates (WCL) were also included. Successful VPS35 IP was verified by co-immunoprecipitation of known interactors VPS29, VPS26, and TBC1D5. As reported by other groups, we do not observe differential interaction of retromer members (VPS29 and 26A) by VPS35 D620N.

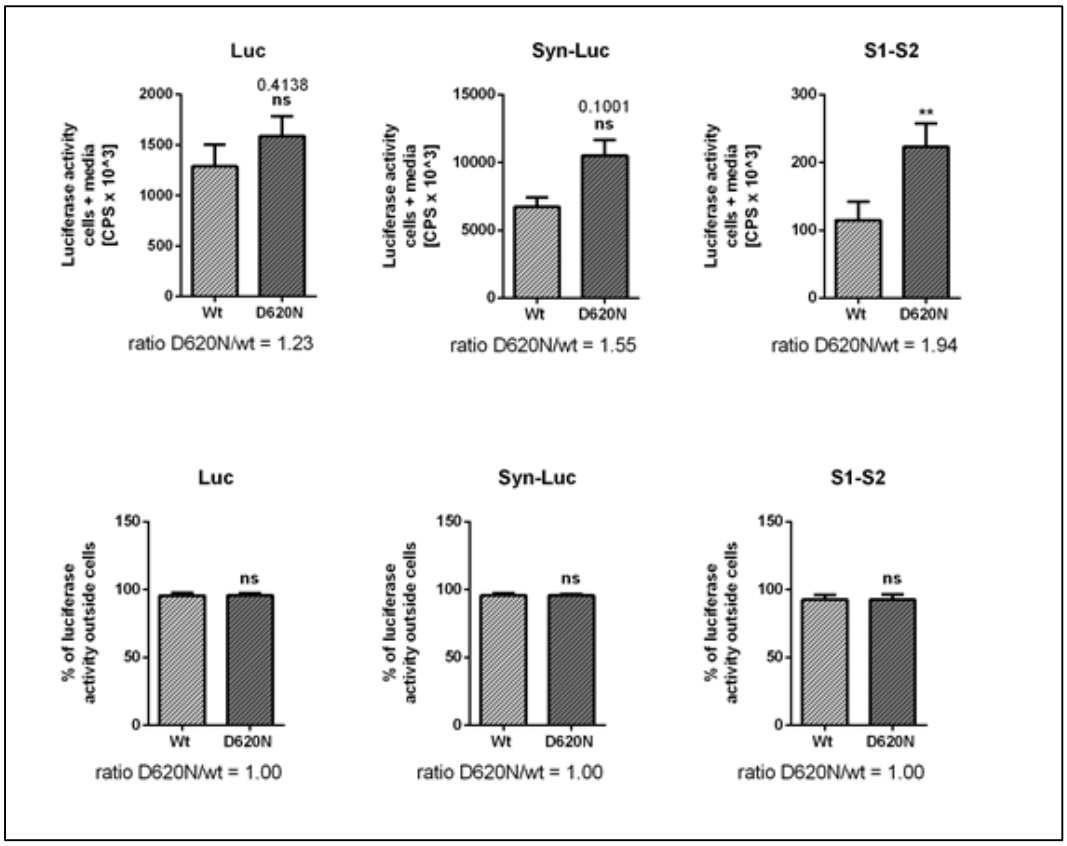

Figure S2. VPS35 D620N enhances intracellular alpha-synuclein aggregation.

WT or VPS35 D20N-expressing SH-SY5Y cells were transfected with S1 and S2 plasmids containing full-length alpha-synuclein fused to N-or C-terminal parts of human Gaussia luciferase. Upon aggregation of alpha-synuclein, complementary portions of luciferase produced a functional enzyme that was detected by adding luciferase substrate coelenterazine. Full length luciferase as well as alpha-synuclein conjugated to full-length luciferase were also examined as basal expression controls. VPS35 D620N-expressing cells exhibited significantly elevated alpha-synuclein aggregation compared to WT cells (student's *t*-test; **P>0.05; Error bars = SEM).

A

	Total number of reads	The number of aligned read pairs	Multiple alignments among the aligned reads	Discordant alignments the aligned reads
wt_0	27,425,148	25,371,327	2,110,170 (8.3%)	1,272,367 (5.0%)
wt_1	27,269,414	25,072,148	2,078,745 (8.3%)	1,240,381 (4.9%)
wt_2	26,202,389	23,913,838	2,313,669 (9.7%)	1,316,808 (5.5%)
mut_0	26,973,387	25,266,403	2,319,494 (9.2%)	1,216,364 (4.8%)
mut_1	27,350,224	25,217,143	2,170,229 (8.6%)	1,248,968 (5.0%)
mut_2	25,351,773	23,124,643	2,184,494 (9.4%)	1,180,550 (5.1%)

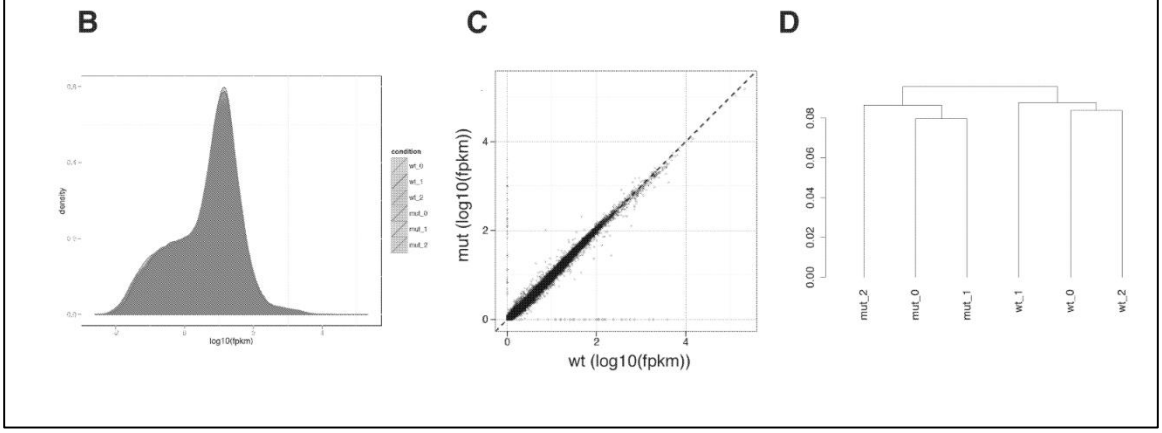

Figure S3. RNA-Seq data assessment.

Alignment statistics for sequencing reads (A). mRNA abundance distribution (density plot) for all genes quantified from RNA-seq analysis (B). Each replicate was plotted individually and overlaid to show there is no distribution bias. FPKM ≥ 0.5 was arbitrary set as cut-off value for downstream analysis. A scatter plot comparing wild-type (wt) and VPS35-D620N (mut) samples (C). Each dot represents the average expression level (FPKM) of individual genes calculated from three biological replicates. The vast majority of genes were expressed similarly in wt (VPS35) and mut (VPS35-D620N) cells, but genes that were differentially expressed are remotely located from the diagonal dashed-line. A dendrogram of all quantified mRNA from RNA-seq analysis (D). Clustering among biological replicates suggested that the D620N mutation causes differential gene expression.

REFERENCES

Ando, M., Funayama, M., Li, Y., Kashihara, K., Murakami, Y., Ishizu, N., … Hattori, N. (2012). *VPS35* mutation in Japanese patients with typical Parkinson's disease. *Movement Disorders*, *27*(11), 1413–1417. http://doi.org/10.1002/mds.25145

Appel, J. R., Ye, S., Tang, F., Sun, D., Zhang, H., Mei, L., & Xiong, W.-C. (2018). Increased Microglial Activity, Impaired Adult Hippocampal Neurogenesis, and Depressive-like Behavior in Microglial VPS35-Depleted Mice. *The Journal of Neuroscience*, *38*(26), 5949–5968. http://doi.org/10.1523/JNEUROSCI.3621-17.2018

Arighi, C. N., Hartnell, L. M., Aguilar, R. C., Haft, C. R., & Bonifacino, J. S. (2004). Role of the mammalian retromer in sorting of the cation-independent mannose 6-phosphate receptor. *The Journal of Cell Biology*, *165*(1), 123–133. http://doi.org/10.1083/jcb.200312055

Ascherio, A., & Schwarzschild, M. A. (2016). The epidemiology of Parkinson's disease: risk factors and prevention. *The Lancet Neurology*, *15*(12), 1257–1272. http://doi.org/10.1016/S1474-4422(16)30230-7

Ashrafi, G., & Schwarz, T. L. (2013). The pathways of mitophagy for quality control and clearance of mitochondria. *Cell Death & Differentiation*, *20*(1), 31–42. http://doi.org/10.1038/cdd.2012.81

Assmann, V., Jenkinson, D., Marshall, J. F., & Hart, I. R. (1999). The intracellular hyaluronan receptor RHAMM/IHABP interacts with microtubules and actin filaments. *Journal of Cell Science*, *112 (Pt 2*, 3943–3954.

Aufschnaiter, A., Kohler, V., & Büttner, S. (2017). Taking out the garbage: cathepsin D and calcineurin in neurodegeneration. *Neural Regeneration Research*, *12*(11), 1776. http://doi.org/10.4103/1673-5374.219031

Bahouth, S. W., & Nooh, M. M. (2017). Barcoding of GPCR trafficking and signaling through the various trafficking roadmaps by compartmentalized signaling networks. *Cellular Signalling*, *36*, 42–55. http://doi.org/10.1016/j.cellsig.2017.04.015

Barth, S., Glick, D., & Macleod, K. F. (2010). Autophagy: Assays and artifacts. *Journal of Pathology*, *221*(2), 117–124. http://doi.org/10.1002/path.2694

Beard, J. D., Steege, A. L., Ju, J., Lu, J., Luckhaupt, S. E., & Schubauer-Berigan, M. K. (2017). Mortality from Amyotrophic Lateral Sclerosis and Parkinson's Disease Among Different Occupation Groups - United States, 1985-2011. *MMWR. Morbidity and Mortality Weekly Report*, *66*(27), 718–722. http://doi.org/10.15585/mmwr.mm6627a2

Belenkaya, T. Y., Wu, Y., Tang, X., Zhou, B., Cheng, L., Sharma, Y. V., … Lin, X. (2008). The Retromer Complex Influences Wnt Secretion by Recycling Wntless from Endosomes to the Trans-Golgi Network. *Developmental Cell*, *14*(1), 120–131. http://doi.org/10.1016/j.devcel.2007.12.003

Blanckenberg, J., Ntsapi, C., Carr, J. A., & Bardien, S. (2014). EIF4G1 R1205H and VPS35 D620N mutations are rare in Parkinson's disease from South Africa. *Neurobiology of Aging*, *35*(2), 445.e1-3. http://doi.org/10.1016/j.neurobiolaging.2013.08.023

Bonifacino, J. S., & Hurley, J. H. (2008). Retromer. *Current Opinion in Cell Biology*, *20*(4), 427–436. http://doi.org/10.1016/j.ceb.2008.03.009

Bowman, S. L., Shiwarski, D. J., & Puthenveedu, M. A. (2016). Distinct G protein-coupled receptor recycling pathways allow spatial control of downstream G protein signaling. *The Journal of Cell Biology*, *214*(7), 797–806. http://doi.org/10.1083/jcb.201512068

Braschi, E., Goyon, V., Zunino, R., Mohanty, A., Xu, L., & McBride, H. M. (2010). Vps35 mediates vesicle transport between the mitochondria and peroxisomes. *Current Biology : CB*, *20*(14), 1310–5. http://doi.org/10.1016/j.cub.2010.05.066

Brundin, P., & Melki, R. (2017). Prying into the Prion Hypothesis for Parkinson's Disease. *The Journal of Neuroscience*, *37*(41), 9808–9818. http://doi.org/10.1523/JNEUROSCI.1788-16.2017

Bunnett, N. W., & Cottrell, G. S. (2010). Trafficking and signaling of G protein-coupled receptors in the nervous system: implications for disease and therapy. *CNS & Neurological Disorders Drug Targets*, *9*(5), 539–56.

Calatayud, C., Carola, G., Consiglio, A., & Raya, A. (2017). Modeling the genetic complexity of Parkinson's disease by targeted genome edition in iPS cells. *Current Opinion in Genetics & Development*, *46*, 123–131. http://doi.org/10.1016/j.gde.2017.06.002

Cataldi, S., Follett, J., Fox, J. D., Tatarnikov, I., Kadgien, C., Gustavsson, E. K., … Farrer, M. J. (2018). Altered dopamine release and monoamine transporters in Vps35 p.D620N knock-in mice. *Npj Parkinson's Disease*, *4*(1), 27. http://doi.org/10.1038/s41531-018-0063-3

Chan, A. S. M., Clairfeuille, T., Landao-Bassonga, E., Kinna, G., Ng, P. Y., Loo, L. S., … Pavlos, N. J. (2016). Sorting nexin 27 couples PTHR trafficking to retromer for signal regulation in osteoblasts during bone growth. *Molecular Biology of the Cell*, *27*(8), 1367–82. http://doi.org/10.1091/mbc.E15-12-0851

Chen, Y., Chen, K., Song, W., Chen, X., Cao, B., Huang, R., … Shang, H.-F. (2013). VPS35 Asp620Asn and EIF4G1 Arg1205His mutations are rare in Parkinson disease from Southwest China. *Neurobiology of Aging*, *34*(6), 1709.e7-1709.e8. http://doi.org/10.1016/j.neurobiolaging.2012.11.003

Choi, A. M. K., Ryter, S. W., & Levine, B. (2013). Autophagy in human health and disease. *The New England Journal of Medicine*, *368*(19), 1845–1846. http://doi.org/10.1056/NEJMc1303158

Choi, K. H., Basma, H., Singh, J., & Cheng, P.-W. (2005). Activation of CMV promoter-controlled glycosyltransferase and beta -galactosidase glycogenes by butyrate, tricostatin A, and 5-aza-2'-deoxycytidine. *Glycoconjugate Journal*, *22*(1–2), 63–69. http://doi.org/10.1007/s10719-005-0326-1

Choy, R. W.-Y., Park, M., Temkin, P., Herring, B. E., Marley, A., Nicoll, R. A., & von Zastrow, M. (2014). Retromer Mediates a Discrete Route of Local Membrane Delivery to Dendrites. *Neuron*, *82*(1), 55–62. http://doi.org/10.1016/j.neuron.2014.02.018

Dehay, B., Bové, J., Rodríguez-Muela, N., Perier, C., Recasens, A., Boya, P., & Vila, M. (2010). Pathogenic lysosomal depletion in Parkinson's disease. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *30*(37), 12535–12544. http://doi.org/10.1523/JNEUROSCI.1920-10.2010

Dehay, B., Martinez-Vicente, M., Caldwell, G. A., Caldwell, K. A., Yue, Z., Cookson, M. R., … Bezard, E. (2013). Lysosomal impairment in Parkinson's disease. *Movement Disorders: Official Journal of the Movement Disorder Society*, *28*(6), 725–732. http://doi.org/10.1002/mds.25462

Deng, H., Gao, K., & Jankovic, J. (2013). The *VPS35* gene and Parkinson's disease. *Movement Disorders*, *28*(5), 569–575. http://doi.org/10.1002/mds.25430

Deng, H., Xu, H., Deng, X., Song, Z., Zheng, W., Gao, K., … Tang, J. (2012). VPS35 mutation in Chinese Han patients with late-onset Parkinson's disease. *European Journal of Neurology*, *19*(9), e96-7. http://doi.org/10.1111/j.1468-1331.2012.03800.x

Dengjel, J., Høyer-Hansen, M., Nielsen, M. O., Eisenberg, T., Harder, L. M., Schandorff, S., … Andersen, J. S. (2012). Identification of autophagosome-associated proteins and regulators by quantitative proteomic analysis and genetic screens. *Molecular & Cellular Proteomics: MCP*, *11*(3), M111.014035. http://doi.org/10.1074/mcp.M111.014035

Dhungel, N., Eleuteri, S., Li, L., Kramer, N. J., Chartron, J. W., Spencer, B., … Gitler, A. D. (2015). Parkinson's Disease Genes VPS35 and EIF4G1 Interact Genetically and Converge on α-Synuclein. *Neuron*, *85*(1), 76–87. http://doi.org/10.1016/j.neuron.2014.11.027

Draghici, S., Khatri, P., Bhavsar, P., Shah, A., Krawetz, S. A., & Tainsky, M. A. (2003). Onto-Tools, the toolkit of the modern biologist: Onto-Express, Onto-Compare, Onto-Design and Onto-Translate. *Nucleic Acids Research, 31*(13), 3775–3781.

