TRANSCRIPTIONAL REGULATION OF ALPHA-SYNUCLEIN BY GATA2 IN SUBSTANTIA NIGRA

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Max Parven Horowitz

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This dissertation was presented

by

Max Parven Horowitz

It was defended on

May 17, 2012

and approved by

Elias Aizenman, Ph.D., Department of Neurobiology

Edward A. Burton, M.D., D.Phil., Department of Neurology

Teresa G. Hastings, Ph.D., Department of Neurology

David G. Standaert, M.D., Ph.D., Department of Neurology, University of Alabama

Alan F. Sved, Ph.D., Department of Neuroscience

Dissertation Advisor: J. Timothy Greenamyre, M.D., Ph.D., Department of Neurology

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Parkinson's disease (PD) is a chronic, progressive movement disorder that affects millions of patients and their families worldwide. Treatment options address some of the symptoms but do not affect the progression of the disease. Central to the motor symptoms of PD are due, in part, to the slow, progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) and the consequent depletion of dopaminergic neurotransmission in the striatum. As these cells die, they accumulate toxic levels of various substances, such as the aggregation-prone protein alpha-synuclein (a-syn, *SNCA*) and iron. It is known that expression of *SNCA* is aberrantly high in SNc dopaminergic neurons in the PD brain, but the transcriptional mechanisms that participate in this dysregulation are poorly understood. Recently, GATA2, a transcription factor known for its critical role in hematopoiesis, was shown to regulate *SNCA* positively and directly *in vitro*; however, it is unknown whether this mode of regulation occurs *in vivo* and is thereby relevant to PD. In this dissertation project, we assessed the relevance of GATA2 in PD by testing two hypotheses in a mammalian model: 1) GATA2 positively regulates *SNCA in vivo* in SNc neurons, and 2) silencing *GATA2* expression in SNc neurons confers protection against the parkinsonian neurotoxin, rotenone. To test these hypotheses, it was necessary first to validate rat

as a suitable model for investigating GATA2 function in adult brain and to develop reagents for silencing *GATA2* expression *in vivo*. Using a viral-mediated gene silencing approach, we found that GATA2 positively regulates *SNCA* in SNc dopaminergic neurons in adult rat. However, silencing *GATA2* was not protective against rotenone treatment in the rat model of PD. Our findings are significant in that they provide the first *in vivo* demonstration of a transcription factor that regulates *SNCA*, a gene of central importance to PD pathogenesis. Although our data suggest that GATA2 is not a useful therapeutic target for PD, our findings shed some light on the role of GATA2 in adult SNc dopaminergic neurons, thus contributing to our understanding of GATA transcription factor biology in the adult brain.

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PREFACE

Throughout graduate school, I was fortunate to have received an enormous amount of help, guidance, and support from many people. The work I have done for this dissertation project is the product of input and technical assistance from many people whom I would like to take the opportunity to thank.

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1.0 GENERAL INTRODUCTION

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1.1 PARKINSON'S DISEASE

1.1.1 Disease Burden

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease (Thomas, 2009), and is a devastating, chronic illness for patients and their families. Advanced age is the most significant risk factor for developing PD. While overall PD prevalence is 100-200 per 100,000, it is estimated that 1.5-2% of the population over 65 years old is afflicted (de Rijk et al., 2000; 1997; Lang & Lozano, 1998a; 1998b). This corresponds to about 6 million PD patients worldwide, 1.5 million of whom reside in the United States (Thomas & Beal, 2007). These numbers are expected to increase in the next 20 years as the median age in the western populations rises.

PD affects all ethnic and racial groups, and is found throughout the world where life expectancy is sufficiently long for onset to occur (de Rijk et al., 1997; 2000; Lang & Lozano, 1998a; 1998b; Nutt & Wooten, 2005; Thomas, 2009). Men are at ~1.5 times higher risk for developing PD than women in most populations, which has been attributed to either increased occupational risk among men and/or risk-reducing biological factors in women (Elbaz & Tranchant, 2007; Elbaz et al., 2002). Because current therapies do not modify disease progression, patients typically live in declining health for 15 years after diagnosis (Lees, Hardy, & Revesz, 2009), and they frequently develop dementia, requiring institutionalization, and succumb to early death, most often from pneumonia (Elbaz & Tranchant, 2007; Lees et al., 2009). Given its chronic and invariably progressive nature, PD carries a significant burden for patients, their families, and the larger community.

1.1.2 Clinical Features

PD typically presents late in life – the median age of onset is 60 years old – with cardinal motor features, which include bradykinesia, rigidity, and tremor at rest (Savitt, Dawson, & Dawson, 2006). These motor features usually begin unilaterally, but progressively worsen and become bilateral over the course of disease. Postural instability (with consequent falls), as well as swallowing difficulties related to bulbar dysfunction, can become prominent and severely debilitating motor complications.

Although PD is considered to be a movement disorder based on its common presenting motor symptoms, many other central and peripheral nervous system domains are affected, and there is often significant autonomic dysfunction preceding diagnosis and occurring

concomitantly with motor symptoms (Chaudhuri & Schapira, 2009; Dubow, 2007; Lees et al., 2009; van Rooden et al., 2011). Common non-motor symptoms in PD include: cognitive impairment or dementia, depression, hallucinations, REM behavioral sleep disorder, fatigue, urinary urge incontinence, constipation, impotence, orthostatic hypotension, and anosmia (Lees et al., 2009). These symptoms represent a significant source of disability for patients and their care-providers since they typically do not respond to standard PD therapies.