Elbaz, A., Bower, J. H., Maraganore, D. M., McDonnell, S. K., Peterson, B. J., Ahlskog, J. E., … Rocca, W. A. (2002). Risk tables for parkinsonism and Parkinson's disease. *Journal of Clinical Epidemiology*, *55*(1), 25–31. http://doi.org/10.1016/S0895-4356(01)00425-5

Feinstein, T. N., Wehbi, V. L., Ardura, J. A., Wheeler, D. S., Ferrandon, S., Gardella, T. J., & Vilardaga, J.-P. (2011). Retromer terminates the generation of cAMP by internalized PTH receptors. *Nature Chemical Biology*, *7*(5), 278–284. http://doi.org/10.1038/nchembio.545

Follett, J., Bugarcic, A., Collins, B. M., & Teasdale, R. D. (2017). Retromer's Role in Endosomal Trafficking and Impaired Function in Neurodegenerative Diseases. *Current Protein & Peptide Science*, *18*(7), 687–701. http://doi.org/10.2174/1389203717666160311121246

Follett, J., Norwood, S. J., Hamilton, N. A., Mohan, M., Kovtun, O., Tay, S., … Teasdale, R. D. (2014a). The Vps35 D620N Mutation Linked to Parkinson's Disease Disrupts the Cargo Sorting Function of Retromer. *Traffic*, *15*(2), 230–244. http://doi.org/10.1111/tra.12136

Follett, J., Norwood, S. J., Hamilton, N. a, Mohan, M., Kovtun, O., Tay, S., … Teasdale, R. D. (2014b). The Vps35 D620N mutation linked to Parkinson's disease disrupts the cargo sorting function of retromer. *Traffic (Copenhagen, Denmark)*, *15*(2), 230–44. http://doi.org/10.1111/tra.12136

Fuse, A., Furuya, N., Kakuta, S., Inose, A., Sato, M., Koike, M., … Hattori, N. (2015). VPS29-VPS35 intermediate of retromer is stable and may be involved in the retromer complex assembly process. *FEBS Letters*, *589*(13), 1430–1436. http://doi.org/10.1016/j.febslet.2015.04.040

Gagliardi, M., Annesi, G., Tarantino, P., Nicoletti, G., & Quattrone, A. (2014). Frequency of the ASP620ASN mutation in VPS35 and Arg1205His mutation in

EIF4G1 in familial Parkinson's disease from South Italy. *Neurobiology of Aging*, *35*(10), 2422.e1-2422.e2. http://doi.org/10.1016/j.neurobiolaging.2014.04.020

Gallon, M., & Cullen, P. J. (2015). Retromer and sorting nexins in endosomal sorting. *Biochemical Society Transactions*, *43*(1), 33–47. http://doi.org/10.1042/BST20140290

Gambardella, S., Biagioni, F., Ferese, R., Busceti, C. L., Frati, A., Novelli, G., … Fornai, F. (2016). Vacuolar Protein Sorting Genes in Parkinson's Disease: A Re-appraisal of Mutations Detection Rate and Neurobiology of Disease. *Frontiers in Neuroscience*, *10*, 532. http://doi.org/10.3389/fnins.2016.00532

Gomez, T. S., & Billadeau, D. D. (2009). A FAM21-containing WASH complex regulates retromer-dependent sorting. *Developmental Cell*, *17*(5), 699–711. http://doi.org/10.1016/j.devcel.2009.09.009

Goswami, P., Joshi, N., & Singh, S. (2017). Neurodegenerative signaling factors and mechanisms in Parkinson's pathology. *Toxicology in Vitro*, *43*(December 2016), 104–112. http://doi.org/10.1016/j.tiv.2017.06.008

Guella, I., Soldà, G., Cilia, R., Pezzoli, G., Asselta, R., Duga, S., & Goldwurm, S. (2012). The Asp620asn mutation in VPS35 is not a common cause of familial Parkinson's disease. *Movement Disorders*, *27*(6), 800–801. http://doi.org/10.1002/mds.24927

Guo, J., Sun, Q., Lv, Z., Yu, R., Li, K., Zhang, Y., … Tang, B. (2012). VPS35 gene variants are not associated with Parkinson's disease in the mainland Chinese population. *Parkinsonism & Related Disorders*, *18*(8), 983–985. http://doi.org/10.1016/j.parkreldis.2012.05.002

Gustavsson, E. K., Guella, I., Trinh, J., Szu-Tu, C., Rajput, A., Rajput, A. H., … Farrer, M. J. (2015). Genetic variability of the retromer cargo recognition complex in parkinsonism. *Movement Disorders*, *30*(4), 580–584. http://doi.org/10.1002/mds.26104

Haelterman, N. A., Yoon, W. H., Sandoval, H., Jaiswal, M., Shulman, J. M., & Bellen, H. J. (2014). A Mitocentric View of Parkinson's Disease. *Annual Review of*

Neuroscience, *37*(1), 137–159. http://doi.org/10.1146/annurev-neuro-071013-014317

Hao, Y.-H., Doyle, J. M., Ramanathan, S., Gomez, T. S., Jia, D., Xu, M., … Potts, P. R. (2013). Regulation of WASH-dependent actin polymerization and protein trafficking by ubiquitination. *Cell*, *152*(5), 1051–1064. http://doi.org/10.1016/j.cell.2013.01.051

Hauser, D. N., & Hastings, T. G. (2013). Mitochondrial dysfunction and oxidative stress in Parkinson's disease and monogenic parkinsonism. *Neurobiology of Disease*, *51*, 35–42. http://doi.org/10.1016/j.nbd.2012.10.011

He, C., & Klionsky, D. J. (2009). Regulation mechanisms and signaling pathways of autophagy. *Annual Review of Genetics*, *43*, 67–93. http://doi.org/10.1146/annurev-genet-102808-114910

Helley, M. P., Pinnell, J., Sportelli, C., & Tieu, K. (2017). Mitochondria: A Common Target for Genetic Mutations and Environmental Toxicants in Parkinson's Disease. *Frontiers in Genetics*, *8*, 177. http://doi.org/10.3389/fgene.2017.00177

Inoshita, T., Arano, T., Hosaka, Y., Meng, H., Umezaki, Y., Kosugi, S., … Hattori, N. (2017). Vps35 in cooperation with LRRK2 regulates synaptic vesicle endocytosis through the endosomal pathway in Drosophila. *Human Molecular Genetics*, *26*(15), 2933–2948. http://doi.org/10.1093/hmg/ddx179

Ishizu, N., Yui, D., Hebisawa, A., Aizawa, H., Cui, W., Fujita, Y., … Watase, K. (2016). Impaired striatal dopamine release in homozygous Vps35 D620N knock-in mice. *Human Molecular Genetics*, *25*(20), ddw279. http://doi.org/10.1093/hmg/ddw279

Jankovic, J., & Stacy, M. (2007). Medical management of levodopa-associated motor complications in patients with Parkinson's disease. *CNS Drugs*, *21*(8), 677–692.

Janku, F., McConkey, D. J., Hong, D. S., & Kurzrock, R. (2011). Autophagy as a target for anticancer therapy. *Nature Reviews. Clinical Oncology*, *8*(9), 528–539. http://doi.org/10.1038/nrclinonc.2011.71

Kanehisa, M., Goto, S., Sato, Y., Kawashima, M., Furumichi, M., & Tanabe, M. (2014). Data, information, knowledge and principle: back to metabolism in KEGG.

Nucleic Acids Research, *42*(Database issue), D199-205. http://doi.org/10.1093/nar/gkt1076

Khatri, P., Draghici, S., Tarca, A. L., Hassan, S. S., & Romero, R. (2007). A System Biology Approach for the Steady-State Analysis of Gene Signaling Networks. In *Progress in Pattern Recognition, Image Analysis and Applications* (pp. 32–41). Springer, Berlin, Heidelberg. http://doi.org/10.1007/978-3-540-76725-1_4

Klionsky, D. J., Abdalla, F. C., Abeliovich, H., Abraham, R. T., Acevedo-Arozena, A., Adeli, K., … Zuckerbraun, B. (2012). Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy*, *8*(4), 445–544. http://doi.org/10.4161/auto.19496

Kordower, J. H., Chu, Y., Hauser, R. A., Freeman, T. B., & Olanow, C. W. (2008). Lewy body–like pathology in long-term embryonic nigral transplants in Parkinson's disease. *Nature Medicine*, *14*(5), 504–506. http://doi.org/10.1038/nm1747

Korecka, J. A., van Kesteren, R. E., Blaas, E., Spitzer, S. O., Kamstra, J. H., Smit, A. B., Bossers, K. (2013). Phenotypic characterization of retinoic acid differentiated SH-SY5Y cells by transcriptional profiling. *PloS One*, *8*(5), e63862. http://doi.org/10.1371/journal.pone.0063862

Korolchuk, V. I., Schutz, M. M., Gomez-Llorente, C., Rocha, J., Lansu, N. R., Collins, S. M., … O'Kane, C. J. (2007). Drosophila Vps35 function is necessary for normal endocytic trafficking and actin cytoskeleton organisation. *Journal of Cell Science*, *120*(24), 4367–4376. http://doi.org/10.1242/jcs.012336

Koschmidder, E., Mollenhauer, B., Kasten, M., Klein, C., & Lohmann, K. (2014). Mutations in VPS26A are not a frequent cause of Parkinson's disease. *Neurobiology of Aging*, *35*(6), 1512.e1-2. http://doi.org/10.1016/j.neurobiolaging.2013.12.016

Krishna, A., Biryukov, M., Trefois, C., Antony, P. M. A., Hussong, R., Lin, J., … May, P. (2014). Systems genomics evaluation of the SH-SY5Y neuroblastoma cell line as a model for Parkinson's disease. *BMC Genomics*, *15*(1), 1154. http://doi.org/10.1186/1471-2164-15-1154

Kumar, K. R., Weissbach, A., Heldmann, M., Kasten, M., Tunc, S., Sue, C. M., … Lohmann, K. (2012). Frequency of the D620N mutation in VPS35 in Parkinson disease. *Archives of Neurology*, *69*(10), 1360–4. http://doi.org/10.1001/archneurol.2011.3367

La Spada, A. R., & Taylor, J. P. (2010). Repeat expansion disease: progress and puzzles in disease pathogenesis. *Nature Reviews. Genetics*, *11*(4), 247–258. http://doi.org/10.1038/nrg2748

Lau, L. M. L. De, & Breteler, M. M. B. (2006). Epidemiology of Parkinson ' s disease. *Lancet Neurol*, *5*(June), 525–535. http://doi.org/18(3) : 231 – 238

Lesage, S., Condroyer, C., Klebe, S., Honore, A., Tison, F., Brefel-Courbon, C., … Brice, A. (2012). Identification of VPS35 mutations replicated in French families with Parkinson disease. *Neurology*, *78*(18), 1449–1450. http://doi.org/10.1212/WNL.0b013e318253d5f2

Levine, B., & Kroemer, G. (2008). Autophagy in the Pathogenesis of Disease. *Cell*, *132*(1), 27–42. http://doi.org/10.1016/j.cell.2007.12.018

Li, C., Shah, S. Z. A., Zhao, D., & Yang, L. (2016). Role of the Retromer Complex in Neurodegenerative Diseases. *Frontiers in Aging Neuroscience*, *8*, 42. http://doi.org/10.3389/fnagi.2016.00042

Li, J.-Y., Englund, E., Holton, J. L., Soulet, D., Hagell, P., Lees, A. J., … Brundin, P. (2008). Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. *Nature Medicine*, *14*(5), 501–503. http://doi.org/10.1038/nm1746

Lin, S.-L., Chang, D., & Ying, S.-Y. (2007). Hyaluronan stimulates transformation of androgen-independent prostate cancer. *Carcinogenesis*, *28*(2), 310–320. http://doi.org/10.1093/carcin/bgl134

Linhart, R., Wong, S., Cao, J., Tran, M., Huynh, A., Ardrey, C., … Venderova, K. (2014). Vacuolar protein sorting 35 (Vps35) rescues locomotor deficits and shortened lifespan in Drosophila expressing a Parkinson's disease mutant of

Leucine-rich repeat kinase 2 (LRRK2). *Molecular Neurodegeneration*, *9*(1), 23. http://doi.org/10.1186/1750-1326-9-23

Loring, J. F. (2018). Autologous Induced Pluripotent Stem Cell-Derived Neurons to Treat Parkinson's Disease. *Stem Cells and Development*, *27*(14), 958–959. http://doi.org/10.1089/scd.2018.0107

Lucas, M., Gershlick, D. C., Vidaurrazaga, A., Rojas, A. L., Bonifacino, J. S., & Hierro, A. (2016). Structural Mechanism for Cargo Recognition by the Retromer Complex. *Cell*, *167*(6), 1623–1635.e14. http://doi.org/10.1016/j.cell.2016.10.056

Lucin, K. M., O'Brien, C. E., Bieri, G., Czirr, E., Mosher, K. I., Abbey, R. J., … Wyss-Coray, T. (2013). Microglial Beclin 1 Regulates Retromer Trafficking and Phagocytosis and Is Impaired in Alzheimer's Disease. *Neuron*, *79*(5), 873–886. http://doi.org/10.1016/j.neuron.2013.06.046

Luo, W., Friedman, M. S., Shedden, K., Hankenson, K. D., & Woolf, P. J. (2009). GAGE: generally applicable gene set enrichment for pathway analysis. *BMC Bioinformatics*, *10*, 161. http://doi.org/10.1186/1471-2105-10-161

Lynch-Day, M. A., Mao, K., Wang, K., Zhao, M., & Klionsky, D. J. (2012). The role of autophagy in Parkinson's disease. *Cold Spring Harbor Perspectives in Medicine*, *2*(4). http://doi.org/10.1101/cshperspect.a009357

MacLeod, D. A., Rhinn, H., Kuwahara, T., Zolin, A., Di Paolo, G., McCabe, B. D., … Abeliovich, A. (2013). RAB7L1 interacts with LRRK2 to modify intraneuronal protein sorting and Parkinson's disease risk. *Neuron*, *77*(3), 425–39. http://doi.org/10.1016/j.neuron.2012.11.033

Maharjan, A. S., Pilling, D., & Gomer, R. H. (2011). High and low molecular weight hyaluronic acid differentially regulate human fibrocyte differentiation. *PloS One*, *6*(10), e26078. http://doi.org/10.1371/journal.pone.0026078

Malgaroli, A., Vallar, L., & Zimarino, V. (2006). Protein homeostasis in neurons and its pathological alterations. *Current Opinion in Neurobiology*, *16*(3), 270–274. http://doi.org/10.1016/j.conb.2006.05.009

Marras, C., Beck, J. C., Bower, J. H., Roberts, E., Ritz, B., Ross, G. W., … Tanner, C. (2018). Prevalence of Parkinson's disease across North America. *Npj Parkinson's Disease*, *4*(1), 21. http://doi.org/10.1038/s41531-018-0058-0

Maxwell, C. A., Keats, J. J., Crainie, M., Sun, X., Yen, T., Shibuya, E., … Pilarski, L. M. (2003). RHAMM is a centrosomal protein that interacts with dynein and maintains spindle pole stability. *Molecular Biology of the Cell*, *14*(6), 2262–2276. http://doi.org/10.1091/mbc.E02-07-0377

Maxwell, C. A., McCarthy, J., & Turley, E. (2008). Cell-surface and mitotic-spindle RHAMM: moonlighting or dual oncogenic functions? *Journal of Cell Science*, *121*(Pt 7), 925–932. http://doi.org/10.1242/jcs.022038

Maxwell, C. A., Rasmussen, E., Zhan, F., Keats, J. J., Adamia, S., Strachan, E., … Reiman, T. (2004). RHAMM expression and isoform balance predict aggressive disease and poor survival in multiple myeloma. *Blood*, *104*(4), 1151–1158. http://doi.org/10.1182/blood-2003-11-4079

McGarvey, J. C., Xiao, K., Bowman, S. L., Mamonova, T., Zhang, Q., Bisello, A., … Friedman, P. A. (2016). Actin-Sorting Nexin 27 (SNX27)-Retromer Complex Mediates Rapid Parathyroid Hormone Receptor Recycling. *Journal of Biological Chemistry*, *291*(21), 10986–11002. http://doi.org/10.1074/jbc.M115.697045

McGough, I. J., & Cullen, P. J. (2011). Recent advances in retromer biology. *Traffic (Copenhagen, Denmark)*, *12*(8), 963–71. http://doi.org/10.1111/j.1600-0854.2011.01201.x

McGough, I. J., Steinberg, F., Jia, D., Barbuti, P. A., Mcmillan, K. J., Heesom, K. J., … Cullen, P. J. (2014). Report Retromer Binding to FAM21 and the WASH Complex Is Perturbed by the Parkinson Disease-Linked VPS35 (D620N) Mutation. *Current Biology*, *24*(14), 1670–1676. http://doi.org/10.1016/j.cub.2014.06.024

McGough, I. J., Steinberg, F., Jia, D., Barbuti, P. A., McMillan, K. J., Heesom, K. J., … Cullen, P. J. (2014). Retromer binding to FAM21 and the WASH complex is

perturbed by the Parkinson disease-linked VPS35(D620N) mutation. *Current Biology: CB*, *24*(14), 1670–1676. http://doi.org/10.1016/j.cub.2014.06.024

Mir, R., Tonelli, F., Lis, P., Macartney, T., Polinski, N. K., Martinez, T. N., … Alessi, D. R. (2018). The Parkinson's disease VPS35[D620N] mutation enhances LRRK2-mediated Rab protein phosphorylation in mouse and human. *Biochemical Journal*, *475*(11), 1861–1883. http://doi.org/10.1042/BCJ20180248

Miura, E., Hasegawa, T., Konno, M., Suzuki, M., Sugeno, N., Fujikake, N., … Aoki, M. (2014). VPS35 dysfunction impairs lysosomal degradation of α-synuclein and exacerbates neurotoxicity in a Drosophila model of Parkinson's disease. *Neurobiology of Disease*, *71*, 1–13. http://doi.org/10.1016/j.nbd.2014.07.014

Mohapatra, S., Yang, X., Wright, J. A., Turley, E. A., & Greenberg, A. H. (1996). Soluble hyaluronan receptor RHAMM induces mitotic arrest by suppressing Cdc2 and cyclin B1 expression. *The Journal of Experimental Medicine*, *183*(4), 1663–1668.

Mootha, V. K., Lindgren, C. M., Eriksson, K.-F., Subramanian, A., Sihag, S., Lehar, J., … Groop, L. C. (2003). PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nature Genetics*, *34*(3), 267–273. http://doi.org/10.1038/ng1180

Mullin, S., & Schapira, A. (2013). α-Synuclein and Mitochondrial Dysfunction in Parkinson's Disease. *Molecular Neurobiology*, *47*(2), 587–597. http://doi.org/10.1007/s12035-013-8394-x

Munsie, L. N., Milnerwood, A. J., Seibler, P., Beccano-Kelly, D. A., Tatarnikov, I., Khinda, J., … Farrer, M. J. (2015). Retromer-dependent neurotransmitter receptor trafficking to synapses is altered by the Parkinson's disease VPS35 mutation p.D620N. *Human Molecular Genetics*, *24*(6), 1691–1703. http://doi.org/10.1093/hmg/ddu582

Nayerossadat, N., Maedeh, T., & Ali, P. A. (2012). Viral and nonviral delivery systems for gene delivery. *Advanced Biomedical Research*, *1*, 27. http://doi.org/10.4103/2277-9175.98152

Neame, S. J., Uff, C. R., Sheikh, H., Wheatley, S. C., & Isacke, C. M. (1995). CD44 exhibits a cell type dependent interaction with triton X-100 insoluble, lipid rich, plasma membrane domains. *Journal of Cell Science*, *108 (Pt 9*, 3127–3135.

Onodera, Y., Teramura, T., Takehara, T., & Fukuda, K. (2015). Hyaluronic acid regulates a key redox control factor Nrf2 via phosphorylation of Akt in bovine articular chondrocytes. *FEBS Open Bio*, *5*, 476–484. http://doi.org/10.1016/j.fob.2015.05.007

Outeiro, T. F., Putcha, P., Tetzlaff, J. E., Spoelgen, R., Koker, M., Carvalho, F., … McLean, P. J. (2008). Formation of toxic oligomeric alpha-synuclein species in living cells. *PloS One*, *3*(4), e1867. http://doi.org/10.1371/journal.pone.0001867

Patel, D., & Witt, S. N. (2018). Sorting Out the Role of α-Synuclein in Retromer-Mediated Endosomal Protein Sorting. *Journal of Experimental Neuroscience*, *12*, 117906951879621. http://doi.org/10.1177/1179069518796215

Pavlos, N. J., & Friedman, P. A. (2017). GPCR Signaling and Trafficking: The Long and Short of It. *Trends in Endocrinology & Metabolism*, *28*(3), 213–226. http://doi.org/10.1016/j.tem.2016.10.007

Pringsheim, T., Jette, N., Frolkis, A., & Steeves, T. D. L. (2014). The prevalence of Parkinson's disease: A systematic review and meta-analysis. *Movement Disorders*, *29*(13), 1583–1590. http://doi.org/10.1002/mds.25945

Reitz, C. (2018). Retromer Dysfunction and Neurodegenerative Disease. *Current Genomics*, *19*(4), 279–288. http://doi.org/10.2174/1389202919666171024122809

Riedel, M., Goldbaum, O., Schwarz, L., Schmitt, S., & Richter-Landsberg, C. (2010). 17-AAG induces cytoplasmic alpha-synuclein aggregate clearance by induction of autophagy. *PloS One*, *5*(1), e8753. http://doi.org/10.1371/journal.pone.0008753

Savani, R. C., Cao, G., Pooler, P. M., Zaman, A., Zhou, Z., & DeLisser, H. M. (2001). Differential involvement of the hyaluronan (HA) receptors CD44 and receptor for HA-mediated motility in endothelial cell function and angiogenesis. *The Journal of Biological Chemistry*, *276*(39), 36770–36778. http://doi.org/10.1074/jbc.M102273200

Schütze, A., Vogeley, C., Gorges, T., Twarock, S., Butschan, J., Babayan, A., … Röck, K. (2016). RHAMM splice variants confer radiosensitivity in human breast cancer cell lines. *Oncotarget*, *7*(16), 21428–21440. http://doi.org/10.18632/oncotarget.7258

Scott, K. L., Kabbarah, O., Liang, M.-C., Ivanova, E., Anagnostou, V., Wu, J., … Chin, L. (2009). GOLPH3 modulates mTOR signalling and rapamycin sensitivity in cancer. *Nature*, *459*(7250), 1085–1090. http://doi.org/10.1038/nature08109

Seaman, M. N. J. (2012). The retromer complex - endosomal protein recycling and beyond. *Journal of Cell Science*, *125*(Pt 20), 4693–702. http://doi.org/10.1242/jcs.103440

Seaman, M. N. J., Harbour, M. E., Tattersall, D., Read, E., & Bright, N. (2009). Membrane recruitment of the cargo-selective retromer subcomplex is catalysed by the small GTPase Rab7 and inhibited by the Rab-GAP TBC1D5. *Journal of Cell Science*, *122*(14), 2371–2382. http://doi.org/10.1242/jcs.048686

Seaman, M. N., McCaffery, J. M., & Emr, S. D. (1998). A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast. *The Journal of Cell Biology*, *142*(3), 665–81.

Shannon, B., Soto-Ortolaza, A., Rayaprolu, S., Cannon, H. D., Labbé, C., Benitez, B. A., … Ross, O. A. (2014). Genetic variation of the retromer subunits VPS26A/B-VPS29 in Parkinson's disease. *Neurobiology of Aging*, *35*(8), 1958.e1-1958.e2. http://doi.org/10.1016/j.neurobiolaging.2014.03.004

Sharma, M., Ioannidis, J. P. A., Aasly, J. O., Annesi, G., Brice, A., Bertram, L., … Krüger, R. (2012). A multi-centre clinico-genetic analysis of the VPS35 gene in Parkinson disease indicates reduced penetrance for disease-associated variants. *Journal of Medical Genetics*, *49*(11), 721–726. http://doi.org/10.1136/jmedgenet-2012-101155

Sheerin, U.-M., Charlesworth, G., Bras, J., Guerreiro, R., Bhatia, K., Foltynie, T., … Wood, N. (2012). Screening for VPS35 mutations in Parkinson's disease.

Neurobiology of Aging, *33*(4), 838.e1-5. http://doi.org/10.1016/j.neurobiolaging.2011.10.032

Shipley, M. M., Mangold, C. A., & Szpara, M. L. (2016). Differentiation of the SH-SY5Y Human Neuroblastoma Cell Line. *Journal of Visualized Experiments*, (108), 1–11. http://doi.org/10.3791/53193

Small, S. A., Kent, K., Pierce, A., Leung, C., Kang, M. S., Okada, H., … Kim, T.-W. (2005). Model-guided microarray implicates the retromer complex in Alzheimer's disease. *Annals of Neurology*, *58*(6), 909–19. http://doi.org/10.1002/ana.20667

Small, S. A., & Petsko, G. A. (2015). Retromer in Alzheimer disease, Parkinson disease and other neurological disorders. *Nature Reviews Neuroscience*, *16*(3), 126–132. http://doi.org/10.1038/nrn3896

Sohr, S., & Engeland, K. (2008). RHAMM is differentially expressed in the cell cycle and downregulated by the tumor suppressor p53. *Cell Cycle (Georgetown, Tex.)*, *7*(21), 3448–3460. http://doi.org/10.4161/cc.7.21.7014

Sposini, S., Jean-Alphonse, F. G., Ayoub, M. A., Oqua, A., West, C., Lavery, S., … Hanyaloglu, A. C. (2017). Integration of GPCR Signaling and Sorting from Very Early Endosomes via Opposing APPL1 Mechanisms. *Cell Reports*, *21*(10), 2855–2867. http://doi.org/10.1016/j.celrep.2017.11.023

Subramaniam, S. R., & Chesselet, M.-F. (2013). Mitochondrial dysfunction and oxidative stress in Parkinson's disease. *Progress in Neurobiology*, *106–107*, 17–32. http://doi.org/10.1016/j.pneurobio.2013.04.004

Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., … Mesirov, J. P. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(43), 15545–15550. http://doi.org/10.1073/pnas.0506580102

Sudhaman, S., Behari, M., Govindappa, S. T., Muthane, U. B., Juyal, R. C., & Thelma, B. K. (2013). VPS35 and EIF4G1 mutations are rare in Parkinson's disease

among Indians. *Neurobiology of Aging*, *34*(10), 2442.e1-2442.e3. http://doi.org/10.1016/j.neurobiolaging.2013.04.025

Sugiura, A., McLelland, G.-L., Fon, E. A., & McBride, H. M. (2014). A new pathway for mitochondrial quality control: mitochondrial-derived vesicles. *The EMBO Journal*, *33*(19), 2142–2156. http://doi.org/10.15252/embj.201488104

Surmeier, D. J., Obeso, J. A., & Halliday, G. M. (2017). Parkinson's Disease Is Not Simply a Prion Disorder. *The Journal of Neuroscience*, *37*(41), 9799–9807. http://doi.org/10.1523/JNEUROSCI.1787-16.2017

Tabuchi, M., Yanatori, I., Kawai, Y., & Kishi, F. (2010). Retromer-mediated direct sorting is required for proper endosomal recycling of the mammalian iron transporter DMT1. *Journal of Cell Science*, *123*(5), 756–766. http://doi.org/10.1242/jcs.060574

Tang, F.-L., Erion, J. R., Tian, Y., Liu, W., Yin, D.-M., Ye, J., … Xiong, W.-C. (2015). VPS35 in Dopamine Neurons Is Required for Endosome-to-Golgi Retrieval of Lamp2a, a Receptor of Chaperone-Mediated Autophagy That Is Critical for -Synuclein Degradation and Prevention of Pathogenesis of Parkinson's Disease. *Journal of Neuroscience*, *35*(29), 10613–10628. http://doi.org/10.1523/JNEUROSCI.0042-15.2015

Tang, F.-L., Liu, W., Hu, J.-X., Erion, J. R., Ye, J., Mei, L., & Xiong, W.-C. (2015). VPS35 Deficiency or Mutation Causes Dopaminergic Neuronal Loss by Impairing Mitochondrial Fusion and Function. *Cell Reports*, *12*(10), 1631–1643. http://doi.org/10.1016/j.celrep.2015.08.001

Tanik, S. A., Schultheiss, C. E., Volpicelli-Daley, L. A., Brunden, K. R., & Lee, V. M. Y. (2013). Lewy body-like α-synuclein aggregates resist degradation and impair macroautophagy. *The Journal of Biological Chemistry*, *288*(21), 15194–15210. http://doi.org/10.1074/jbc.M113.457408

Tarca, A. L., Draghici, S., Khatri, P., Hassan, S. S., Mittal, P., Kim, J.-S., … Romero, R. (2009). A novel signaling pathway impact analysis. *Bioinformatics (Oxford, England)*, *25*(1), 75–82. http://doi.org/10.1093/bioinformatics/btn577

Taylor, J. P., Hardy, J., & Fischbeck, K. H. (2002). Toxic proteins in neurodegenerative disease. *Science (New York, N.Y.)*, *296*(5575), 1991–1995. http://doi.org/10.1126/science.1067122

Taylor, K. S. M., Cook, J. A., & Counsell, C. E. (2007). Heterogeneity in male to female risk for Parkinson's disease. *Journal of Neurology, Neurosurgery, and Psychiatry*, *78*(8), 905–6. http://doi.org/10.1136/jnnp.2006.104695

Tian, Y., Tang, F.-L., Sun, X., Wen, L., Mei, L., Tang, B.-S., & Xiong, W.-C. (2015). VPS35-deficiency results in an impaired AMPA receptor trafficking and decreased dendritic spine maturation. *Molecular Brain*, *8*(1), 70. http://doi.org/10.1186/s13041-015-0156-4

Tofaris, G. K. (2012). Lysosome-dependent pathways as a unifying theme in Parkinson's disease. *Movement Disorders*, *27*(11), 1364–1369. http://doi.org/10.1002/mds.25136

Trapasso, S., & Allegra, E. (2012). Role of CD44 as a marker of cancer stem cells in head and neck cancer. *Biologics: Targets & Therapy*, *6*, 379–383. http://doi.org/10.2147/BTT.S37906

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., … Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature Protocols*, *7*(3), 562–578. http://doi.org/10.1038/nprot.2012.016

Trousdale, C., & Kim, K. (2015). Retromer: Structure, function, and roles in mammalian disease. *European Journal of Cell Biology*, *94*(11), 513–521. http://doi.org/10.1016/j.ejcb.2015.07.002

Tsika, E., Glauser, L., Moser, R., Fiser, A., Daniel, G., Sheerin, U.-M., … Moore, D. J. (2014). Parkinson's disease-linked mutations in VPS35 induce dopaminergic neurodegeneration. *Human Molecular Genetics*, *23*(17), 4621–4638. http://doi.org/10.1093/hmg/ddu178

Tysnes, O. B., & Storstein, A. (2017). Epidemiology of Parkinson's disease. *Journal of Neural Transmission*, *124*(8), 901–905. http://doi.org/10.1007/s00702-017-1686-y

Vilariño-Güell, C., Wider, C., Ross, O. A., Dachsel, J. C., Kachergus, J. M., Lincoln, S. J., … Farrer, M. J. (2011). VPS35 mutations in parkinson disease. *American Journal of Human Genetics*, *89*(1), 162–167. http://doi.org/10.1016/j.ajhg.2011.06.001

Villa, N., Do, A., Hershey, J. W. B., & Fraser, C. S. (2013). Human eukaryotic initiation factor 4G (eIF4G) protein binds to eIF3c, -d, and -e to promote mRNA recruitment to the ribosome. *The Journal of Biological Chemistry*, *288*(46), 32932–40. http://doi.org/10.1074/jbc.M113.517011

Vitting-Seerup, K., Porse, B. T., Sandelin, A., & Waage, J. (2014). spliceR: an R package for classification of alternative splicing and prediction of coding potential from RNA-seq data. *BMC Bioinformatics*, *15*, 81. http://doi.org/10.1186/1471-2105-15-81

Wang, C.-L., Tang, F.-L., Peng, Y., Shen, C.-Y., Mei, L., & Xiong, W.-C. (2012). VPS35 regulates developing mouse hippocampal neuronal morphogenesis by promoting retrograde trafficking of BACE1. *Biology Open*, *1*(12), 1248–1257. http://doi.org/10.1242/bio.20122451

Wang, C., Niu, M., Zhou, Z., Zheng, X., Zhang, L., Tian, Y., … Zhang, Y. (2016). VPS35 regulates cell surface recycling and signaling of dopamine receptor D1. *Neurobiology of Aging*, *46*, 22–31. http://doi.org/10.1016/j.neurobiolaging.2016.05.016

Wang, D., Narula, N., Azzopardi, S., Smith, R. S., Nasar, A., Altorki, N. K., … Du, Y.-C. N. (2016). Expression of the receptor for hyaluronic acid mediated motility (RHAMM) is associated with poor prognosis and metastasis in non-small cell lung carcinoma. *Oncotarget*, *7*(26), 39957–39969. http://doi.org/10.18632/oncotarget.9554

Wang, S., & Bellen, H. J. (2015). The retromer complex in development and disease. *Development*, *142*(14), 2392–2396. http://doi.org/10.1242/dev.123737

Wang, W., Wang, X., Fujioka, H., Hoppel, C., Whone, A. L., Caldwell, M. A., … Zhu, X. (2016). Parkinson's disease–associated mutant VPS35 causes mitochondrial dysfunction by recycling DLP1 complexes. *Nature Medicine*, *22*(1), 54–63. http://doi.org/10.1038/nm.3983

Wen, L., Tang, F.-L., Hong, Y., Luo, S.-W., Wang, C.-L., He, W., … Xiong, W.-C. (2011). VPS35 haploinsufficiency increases Alzheimer's disease neuropathology. *The Journal of Cell Biology*, *195*(5), 765–779. http://doi.org/10.1083/jcb.201105109

Williams-Gray, C. H., Mason, S. L., Evans, J. R., Foltynie, T., Brayne, C., Robbins, T. W., & Barker, R. A. (2013). The CamPaIGN study of Parkinson's disease: 10-year outlook in an incident population-based cohort. *Journal of Neurology, Neurosurgery, and Psychiatry*, *84*(11), 1258–64. http://doi.org/10.1136/jnnp-2013-305277

Williams, E. T., Glauser, L., Tsika, E., Jiang, H., Islam, S., & Moore, D. J. (2018). Parkin mediates the ubiquitination of VPS35 and modulates retromer-dependent endosomal sorting. *Human Molecular Genetics*. http://doi.org/10.1093/hmg/ddy224

Winklhofer, K. F. (2014). Parkin and mitochondrial quality control: toward assembling the puzzle. *Trends in Cell Biology*, *24*(6), 332–41. http://doi.org/10.1016/j.tcb.2014.01.001

Wooten, G. F., Currie, L. J., Bovbjerg, V. E., Lee, J. K., & Patrie, J. (2004). Are men at greater risk for Parkinson's disease than women? *Journal of Neurology, Neurosurgery, and Psychiatry*, *75*(4), 637–9. http://doi.org/10.1136/JNNP.2003.020982

Yang, P.-T., Lorenowicz, M. J., Silhankova, M., Coudreuse, D. Y. M., Betist, M. C., & Korswagen, H. C. (2008). Wnt Signaling Requires Retromer-Dependent

Recycling of MIG-14/Wntless in Wnt-Producing Cells. *Developmental Cell*, *14*(1), 140–147. http://doi.org/10.1016/J.DEVCEL.2007.12.004

Yin, J., Liu, X., He, Q., Zhou, L., Yuan, Z., & Zhao, S. (2016). Vps35-dependent recycling of Trem2 regulates microglial function. *Traffic*, *17*(12), 1286–1296. http://doi.org/10.1111/tra.12451

Yun, S. P., Kim, H., Ham, S., Kwon, S.-H., Lee, G. H., Shin, J.-H., … Lee, Y. (2017). VPS35 regulates parkin substrate AIMP2 toxicity by facilitating lysosomal clearance of AIMP2. *Cell Death & Disease*, *8*(4), e2741–e2741. http://doi.org/10.1038/cddis.2017.157

Zavodszky, E., Seaman, M. N. J., Moreau, K., Jimenez-Sanchez, M., Breusegem, S. Y., Harbour, M. E., & Rubinsztein, D. C. (2014). Mutation in VPS35 associated with Parkinson's disease impairs WASH complex association and inhibits autophagy. *Nature Communications*, *5*(1), 3828. http://doi.org/10.1038/ncomms4828

Zhang, P., Wu, Y., Belenkaya, T. Y., & Lin, X. (2011). SNX3 controls Wingless/Wnt secretion through regulating retromer-dependent recycling of Wntless. *Cell Research*, *21*(12), 1677–1690. http://doi.org/10.1038/cr.2011.167

Zhang, Q., Wu, X., Chen, P., Liu, L., Xin, N., Tian, Y., & Dillin, A. (2018). The Mitochondrial Unfolded Protein Response Is Mediated Cell-Non-autonomously by Retromer-Dependent Wnt Signaling. *Cell*, *174*(4), 870–883.e17. http://doi.org/10.1016/j.cell.2018.06.029

Zhang, Y., Chen, S., Xiao, Q., Cao, L., Liu, J., Rong, T.-Y., … Chen, S.-D. (2012). Vacuolar protein sorting 35 Asp620Asn mutation is rare in the ethnic Chinese population with Parkinson's disease. *Parkinsonism & Related Disorders*, *18*(5), 638–640. http://doi.org/10.1016/j.parkreldis.2012.02.011

Zimprich, A., Benet-Pagès, A., Struhal, W., Graf, E., Eck, S. H., Offman, M. N., … Strom, T. M. (2011). A Mutation in VPS35, Encoding a Subunit of the Retromer Complex, Causes Late-Onset Parkinson Disease. *The American Journal of Human Genetics*, *89*(1), 168–175. http://doi.org/10.1016/j.ajhg.2011.06.008

APPENDIX A

Nestin-Positive/Sox2-Negative Cells Mediate Adult Neurogenesis of Nigral Dopaminergic Neurons in Mice

Citation: Albright, J. E., Stojkovska, I., Rahman, A. A., Brown, C. J., & Morrison, B. E. (2016). Nestin-positive/SOX2-negative cells mediate adult neurogenesis of nigral dopaminergic neurons in mice. Neuroscience Letters, 615, 50–54. http://doi.org/10.1016/j.neulet.2016.01.019

Introduction

Parkinson's disease (PD) is the most common motor disorder and the second most prevalent neurodegenerative disease. PD motor dysfunction (rigidity, tremor, bradykinesia, and postural instability) results from loss of dopaminergic (DA) neurons in the substantia nigra (SN). However, a basic understanding of the mechanism for DA neuron loss remains elusive. Consequently, only symptomatic treatments exist for PD and none that address the underlying neurodegeneration. Determining whether DA neurons are replenished in the adult SN is fundamental to understanding the loss of these neurons during PD. One possibility is that suppression of adult DA neurogenesis could be a driving force toward PD. Additionally, exploiting endogenous neurogenesis could offer potential avenues for therapy as well as better inform stem cell transplantation efforts.

Deficiencies in the process of adult neurogenesis have been strongly associated with Alzheimer's disease (AD), a neurodegenerative disorder sharing many epidemiological features with PD. Hippocampal neurons responsible for memory processing are preferentially lost in AD and evidence suggests this may be due, in part, to decreased neurogenesis (reviewed in [1]). Hippocampal adult neurogenesis occurs at a high rate throughout life but appears to decrease sharply in human AD as well as mouse

models of the disease. Interventions that promote adult neurogenesis improve preclinical model outcomes for histopathology as well as functionality. Neurogenesis of hippocampal neurons in adult humans and rodents begin from stem cell (SC) populations residing in the subgranular zone (SGZ) of the dentate gyrus. SC populations are relatively less differentiated and possess replicative capacity. Therefore, these cells can regenerate the SC pool as well as contribute progeny that can differentiate into neuronal precursor cells (NPCs) that are post-mitotic and committed to neuronal fates.

In addition to hippocampal neurons, olfactory neurons are replenished throughout the life of primates and rodents. SCs responsible for this process reside in the subventricular zone (SVZ). Once born, NPCs migrate a considerable distance to the olfactory bulb before integration and final differentiation into functional neurons. Currently, the SGZ and SVZ are the only known locations for SC generation in the adult mammalian brain. However, whether NPC pools reside elsewhere or transdifferentiation to NPCs from existing cell types occur remains to be determined.

The evidence for a contribution of adult neurogenesis to PD is inconclusive. Reduced proliferation of SVZ cells has been reported in human PD [2,3]. Additionally, a number of studies have reported that wide-spread overexpression of a protein known to be a monogenic cause of rare forms of PD, alpha-synuclein (WT, A53T, E46K, and A30P), in mice results in inhibition of neurogenesis in the SGZ and SVZ [4-8]. Moreover, transgenic mouse overexpression of PD-causing mutations in leucine-rich repeat kinase 2 (LRKK2) hinder proliferation and survival of SCs in the SVZ and SGZ [9]. However, a major caveat is that none of these mouse models induce DA neuron loss in the SN so the relevance to DA neurogenesis or even PD is debated. Administering the

PD model neurotoxins 6-OHDA or MPTP in rats or mice, respectively, does target DA neurons and inhibition of SC generation in the SVZ has been reported [2,10,11]. Conversely, other groups have reported increased neurogenesis following MPTP treatment [12]. In any event, the DA neuron loss in these models is rapid, easily achieving 80-100% loss within two weeks. Such a speedy loss does not mirror human progressive PD and cannot be explained by suppression of neurogenesis making connections between PD and this process using these neurotoxins dubious.

Attempts have been made to monitor adult DA neurogenesis in the SN directly using various cell lineage tracing methods with conflicting results [13-15]. Lack of compelling evidence for DA neurogenesis has reinforced the prevailing notion that the presence of a toxic stimulus or trophic factor withdrawal induces mature DA neurons to undergo death in a slow and progressive manner. Therefore, while still controversial, the predominate viewpoint is that stem cell replacement of adult DA neurons in the SN does not occur at appreciable levels [13]. The most prevalent method of DA neuron lineage tracing utilizes DNA incorporation of the thymidine analog bromodeoxyuridine (Brd-U) or similar reagent to monitor for cell division of SCs. This method is problematic for a number of reasons. Firstly, Brd-U is quite toxic to the organism and to dividing cells. Secondly, Brd-U can yield false-positive results for cells undergoing DNA repair. Additionally, these regimens of Brd-U use are adopted from studies focused in regions of the brain exhibiting high levels of neurogenesis such as the hippocampus. Moreover, use of this chemical requires double-immunolabeling for the DA neuron marker tyrosine hydroxylase (TH) and Brd-U. Resolution of double-positive cells requires great care using confocal microscopy and issues have been reported [15]. This method is also not

readily amenable to large sample numbers. Another common method of lineage tracing is by injection of retrovirus to permanently label SCs and NPCs. However, this requires knowledge of the precursor cell location which is currently unknown. The aim of this study was to overcome these limitations by employing a novel system that utilizes a drug that could be administered for 6-8 weeks without severe health complications, extends the post-labeling duration, and allows for accurate quantitation of DA neurons in the SN of mice by single-labeling.

Methods

All husbandry and study procedures involving mice were performed in accordance with Boise Veterans Affairs Medical Center Institutional Animal Care and Use Committee guidelines. *Th*^{lox} mice [16] were kindly provided by Drs. Richard Palmiter and Martin Darvas at The University of Washington. The *nestin-CRE*^{ERT2} [17] and *Sox2-CRE*^{ERT2} [18] mouse lines were obtained from The Jackson Laboratory. Mice were fed *ad libitum* and maintained on 12hr light/dark cycles. For tamoxifen treatment, three-month-old mice were provided 400mg/kg tamoxifen citrate chow (Envigo) as sole food source for 6 weeks. Mice were then placed back on standard rodent chow for the remainder of the study.

For Immunohistochemistry (IHC), mice (n = 6 per group) were anesthetized with 5% isoflurane and transcardially perfused with 10mL 0.1M phosphate buffer (PB) (pH 7.2) with 1mM EDTA followed by 10mL 4% paraformaldehyde (PFA) in PB. Brains were harvested and then fixed in 4% PFA for 24h at 4°C. Next, brains were cryoprotected in 30% sucrose/PB at 4°C until sunk. The brains were then frozen in OCT media and a Leica CM1950 cryostat used to cut 35 µm sections. Free-floating IHC was performed

with three PB washes between each step. Endogenous peroxidase activity was removed with H_2O_2 (3%) and methanol (10%) for 30min followed by blocking and permeabilization with 0.5% bovine serum albumin/0.2% triton X100/PB solution for 1h. To label DA neurons a primary antibody against tyrosine hydroxylase (EMD Millipore; AB152) (1:2000 dilution) was used overnight at 4°C. A secondary antibody conjugated to biotin (Jackson Immuno Research) (1:500 dilution) was then applied for 1h at room temperature followed by ABC staining (Vector Labs) (A and B solutions at 1:100 dilutions) for 1h. DA neurons were visualized using 3,3' diaminobenzidine (DAB) (Sigma Aldrich) substrate addition at 1mg/mL (pH 7.2) and allowed to develop for 4 min. The reaction was stopped in ddH_2O, sections placed on slides, dried, and coverslips mounted using Vectamount.

All DA neuron somas in the SN for all SN-containing sections in the right hemisphere (average of 38.6 sections per hemisphere) were counted using bright field microscopy. The left hemisphere was punctured with a 30-gauge needle at the time of cryosectioning to identify hemispheres. To correct for bisected DA neuron somas appearing in adjacent sections, the Abercrombie factor was determined and applied to counts [19].

Data is presented as mean ± SEM for groups. Statistical significance ($p < 0.05$) between groups was determined using multiple-way ANOVA followed by a *post hoc* Tukey's test using GraphPad Prism 6 software.

Results

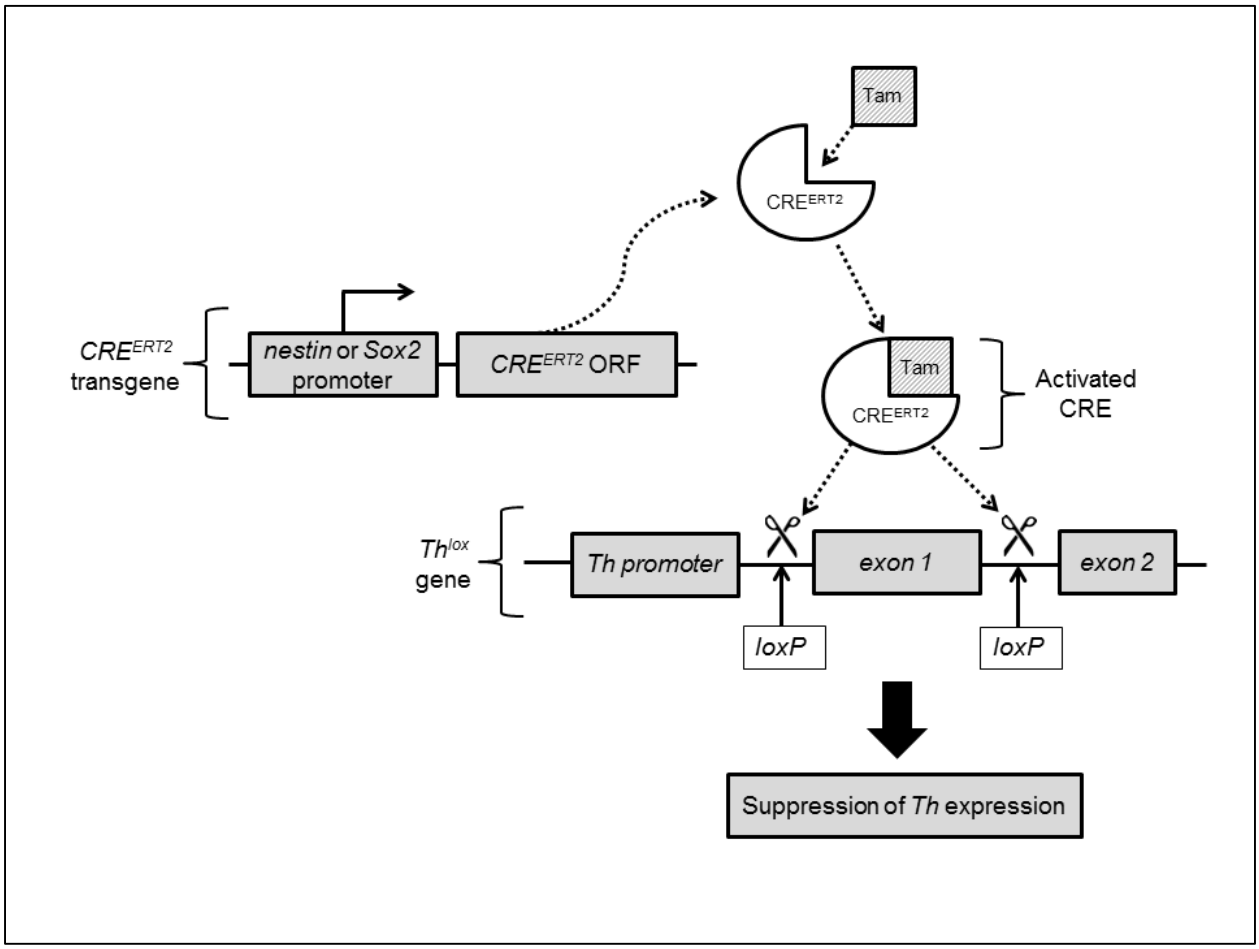

Figure A1. Genetic model to assess adult DA neurogenesis.

Tamoxifen (Tam) treatment activates CRE activity in *nestin* or *Sox2*-expressing cells resulting in *Th* gene silencing.

A genetic approach was developed to remove the DA neuron marker tyrosine hydroxylase (TH) from precursor cells in adult mice **(Fig. A1)**. Consequently, if DA neurons were replenished in adult mice, there should be a gradual loss of TH positive neurons in the SN over time following removal of the *Th* gene from precursor cells. Tamoxifen-inducible *Th* excision was chosen for this drug's ability to readily cross the

blood-brain barrier and good tolerability. To target NPCs for *Th* excision, *nestin* and *Sox2* promoters were selected to drive the expression of a tamoxifen-inducible CRE recombinase (CRE^{ERT2}) in SCs and/or NPCs. *Nestin* and *Sox2* are two of the most well-described neural progenitor markers and are not expressed in mature neurons making them good initial candidates to target DA neuron precursors. Both *nestin-*CRE^{ERT2} [17] and *Sox2-*CRE^{ERT2} [18] mouse lines were crossed with transgenic mice possessing the endogenous *Th* gene engineered with *loxP* sites flanking exon 1 [16] thereby allowing for the excision and silencing of *Th* in the presence of CRE activity. Three-month-old double or single (control) transgenic mice (*nestin*$^{CRE\text{-}ERT2}$***:***$Th^{lox/lox}$, *Sox2*$^{CRE\text{-}ERT2}$***:***$Th^{lox/lox}$, *or* $Th^{lox/lox}$) were treated with or without tamoxifen-laden chow for six weeks **(Fig. A2A)**. Six months following initiation of tamoxifen treatment brains were harvested and DA neurons in the SN counted. Remarkably, tamoxifen treated *nestin*$^{CRE\text{-}ERT2}$***:***$Th^{lox/lox}$ mice exhibited significantly fewer TH+ neurons in the SN than controls **(Fig. A2B, Fig AS1)**. In addition, no reduction in TH+ cells was observed in the SN of untreated *nestin*$^{CRE\text{-}ERT2}$***:***$Th^{lox/lox}$ mice or tamoxifen-treated *Sox2*$^{CRE\text{-}ERT2}$***:***$Th^{lox/lox}$ *or* $Th^{lox/lox}$ mice indicating that the loss in TH signal was due to *Th* gene excision and not the result of non-specific downregulation of *Th* expression by tamoxifen or the presence of the CRE transgene. Therefore, this result indicates that a nestin-positive population of precursors replenishes adult DA neurons in mice. However, it was surprising to find that *Sox2*$^{CRE\text{-}ERT2}$***:***$Th^{lox/lox}$ mice showed no TH+ cell loss given that Sox2 has been widely reported to be a ubiquitous neural SC marker [20,21].

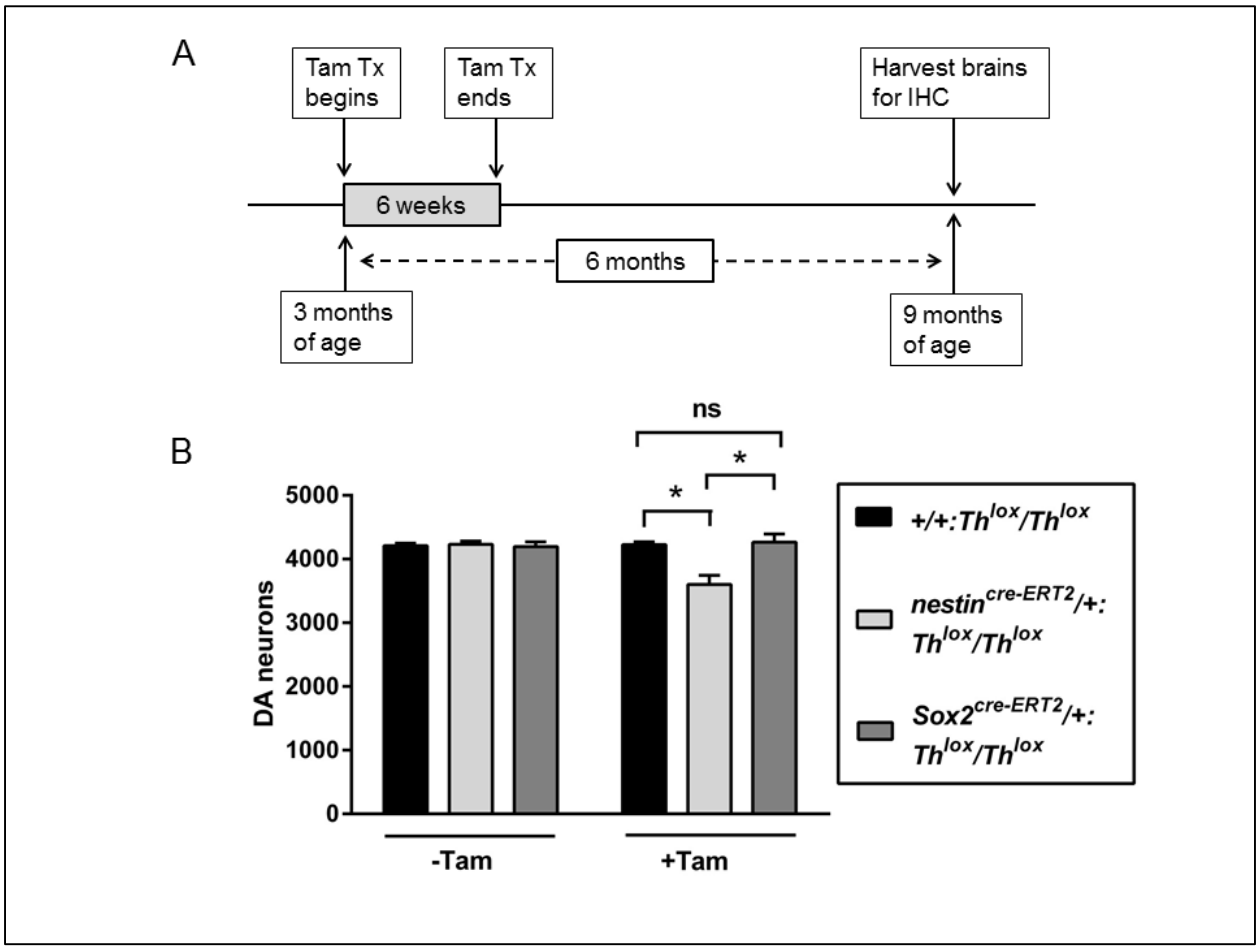

Figure A2. Adult DA neurogenesis by Nestin positive NPCs.

Six-week treatment with tamoxifen (Tam) citrate chow (400mg/kg chow) was used to activate CREERT2 activity in 3-month-old transgenic mice (A). NestinCRE-ERT2:Thlox/lox mice showed decreased DA neurons in the SN following Tam treatment (B). DA neurons in the SN were visualized by IHC (DAB). All sections containing SN from the right hemisphere were counted and neuron totals were corrected using the Abercrombie factor. Multiple-way ANOVA was performed followed by posthoc Tukey's test (6 mice per group; error bars = SEM; * = $p<0.05$; ns = not significant).

Discussion

This study provides compelling evidence for DA neurogenesis in the SN of adult mice by utilizing a novel cell lineage tracing model. Discovery of a nestin+ pool of DA progenitor cells will empower future studies to focus on the process of adult neurogenesis for DA neurons as well as enable locating these cells within the mammalian brain. Interestingly, this study found that Sox2 was not expressed in progenitors that gave rise to DA neurons in the adult SN. Sox2 expression is largely restricted to neural SCs, being turned down following cell cycle exit [22]. Additionally, Sox2 expression is closely linked to SC function, being one of four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) whose overexpression in concert can induce adult somatic cells to become embryo-like SCs [23]. Nestin is an intermediate filament protein expressed by SCs and NPCs *in vivo* and *in vitro* and may persist for a longer period of time than Sox2 expression (reviewed in [24]). Thus, targeting nestin-expressing cells will affect SOX2+ SCs in addition to a more differentiated progenitor population. Taken together, this may suggest that SOX2- cells giving rise to DA neurons in the adult SN exhibit greater differentiation and are not renewed by SCs. The implication would be that there is a limited supply of DA neuron progenitors available for adult neurogenesis. Therefore, depletion of this progenitor pool by normal turnover or by deleterious factors would result in an eventual loss of mature DA neurons. Another possibility is that SCs are not needed due to transdifferentiation of progenitors from another cell type. Investigation of additional neural SC and NPC markers by cell lineage tracing *in vivo* might address this.

The cause of DA neuron loss in PD has remained a mystery despite several decades of intense investigation. Over this time, considerable effort has been placed in identifying factors that induce death of mature DA neurons *in vitro* and *in vivo*. However, if DA neurogenesis is a natural ongoing homeostatic mechanism in the brain as evidence presented here suggests, inhibition of this process could be responsible, at least in part, for the progressive loss of DA neurons observed in PD. Reports of heightened sensitivity for SC and NPC populations in the adult brain to enhanced inflammatory response and other toxic stimuli support this notion [25-27]. Interestingly, the rate for DA neuron loss in the SN using a purely inflammatory model of PD reported by two groups mirrors TH+ cell loss by *nestin*-mediated *Th* excision shown in Fig. 2B. Extrapolating for single brain hemispheres, data presented by Frank-Cannon et al. [28] and Morrison et al. [29] indicate an approximate rate of 14.3 and 12.3 DA neuron loss/day, respectively. If the total TH+ cell loss in the SN observed in Fig. 2B is divided by the tamoxifen treatment duration of six weeks, a rate of 13.9 ± 1.2 is yielded. This is an intriguing correlation that would also suggest that the enhanced inflammatory model might mediate neurodegeneration by impeding adult neurogenesis of DA neurons. Future studies are warranted to further substantiate this association.

Findings from this study may direct stem cell replacement therapy for PD. Growth of stem cell research in the late 1980's generated great interest for use of this technology as a potential PD treatment. However, clinical trials using fetal stem cells to replace lost DA neurons have yielded promising but variable results [30,31]. The variability was believed to result from inconsistent source stem cells. Thorough characterization of neural stem cells *in vivo* may allow for the development of

appropriate and consistent cell types for therapy. Therefore, identification and characterization of the newly discovered nestin+/Sox2- DA neuron precursor population will provide a foundation for these investigations.

In conclusion, this study reveals the existence of a nestin+/SOX2- DA progenitor pool that replenishes mature DA neurons in the adult mouse SN. This finding has broad implications for brain biology and PD pathology and serves as a basis for future investigation of these important cells.

REFERENCES

[1] B. Winner, Z. Kohl, F.H. Gage, Neurodegenerative disease and adult neurogenesis, Eur. J. Neurosci. 33 (2011) 1139–1151. doi:10.1111/j.1460-9568.2011.07613.x.

[2] G.U. Höglinger, P. Rizk, M.P. Muriel, C. Duyckaerts, W.H. Oertel, I. Caille, et al., Dopamine depletion impairs precursor cell proliferation in Parkinson disease, Nat. Neurosci. 7 (2004) 726–735. doi:10.1038/nn1265.

[3] G.C. O'Keeffe, P. Tyers, D. Aarsland, J.W. Dalley, R.A. Barker, M.A. Caldwell, Dopamine-induced proliferation of adult neural precursor cells in the mammalian subventricular zone is mediated through EGF, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 8754–8759. doi:10.1073/pnas.0803955106.

[4] B. Winner, D.C. Lie, E. Rockenstein, R. Aigner, L. Aigner, E. Masliah, et al., Human wild-type alpha-synuclein impairs neurogenesis, J. Neuropathol. Exp. Neurol. 63 (2004) 1155–1166.

[5] L. Crews, H. Mizuno, P. Desplats, E. Rockenstein, A. Adame, C. Patrick, et al., Alpha-synuclein alters Notch-1 expression and neurogenesis in mouse embryonic stem cells and in the hippocampus of transgenic mice, J. Neurosci. Off. J. Soc. Neurosci. 28 (2008) 4250–4260. doi:10.1523/JNEUROSCI.0066-08.2008.

[6] S. Nuber, E. Petrasch-Parwez, B. Winner, J. Winkler, S. von Hörsten, T. Schmidt, et al., Neurodegeneration and motor dysfunction in a conditional model of Parkinson's disease, J. Neurosci. Off. J. Soc. Neurosci. 28 (2008) 2471–2484. doi:10.1523/JNEUROSCI.3040-07.2008.

[7] B. Winner, E. Rockenstein, D.C. Lie, R. Aigner, M. Mante, U. Bogdahn, et al., Mutant alpha-synuclein exacerbates age-related decrease of neurogenesis, Neurobiol. Aging. 29 (2008) 913–925. doi:10.1016/j.neurobiolaging.2006.12.016.

[8] F. Marxreiter, S. Nuber, M. Kandasamy, J. Klucken, R. Aigner, R. Burgmayer, et al., Changes in adult olfactory bulb neurogenesis in mice expressing the A30P mutant form of alpha-synuclein, Eur. J. Neurosci. 29 (2009) 879–890. doi:10.1111/j.1460-9568.2009.06641.x.

[9] B. Winner, H.L. Melrose, C. Zhao, K.M. Hinkle, M. Yue, C. Kent, et al., Adult neurogenesis and neurite outgrowth are impaired in LRRK2 G2019S mice, Neurobiol. Dis. 41 (2011) 706–716. doi:10.1016/j.nbd.2010.12.008.

[10] S.A. Baker, K.A. Baker, T. Hagg, Dopaminergic nigrostriatal projections regulate neural precursor proliferation in the adult mouse subventricular zone, Eur. J. Neurosci. 20 (2004) 575–579. doi:10.1111/j.1460-9568.2004.03486.x.

[11] B. Winner, M. Geyer, S. Couillard-Despres, R. Aigner, U. Bogdahn, L. Aigner, et al., Striatal deafferentation increases dopaminergic neurogenesis in the adult olfactory bulb, Exp. Neurol. 197 (2006) 113–121. doi:10.1016/j.expneurol.2005.08.028.

[12] J. Peng, L. Xie, K. Jin, D.A. Greenberg, J.K. Andersen, Fibroblast growth factor 2 enhances striatal and nigral neurogenesis in the acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease, Neuroscience. 153 (2008) 664–670. doi:10.1016/j.neuroscience.2008.02.063.

[13] O. Arias-Carrión, E. Yamada, N. Freundlieb, M. Djufri, L. Maurer, G. Hermanns, et al., Neurogenesis in substantia nigra of parkinsonian brains?, J. Neural Transm. Suppl. (2009) 279–285.

[14] M. Zhao, S. Momma, K. Delfani, M. Carlen, R.M. Cassidy, C.B. Johansson, et al., Evidence for neurogenesis in the adult mammalian substantia nigra, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 7925–7930. doi:10.1073/pnas.1131955100.

[15] H. Frielingsdorf, K. Schwarz, P. Brundin, P. Mohapel, No evidence for new dopaminergic neurons in the adult mammalian substantia nigra, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 10177–10182. doi:10.1073/pnas.0401229101.

[16] C.R. Jackson, G.-X. Ruan, F. Aseem, J. Abey, K. Gamble, G. Stanwood, et al., Retinal dopamine mediates multiple dimensions of light-adapted vision, J.

Neurosci. Off. J. Soc. Neurosci. 32 (2012) 9359–9368. doi:10.1523/JNEUROSCI.0711-12.2012.

[17] J. Battiste, A.W. Helms, E.J. Kim, T.K. Savage, D.C. Lagace, C.D. Mandyam, et al., Ascl1 defines sequentially generated lineage-restricted neuronal and oligodendrocyte precursor cells in the spinal cord, Dev. Camb. Engl. 134 (2007) 285–293. doi:10.1242/dev.02727.

[18] K. Arnold, A. Sarkar, M.A. Yram, J.M. Polo, R. Bronson, S. Sengupta, et al., Sox2(+) adult stem and progenitor cells are important for tissue regeneration and survival of mice, Cell Stem Cell. 9 (2011) 317–329. doi:10.1016/j.stem.2011.09.001.

[19] M. Abercrombie, Estimation of nuclear population from microtome sections, Anat. Rec. 94 (1946) 239–247.

[20] H. Suh, A. Consiglio, J. Ray, T. Sawai, K.A. D'Amour, F.H. Gage, In vivo fate analysis reveals the multipotent and self-renewal capacities of Sox2+ neural stem cells in the adult hippocampus, Cell Stem Cell. 1 (2007) 515–528. doi:10.1016/j.stem.2007.09.002.

[21] J.B. Kim, H. Zaehres, G. Wu, L. Gentile, K. Ko, V. Sebastiano, et al., Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors, Nature. 454 (2008) 646–650. doi:10.1038/nature07061.

[22] V. Graham, J. Khudyakov, P. Ellis, L. Pevny, SOX2 functions to maintain neural progenitor identity, Neuron. 39 (2003) 749–765.

[23] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell. 126 (2006) 663–676. doi:10.1016/j.cell.2006.07.024.

[24] J. Hsieh, Orchestrating transcriptional control of adult neurogenesis, Genes Dev. 26 (2012) 1010–1021. doi:10.1101/gad.187336.112.

[25] C.T. Ekdahl, J.-H. Claasen, S. Bonde, Z. Kokaia, O. Lindvall, Inflammation is detrimental for neurogenesis in adult brain, Proc. Natl. Acad. Sci. 100 (2003) 13632–13637. doi:10.1073/pnas.2234031100.

[26] M.L. Monje, H. Toda, T.D. Palmer, Inflammatory blockade restores adult hippocampal neurogenesis, Science. 302 (2003) 1760–1765. doi:10.1126/science.1088417.

[27] H. Fujioka, T. Akema, Lipopolysaccharide acutely inhibits proliferation of neural precursor cells in the dentate gyrus in adult rats, Brain Res. 1352 (2010) 35–42. doi:10.1016/j.brainres.2010.07.032.

[28] T.C. Frank-Cannon, T. Tran, K.A. Ruhn, T.N. Martinez, J. Hong, M. Marvin, et al., Parkin deficiency increases vulnerability to inflammation-related nigral degeneration, J. Neurosci. Off. J. Soc. Neurosci. 28 (2008) 10825–10834. doi:10.1523/JNEUROSCI.3001-08.2008.

[29] B.E. Morrison, M.C.G. Marcondes, D.K. Nomura, M. Sanchez-Alavez, A. Sanchez-Gonzalez, I. Saar, et al., Cutting edge: IL-13Rα1 expression in dopaminergic neurons contributes to their oxidative stress-mediated loss following chronic peripheral treatment with lipopolysaccharide, J. Immunol. Baltim. Md 1950. 189 (2012) 5498–5502. doi:10.4049/jimmunol.1102150.

[30] C.R. Freed, P.E. Greene, R.E. Breeze, W.Y. Tsai, W. DuMouchel, R. Kao, et al., Transplantation of embryonic dopamine neurons for severe Parkinson's disease, N. Engl. J. Med. 344 (2001) 710–719. doi:10.1056/NEJM200103083441002.

[31] C.W. Olanow, C.G. Goetz, J.H. Kordower, A.J. Stoessl, V. Sossi, M.F. Brin, et al., A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease, Ann. Neurol. 54 (2003) 403–414. doi:10.1002/ana.10720.

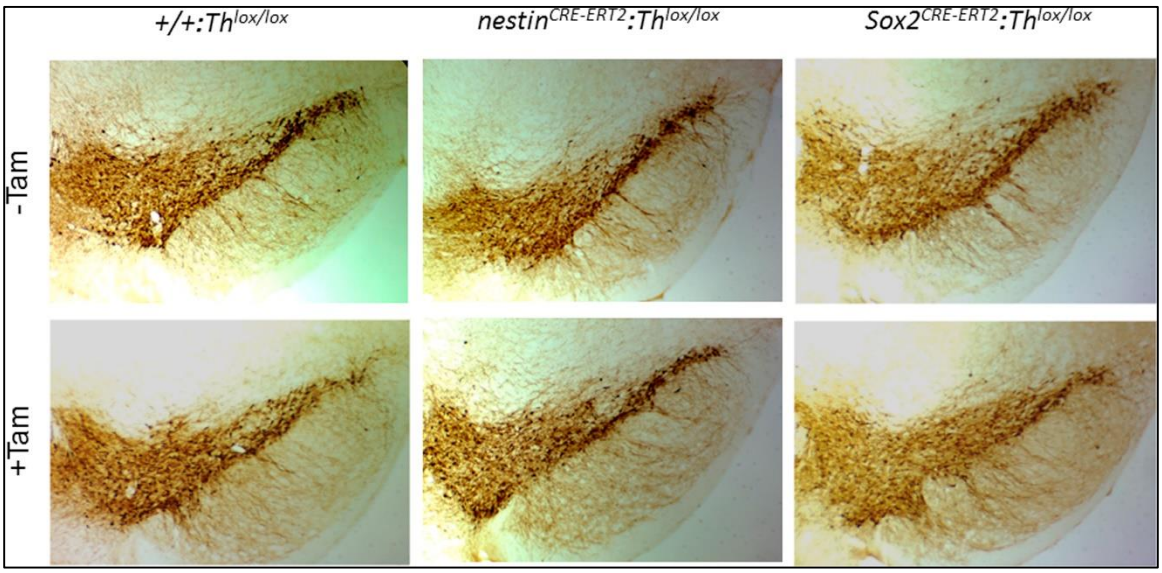

Figure AS1. IHC detection of TH+ cells in the SN.

Transgenic mice were sacrificed six months following treatment with or without tamoxifen (Tam)-laden chow. IHC (DAB) was then performed to quantify DA neurons in the SN using a TH antibody. Representative images from similar levels of the SN are shown. Note that differences in TH+ cell density are not readily apparent from comparison of single sections and require compiling DA neuron counts across all sections of each mouse.

APPENDIX B

Nigral Dopaminergic Neuron Replenishment in Adult Mice Through Ve-Cadherin-Expressing Neural Progenitor Cells

Citation: Rahman, A. A., Lai, N. K., Albright, J. E., Urquhart, P. E., Webb, A. R., & Morrison, B. E. (2017). Nigral dopaminergic neuron replenishment in adult mice through VE-cadherin-expressing neural progenitor cells. Neural Regeneration Research, 12(11), 1865–1869. http://doi.org/10.4103/1673-5374.219050

Abstract

The function of dopaminergic neurons in the substantia nigra is of central importance to the coordination of movement by the brain's basal ganglia circuitry. This is evidenced by the loss of these neurons, resulting in the cardinal motor deficits associated with Parkinson's disease. In order to fully understand the physiology of these key neurons and to craft potential therapies for their loss, it essential to determine if and how dopaminergic neurons are replenished in the adult brain. Recent work has presented evidence for adult neurogenesis of these neurons by Nestin+/Sox2- neural progenitor cells. We sought to further validate this finding and explore a potential atypical origin for these progenitor cells. Since neural progenitor cells have a proximal association with the vasculature of the brain and the subsets of endothelial cells are Nestin+, we hypothesized that dopaminergic neural progenitor cells might share a common cell lineage. Therefore, we employed a *VE-cadherin promoter-driven* CRE^{ERT2}***:***$TH^{lox/lox}$ transgenic mouse line to ablate the *tyrosine hydroxylase* gene function from endothelial cells in adult animals. After six months, but not three months, following the genetic blockade of *tyrosine hydroxylase* expression in VE-cadherin+ cells, we observed a significant reduction in *tyrosine hydroxylase*+ neurons in the substantia nigra. The results from this genetic

lineage tracing study suggest that dopaminergic neurons are replenished in adult mice by a VE-cadherin+ progenitor cell population potentially arising from an endothelial cell lineage.

Introduction

The existence of adult neurogenesis for dopaminergic (DA) neurons is controversial. However, a major limitation of previous studies is their reliance upon nucleotide analog, such as bromodeoxyuridine (BrdU), incorporation to identify replicating neurons (Kay and Blum, 2000; Zhao et al., 2003; Frielingsdorf et al., 2004; Aponso et al., 2008). This approach presumes that DA neurons in adult animals are generated in a replication-dependent manner from neural progenitor cells (NPCs). Recent studies have demonstrated in multiple animal systems, including mice, that quiescent, non-replicative neurogenesis occurs for some neural populations within the adult brain (Nishimura et al., 2011; Barbosa et al., 2015; Fuentealba et al., 2015). Additionally, previous studies that have focused on DA adult neurogenesis employed relatively short labeling and post-labeling periods, thereby decreasing the ability to detect the slow regeneration rates (Zhao et al., 2003; Frielingsdorf et al., 2004; Arias-Carrión et al., 2009). To avoid these potential pitfalls, we have established a transgenic mouse system that utilizes genetic cell lineage tracing observed over a six-month period.

Neurons are believed to arise from ectodermal tissue during embryonic development, while endothelial cells likely arise from the mesoderm. However, a strong connection exists among vascular physiology, NPCs, and Parkinson's disease (PD). NPCs in the adult brain have been reported to reside and differentiate in close association with capillaries, suggesting an intimate relationship with endothelial cells (Siegenthaler

and Pleasure, 2010). One unexplored possibility, supported by mounting evidence, is that specialized endothelial cells give rise to NPCs. For example, a number of factors associated with endothelial physiology (e.g., VEGF and endothelin) have robustly influenced outcomes in pre-clinical models of neurodegenerative disease (Wang et al., 2007; Kirby et al., 2015). Remarkably, similar to DA neurons, subsets of endothelial cells have been demonstrated to produce and respond to dopamine (Basu et al., 2001; Sorriento et al., 2012). In addition, when co-cultured with endothelial cells, it has been reported that mouse NPCs differentiate into endothelial cells (~6% rate) capable of forming capillary networks, which blurs the lines between these cell types (Wurmser et al., 2004).

NPCs express a number of endothelial cell markers and share common niches within the brain. For instance, Pramel7 was recently identified as a marker and mediator for a pre-implantation embryonic stem cell pluripotency ground state that has limited replicative self-renewal capacity (Graf et al., 2017). *In situ* RNA hybridization data on adult mouse brains available from the Allen Institute for Brain Science indicates that Pramel7 is expressed in the meninges, most likely in the endothelial cells (Lein et al., 2007). Moreover, the greatest concentration of positive signal arises from the meninges immediately ventral to the substantia nigra. This is particularly interesting given that Bifari et al. recently reported that quiescent NPCs generated during embryogenesis migrate from the meninges to differentiate into cortical neurons without replication in adult mice (Bifari et al., 2016). Previous work has also shown that DA neurons in the substantia nigra (SN) express IL-13RA1, a histological marker for endothelial cells (Morrison et al., 2012). Additionally, the discovery of a Nestin+/Sox2- DA NPC

population in adult mice reported by Albright and colleagues may indicate an atypical origin for these cells since Sox2 is broadly reported as a canonical marker for NPCs (Albright et al., 2016). Nestin+/Sox2- cells (NeuN-; non-neural) were also described by Hendrickson et al. and reside in a satellite position directly neighboring mature neurons in the adult rat brain. This suggests that these cells may represent previously uncharacterized NPCs (Hendrickson et al., 2011). In addition, Nestin+ endothelial cells are found throughout the body (Suzuki et al., 2010). Whether there is a connection between $Nestin^{+}/Sox2^{-}$ DA NPCs and Nestin+ endothelial cells, Nestin+ endothelial precursor cells, or $Nestin^{+}$ mesenchymal stem cells (Pacini and Petrini, 2014) that are known to regulate endothelial progenitor cell differentiation and which might directly transdifferentiate into endothelial cells (Xie et al., 2015), could be fundamental toward understanding DA neurogenesis. We therefore seek to determine if adult DA NPCs are derived from an endothelial cell lineage.

Materials and Methods

Experimental Animals

Procedures and husbandry for study animals were performed under Boise State University and Boise Veterans Affairs Medical Center Institutional Animal Care and Use Committee guidelines and in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011). The TH^{lox} mouse line (Jackson et al., 2012) used in this study was a kind gift from Dr. Martin Darvas and Dr. Richard Palmiterat (The University of Washington). The *VE-cadherin-*CRE^{ERT2} mice were generously provided by Dr. Luisa Iruela-Arispe (The University of California, Los

Angeles). During the course of this study, mice were provided food *ad libitum* and housed with 12-hour day-night cycles. Tamoxifen treatment was administered to three-month-old mice via standard rodent chow infused with 400 mg/kg tamoxifen citrate (Envigo; TD.130860), which was provided as the lone food source for a duration of six weeks. Following treatment, mice were returned to a standard chow diet.

Tissue processing and immunohistochemistry

Mice were anesthetized by isoflurane inhalation and transcardially perfused with a 50 mL phosphate buffer (PB; pH 7.2) containing heparin sodium salt (20 units/mL) followed by 50 mL of a 4% paraformaldehyde (PFA) in PB solution. Mouse brains were collected and placed in 4% PFA/PB overnight at 4°C. The following day, the brains were placed in 30% sucrose/PB at 4°C until they sunk (~72 hours). Tissue was then rapidly frozen in OCT and stored at -80°C until immunohistochemistry (IHC) was performed.

For IHC, OCT-embedded brains were equilibrated to a cryostat (Leica CM1950) at -20°C overnight. Sections were then cut at a thickness of 35 µm, placed into 12 well plates containing PB, and processed by free-floating IHC. Endogenous HRP activity was quenched with a 3% H_2O_2/10% methanol/PB solution incubated for 15 min at room temperature. Sections were then blocked and permeabilized with 0.5% BSA/0.25% Triton X100/PB. A TH antibody (Millipore; AB152) was used at 1:2,000 (in 0.5% BSA/PB), 4°C overnight. A biotinylated secondary antibody (Jackson Immuno Research; BA-1000) solution (1:500) was then added and incubated at room temperature for 1 h. Next, streptavidin-HRP was added according to manufacturer instructions (Vector Labs; PK-6200). Wash steps were performed using PB. TH labeling was visualized using 50 mg/mL 3,3'-Diaminobenzidine tetrahydrochloride hydrate (DAB; VWR cat # AAJ62216-

09) in PB. Sections were dried overnight, placed on slides, and then coverslips were mounted with Vectamount (Vector Labs; H-5000).

Quantification of DA neurons in the SN

DAB-labeled DA neurons were visualized with bright-field microscopy. For counting purposes, the left hemisphere was marked by piercing with a 20-gauge needle prior to sectioning the SN. Each tissue section containing SN from the right brain hemisphere was quantified following IHC. The SN was represented in an average of 38.9 sections/hemisphere across all samples. DA neuron counts included the substantia nigra pars compacta and excluded TH+ neurons located in the adjoining ventral tegmental area.

Isolation of endothelial cells and assessment of TH excision

Primary mouse endothelial cells were obtained by magnetic-activated cell sorting (MACS) as previously described (Shi et al., 1999). Briefly, 6-month-old mice were sacrificed, one liver lobe harvested, and placed in DMEM on ice. Livers were then minced using sterile razor blades. Each diced liver sample was then transferred to a tube containing 100mg of collagenase Type I (Rockland Immunochemical; MB-118-0100) in 25 mL of HBSS (+Calcium, +Magnesium, +1%BSA). Tubes were incubated with occasional mixing in a 37°C water bath for 60 minutes. Samples were then filtered through a 70µm sterile cell strainer and centrifuged at 300X*g* for 5 min at 4°C. Supernatants were discarded and pellets washed once with 0.1% BSA/PBS and centrifuged at 300 X*g* for 5 min at 4°C. Supernatants were aspirated and pellets resuspended in 0.5% BSA/PBS with 2mM EDTA. MACS was performed according to the manufacturers (MiltenyiBiotec) protocol using positive selection with CD31 microbeads and MS columns. Following endothelial cell isolation, DNA was extracted

using a mouse tissue DNA extraction kit (Biopioneer; MAQ-1). PCR was performed using the following primers: TAGGGAGATGCCAAAGGCTA; CAGGACCCAACAGAAGCATT. Thermocycling was done using the following parameters: annealing temperature = 62C, 30 seconds; extension time = 30 seconds; cycles = 35.PCR products were labeled with SYBR safe and resolved on a 1.5% agarose gel.

Statistical analysis

DA neuron counts were analyzed for significance ($p < 0.05$) among groups using a multiple-way ANOVA in conjunction with a *post hoc* Tukey's test using Graphpad Prism 6 software. Means are shown and the standard error of the mean is represented by error bars.

Results

DA NPCs express VE-cadherin

To test whether DA neurons arise from an endothelial cell lineage in adult animals, we generated the transgenic mouse shown in **Figure B1** where, in effect, the *TH* gene is being utilized as a genetic cell lineage tracing marker.

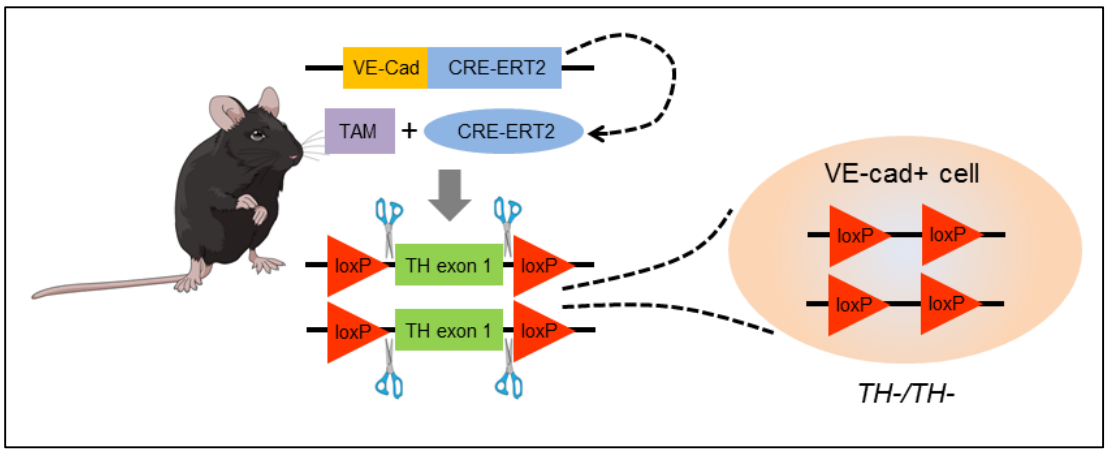

Figure B1. Inducible transgenic mouse model for adult DA neurogenesis assessment.

Three-month-old mice expressing CRE-ERT2 under the control of a VE-cadherin promoter were given tamoxifen (Tam)-laden chow, resulting in excision and silencing of the loxP-containing TH gene (homozygous) in VE-cadherin+ cells.

A Vascular Endothelial Cadherin promoter was used to drive expression of a tamoxifen-activatable CRE recombinase (*VE-cadherin CRE^{ERT2}*) in endothelial cells (Monvoisin et al., 2006). Upon activation of CRE activity by tamoxifen treatment in adult mice (three months of age), the *tyrosine hydroxylase* (TH)gene was silenced by excision in VE-cadherin positive cells. Six months after initiation of a six-week tamoxifen treatment, the mice were assessed for loss of nigral DA (TH+) neurons **(Figure B2A)**. Interestingly, *VE-cadherin$^{CRE-ERT2}$* mice receiving tamoxifen displayed a reduction in DA neurons within the SN **(Figure B2C)**. To verify whether this loss resulted from TH excision in DA progenitors or from existing mature DA neurons, we assessed DA neurons in a cohort of *VE-cadherin promoter-driven CRE^{ERT2}:TH$^{lox/lox}$* mice three months after tamoxifen administration **(Figure B2B)**. We observed no loss in nigral DA neurons in this group, indicating that TH excision occurred in cells other than mature DA neurons.

In addition, we confirmed successful TH excision from endothelial cells isolated from tamoxifen treated *VE-cadherin promoter-driven CRE^{ERT2}:TH$^{lox/lox}$* mice**(Figure 3)**.

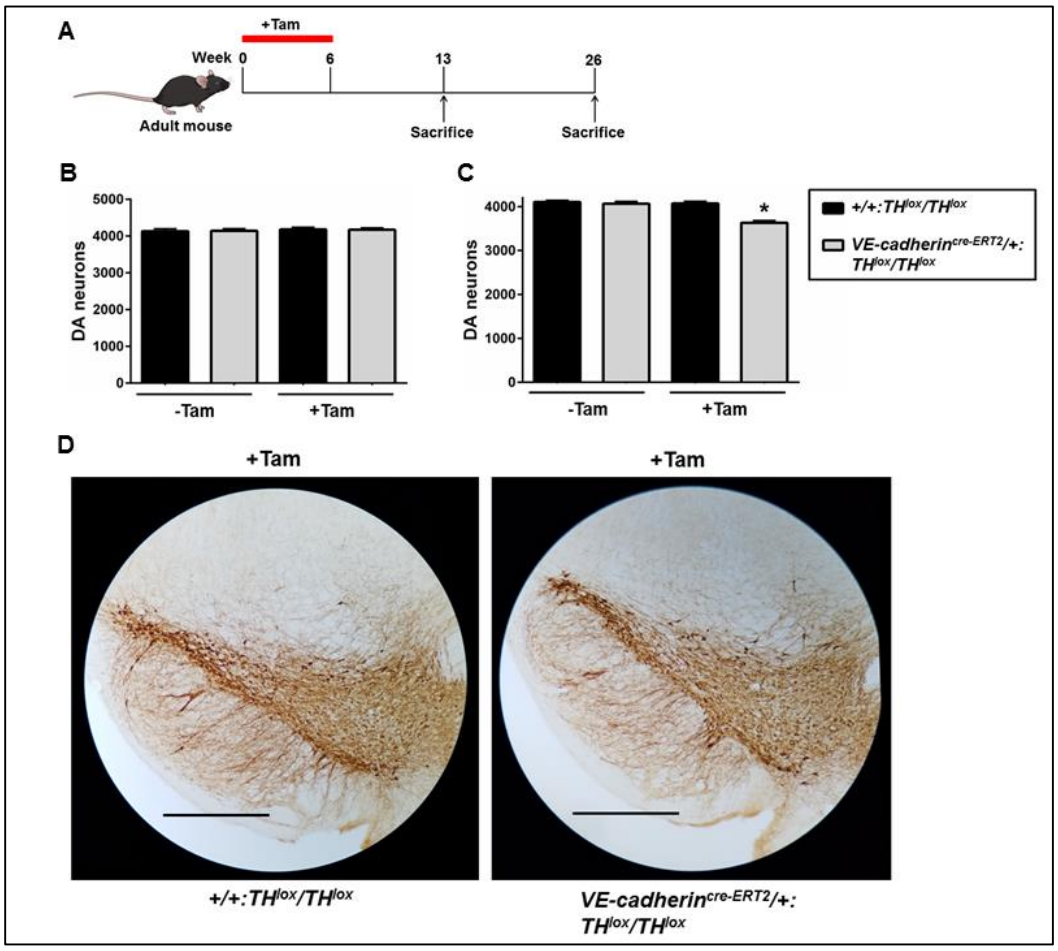

Figure B2. Adult nigral DA neurogenesis by VE-cadherin+ progenitor cells.

Tamoxifen (Tam) citrate-laden chow (400mg/kg food pellet) was fed *ad libitum* to three-month-old transgenic mice **(A)**. Brains were harvested, IHC performed for TH immunoreactivity, and DA neurons quantified at 13 and 26 weeks following Tam administration. No difference was observed among groups at three months following Tam treatment **(B)**. However, at 6 months post-treatment, *VE-cadherin$^{CRE-ERT2}$:TH$^{lox/lox}$* mice showed significantly reduced DA neurons in the SN compared with control groups **(C)**. Every tissue section in the right hemisphere containing SN was counted. Statistical analysis was performed using a multiple-way ANOVA in conjunction with a *post hoc* Tukey's test (group size = 6 mice; error bars = SEM; * = $p<0.05$ compared across all other groups). Nigral DA neurons were observed by IHC using a TH antibody (DAB). Representative images are shown (scale bar = 500 µm) **(D)**.

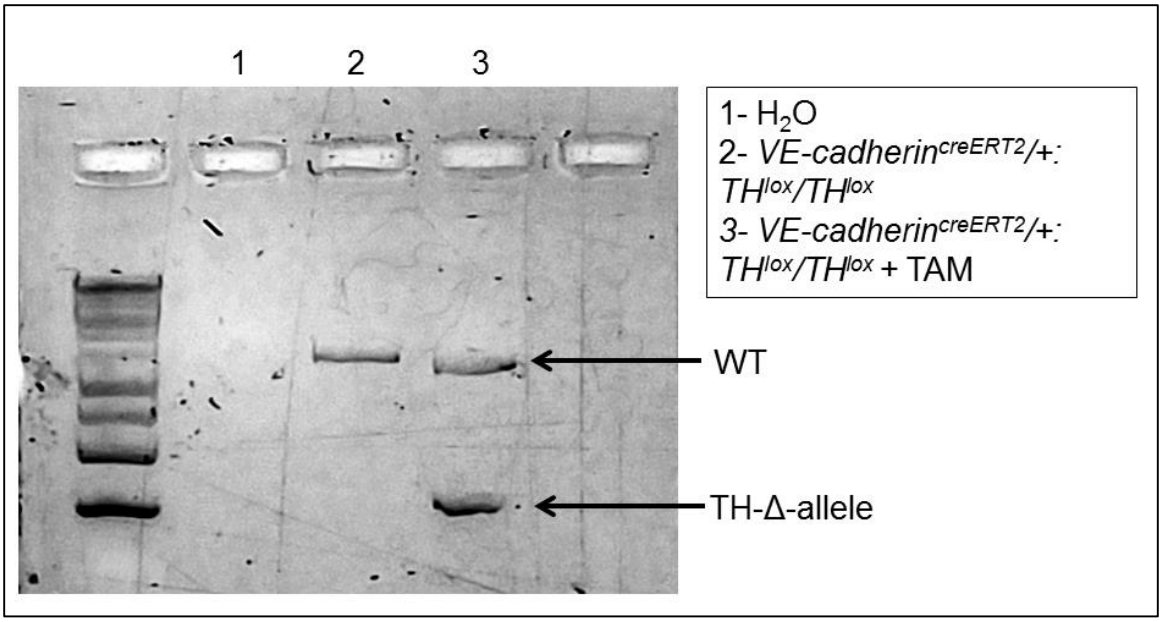

Figure B3. Endothelial TH excision from *VE-cadherin^{creERT2}: TH$^{lox/ox}$* transgenic mouse line treated with Tamoxifen.

Endothelial cells were isolated by MACS from livers harvested from untreated or TAM treated (400 mg/kg, 6 weeks chow) mice. DNA was then extracted and PCR performed to verify excision of TH in endothelial cells.

Discussion

Determining whether DA neurons undergo adult neurogenesis is of central importance to understanding fundamental brain physiology as well as developing potential therapies to combat their loss. Our current study has presented evidence in favor of dopaminergic neuron regeneration in adult mice through VE-cadherin-expressing NPCs. This finding builds upon previous work demonstrating that Nestin+/Sox2- NPCs in the adult mouse brain regenerate nigral DA neurons in a slow, progressive manner (Albright et al., 2016). These results are also consistent with the possibility of an endothelial cell-derived DA NPC population in adult mice.

The data presented here might also explain the mounting evidence linking DA neuron and endothelial physiology. In addition to the ability to produce and respond to dopamine, endothelial cells exhibit acute sensitivity to inflammatory response and oxidative stress (Pober and Sessa, 2007). Interestingly, oxidative stress has been shown to cause mesenchymal transdifferentiation of endothelial cells *in vivo*, providing further support for the plasticity of endothelial cells (Montorfano et al., 2014). If DA neurons share a close lineage with endothelial cells, our findings offer an explanation for the unique sensitivity of DA neurons toward oxidative and inflammatory response-based systemic insults. For example, peripheral administration of the potent oxidizing agent paraquat has been reported to cause the specific loss of nigrostriatal DA neurons (McCormack et al., 2002). In addition, systemic (intraperitoneal) administration of bacterial lipopolysaccharide twice weekly for six months results in chronic inflammatory response and in a selective, slow, and progressive loss of DA neurons in the SN (Qin et al., 2007; Frank-Cannon et al., 2008). Furthermore, the neurotoxic effect of MPTP on DA neurons can be completely halted with anti-inflammatory therapy (Aubin et al., 1998; Nomura et al., 2011). Similarly, NSAID use, particularly ibuprofen, has been correlated with a reduced incidence for PD suggesting the presence of an inflammatory instigator in human disease that targets these neurons (Chen et al., 2003; Gao et al., 2011). Therefore, a growing body of data indicates that DA neurons experience heightened sensitivity, compared with other neural populations, to insults that could, in part, be explained by physiology shared with endothelial cells.

Recent work by multiple groups in diverse systems has revealed the existence of replication-independent adult neurogenesis (Nishimura et al., 2011; Barbosa et al., 2015; Fuentealba et al., 2015; Bifari et al., 2016). These studies have shown that quiescent NPCs, produced and expanded during embryogenesis, directly differentiate into neurons as needed in the adult animal. If a similar mechanism is responsible for the generation of DA neurons in adults, it would provide an explanation for an inability to detect DA neurogenesis using assays that rely upon nucleotide analog incorporation (e.g., Brd-U) (Frielingsdorf et al., 2004). Additionally, previous studies employing a nucleotide labeling strategy have utilized a relatively short post-labeling period before brain harvesting, ranging from 8 hours to 6 weeks (Kay and Blum, 2000; Zhao et al., 2003; Frielingsdorf et al., 2004; Aponso et al., 2008). This approach could dramatically limit the ability to detect DA neurogenesis if, as our results suggest, this process occurs at a very slow rate and is, using our method, undetectable at three months post-labeling. If a replicative population of DA NPCs exists, then it will be essential to allow enough time for differentiation into a TH+ neuron to occur before assessment. Therefore, further investigations using alternative strategies, like the one demonstrated here, are warranted to identify the precise source of DA NPCs and characterize this regenerative process so that it can be harnessed for therapeutic benefit.

REFERENCES

Albright JE, Stojkovska I, Rahman AA, Brown CJ, Morrison BE (2016) Nestin-positive/SOX2-negative cells mediate adult neurogenesis of nigral dopaminergic neurons in mice. Neurosci Lett.

Aponso PM, Faull RLM, Connor B (2008) Increased progenitor cell proliferation and astrogenesis in the partial progressive 6-hydroxydopamine model of Parkinson's disease. Neuroscience 151:1142–1153.

Arias-Carrión O, Yamada E, Freundlieb N, Djufri M, Maurer L, Hermanns G, Ipach B, Chiu W-H, Steiner C, Oertel WH, Höglinger GU (2009) Neurogenesis in substantia nigra of parkinsonian brains? J Neural Transm Suppl:279–285.

Aubin N, Curet O, Deffois A, Carter C (1998) Aspirin and salicylate protect against MPTP-induced dopamine depletion in mice. J Neurochem 71:1635–1642.

Barbosa JS, Sanchez-Gonzalez R, Di Giaimo R, Baumgart EV, Theis FJ, Götz M, Ninkovic J (2015) Neurodevelopment. Live imaging of adult neural stem cell behavior in the intact and injured zebrafish brain. Science 348:789–793.

Basu S, Nagy JA, Pal S, Vasile E, Eckelhoefer IA, Bliss VS, Manseau EJ, Dasgupta PS, Dvorak HF, Mukhopadhyay D (2001) The neurotransmitter dopamine inhibits angiogenesis induced by vascular permeability factor/vascular endothelial growth factor. Nat Med 7:569–574.

Bifari F, Decimo I, Pino A, Llorens-Bobadilla E, Zhao S, Lange C, Panuccio G, Boeckx B, Thienpont B, Vinckier S, Wyns S, Bouche A, Lambrechts D, Giugliano M, Dewerchin M, Martin-Villalba A, Carmeliet P (2016) Neurogenic Radial Glia-like Cells in Meninges Migrate and Differentiate into Functionally Integrated Neurons in the Neonatal Cortex. Cell Stem Cell.

Chen H, Zhang SM, Hernán MA, Schwarzschild MA, Willett WC, Colditz GA, Speizer FE, Ascherio A (2003) Nonsteroidal anti-inflammatory drugs and the risk of Parkinson disease. Arch Neurol 60:1059–1064.

Frank-Cannon TC, Tran T, Ruhn KA, Martinez TN, Hong J, Marvin M, Hartley M, Treviño I, O'Brien DE, Casey B, Goldberg MS, Tansey MG (2008) Parkin deficiency increases vulnerability to inflammation-related nigral degeneration. J Neurosci Off J Soc Neurosci 28:10825–10834.

Frielingsdorf H, Schwarz K, Brundin P, Mohapel P (2004) No evidence for new dopaminergic neurons in the adult mammalian substantia nigra. Proc Natl Acad Sci U S A 101:10177–10182.

Fuentealba LC, Rompani SB, Parraguez JI, Obernier K, Romero R, Cepko CL, Alvarez-Buylla A (2015) Embryonic Origin of Postnatal Neural Stem Cells. Cell 161:1644–1655.

Gao X, Chen H, Schwarzschild MA, Ascherio A (2011) Use of ibuprofen and risk of Parkinson disease. Neurology 76:863–869.

Graf U, Casanova EA, Wyck S, Dalcher D, Gatti M, Vollenweider E, Okoniewski MJ, Weber FA, Patel SS, Schmid MW, Li J, Sharif J, Wanner GA, Koseki H, Wong J, Pelczar P, Penengo L, Santoro R, Cinelli P (2017) Pramel7 mediates ground-state pluripotency through proteasomal-epigenetic combined pathways. Nat Cell Biol 19:763–773.

Hendrickson ML, Rao AJ, Demerdash ONA, Kalil RE (2011) Expression of nestin by neural cells in the adult rat and human brain. PloS One 6:e18535.

Jackson CR, Ruan G-X, Aseem F, Abey J, Gamble K, Stanwood G, Palmiter RD, Iuvone PM, McMahon DG (2012) Retinal dopamine mediates multiple dimensions of light-adapted vision. J Neurosci Off J Soc Neurosci 32:9359–9368.

Kay JN, Blum M (2000) Differential response of ventral midbrain and striatal progenitor cells to lesions of the nigrostriatal dopaminergic projection. Dev Neurosci 22:56–67.

Kirby ED, Kuwahara AA, Messer RL, Wyss-Coray T (2015) Adult hippocampal neural stem and progenitor cells regulate the neurogenic niche by secreting VEGF. Proc Natl Acad Sci U S A 112:4128–4133.

Lein ES et al. (2007) Genome-wide atlas of gene expression in the adult mouse brain. Nature 445:168–176.

McCormack AL, Thiruchelvam M, Manning-Bog AB, Thiffault C, Langston JW, Cory-Slechta DA, Di Monte DA (2002) Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. Neurobiol Dis 10:119–127.

Montorfano I, Becerra A, Cerro R, Echeverría C, Sáez E, Morales MG, Fernández R, Cabello-Verrugio C, Simon F (2014) Oxidative stress mediates the conversion of endothelial cells into myofibroblasts via a TGF-β1 and TGF-β2-dependent pathway. Lab Investig J Tech Methods Pathol 94:1068–1082.

Monvoisin A, Alva JA, Hofmann JJ, Zovein AC, Lane TF, Iruela-Arispe ML (2006) VE-cadherin-CreERT2 transgenic mouse: a model for inducible recombination in the endothelium. Dev Dyn Off Publ Am Assoc Anat 235:3413–3422.

Morrison BE, Marcondes MCG, Nomura DK, Sanchez-Alavez M, Sanchez-Gonzalez A, Saar I, Kim K-S, Bartfai T, Maher P, Sugama S, Conti B (2012) Cutting edge: IL-13Rα1 expression in dopaminergic neurons contributes to their oxidative stress-mediated loss following chronic peripheral treatment with lipopolysaccharide. J Immunol Baltim Md 1950 189:5498–5502.

National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011) Guide for the Care and Use of Laboratory Animals, 8th ed. Washington (DC): National Academies Press (US). Available at: http://www.ncbi.nlm.nih.gov/books/NBK54050/.

Nishimura K, Inoue T, Yoshimoto K, Taniguchi T, Kitamura Y, Agata K (2011) Regeneration of dopaminergic neurons after 6-hydroxydopamine-induced lesion in planarian brain. J Neurochem 119:1217–1231.

Nomura DK, Morrison BE, Blankman JL, Long JZ, Kinsey SG, Marcondes MCG, Ward AM, Hahn YK, Lichtman AH, Conti B, Cravatt BF (2011) Endocannabinoid hydrolysis generates brain prostaglandins that promote neuroinflammation. Science 334:809–813.

Pacini S, Petrini I (2014) Are MSCs angiogenic cells? New insights on human nestin-positive bone marrow-derived multipotent cells. Front Cell Dev Biol 2:20.

Pober JS, Sessa WC (2007) Evolving functions of endothelial cells in inflammation. Nat Rev Immunol 7:803–815.

Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong J-S, Knapp DJ, Crews FT (2007) Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia 55:453–462.

Shi GP, Villadangos JA, Dranoff G, Small C, Gu L, Haley KJ, Riese R, Ploegh HL, Chapman HA (1999) Cathepsin S required for normal MHC class II peptide loading and germinal center development. Immunity 10:197–206.

Siegenthaler JA, Pleasure SJ (2010) There's no place like home for a neural stem cell. Cell Stem Cell 7:141–143.

Sorriento D, Santulli G, Del Giudice C, Anastasio A, Trimarco B, Iaccarino G (2012) Endothelial cells are able to synthesize and release catecholamines both in vitro and in vivo. Hypertens Dallas Tex 1979 60:129–136.

Suzuki S, Namiki J, Shibata S, Mastuzaki Y, Okano H (2010) The neural stem/progenitor cell marker nestin is expressed in proliferative endothelial cells, but not in mature vasculature. J Histochem Cytochem Off J Histochem Soc 58:721–730.

Wang Y, Jin K, Greenberg DA (2007) Neurogenesis associated with endothelin-induced cortical infarction in the mouse. Brain Res 1167:118–122.

Wurmser AE, Nakashima K, Summers RG, Toni N, D'Amour KA, Lie DC, Gage FH (2004) Cell fusion-independent differentiation of neural stem cells to the endothelial lineage. Nature 430:350–356.

Xie L, Zeng X, Hu J, Chen Q (2015) Characterization of Nestin, a Selective Marker for Bone Marrow Derived Mesenchymal Stem Cells. Stem Cells Int 2015:762098.

Zhao M, Momma S, Delfani K, Carlen M, Cassidy RM, Johansson CB, Brismar H, Shupliakov O, Frisen J, Janson AM (2003) Evidence for neurogenesis in the adult mammalian substantia nigra. Proc Natl Acad Sci U S A 100:7925–7930.