PD is clinically heterogeneous in its presenting signs and symptoms, as well as the severity of its course, leading to the view that PD is in fact numerous distinct disease entities with a similar phenotype (van Rooden et al., 2011). As discussed below, the genetics of PD are consistent with this view. Studies have been undertaken to categorize PD sub-types based on motor and non-motor signs and symptoms. One recent article described four "clusters" of PD cases, which differ based on their non-dopaminergic features and the prevalence of motor complications during disease course (van Rooden et al., 2011). The goal of identifying clinical PD subtypes is to determine prognosis more accurately, to refine inclusion and exclusion criteria in clinical trials so that they have a better likelihood of success, and eventually to tailor the therapeutic approach more effectively.

1.1.3 Etiology & Neuropathology

In part, the motor symptoms of PD arise from progressive degeneration of neuromelanin-containing, dopaminergic neurons in the substantia nigra pars compacta (SNc) and a resultant depletion of dopamine in the striatum, where their axons terminate. The demise of these neurons is insidious and motor signs do not appear until 50-70% of these cells have died (Lesage &

Brice, 2009). However, meticulous pathological analysis performed by Braak suggests that the earliest signs of PD pathology (*i.e.* Lewy bodies) are found outside of the SNc – in ganglia within the digestive tract and in the olfactory bulb – and only later progress to SNc and cortex (Braak et al., 2003). The finding of extra-nigral pathology may explain some non-motor features of PD, however the degree to which staging of Lewy pathology reflects severity of symptoms remains a matter of debate (Jellinger, 2009).

What starts the process of neuronal dysfunction and death in PD is unclear. The vast majority of PD cases (~90%) are currently believed to be sporadic (idiopathic), meaning that they lack a clearly defined basis. Most cases of PD are thought to arise from a multifactorial interaction of environmental exposures, genetic predisposition, and aging (Horowitz & Greenamyre, 2010a). This means that there is not a single etiologic cause of PD. However, these factors appear to converge on a limited set of subcellular organelles and mechanisms (Greenamyre & Hastings, 2004). A minority of PD cases can be ascribed to mutations in single genes that have been convincingly demonstrated to be pathogenic (monogenic PD). Characterization of these causative genes has begun to lead to important insights into disease mechanisms.

1.1.3.1 Monogenic Forms of PD

The identification of monogenic forms of PD has led to major advances in our understanding of PD pathophysiology. To date, 16 loci (PARK1-16) have been associated with PD (Lesage & Brice, 2009). Of these, mutations in 5 genes have been confirmed to cause parkinsonian syndromes that resemble PD: the dominantly inherited alpha-synuclein (*SNCA*) and *LRRK2*, and the recessively inherited *parkin*, *PINK1*, and *DJ-1*. The remaining 11 loci require further

investigation to determine precisely the extent of their contribution to PD. Much of what is known about PD pathogenesis has come from work investigating monogenic forms of PD *in vitro* and *in vivo*. However, it should be noted that genetic mutations may give rise to clinical parkinsonism that does not necessarily involve the same pathogenic events as sporadic PD. In this context, it is prudent to exercise caution when extrapolating from monogenic cases of PD to pathogenic mechanisms in sporadic PD.

Alpha-synuclein (SNCA)

The first monogenic form of PD was discovered in families with autosomal dominant transmission of PD and missense mutations in the alpha-synuclein gene (*SNCA*) (Polymeropoulos et al., 1997). SNCA contains 7 exons and encodes a 140-amino acid protein (a-syn) that localizes mostly to pre-synaptic terminals (Breydo, Wu, & Uversky, 2012). Though the physiologic function of a-syn is incompletely understood, it appears to function in vesicle recycling via interacting with membranes (Cheng, Vivacqua, & Yu, 2011). Its natively unfolded structure renders the protein prone to aggregation under various pathophysiological conditions, including the three PD-related mutations (Breydo et al., 2012). Examination of the pathological hallmark of PD – the Lewy body – revealed that they contain abundant a-syn (Spillantini et al., 1997). This finding placed a-syn at the center of both familial and sporadic PD pathogenesis. Indeed, other PD-relevant factors were subsequently found to enhance a-syn aggregation and toxicity, including: oxidative modification; phosphorylation at serine 129; C-terminal truncations; interactions with metals, certain proteins or lipids; pesticides; and increased steady-state levels of a-syn *per se* (Breydo et al., 2012; Lundvig, Lindersson, & Jensen, 2005). Interaction with dopamine stabilizes a particularly toxic aggregation intermediate (protofibril)

(Conway, Rochet, Bieganski, & Lansbury, 2001). Under pathological conditions, a-syn is thought to exert cellular toxicity through a variety of mechanisms, largely involving interactions with cellular membranes (Breydo et al., 2012; Vekrellis, Xilouri, Emmanouilidou, Rideout, & Stefanis, 2011).

Intriguingly, while it was recognized that mutations in the *SNCA* cause PD, it was subsequently found that duplications and triplications of the locus containing *wild-type* (WT) *SNCA* also cause PD (Singleton et al., 2003). These locus multiplications lead to 1.5- to 2-fold increases in WT SNCA mRNA and protein levels relative to normal *SNCA* expression levels (Miller et al., 2004). Individuals with *SNCA* locus triplication develop disease with an earlier onset and more severe phenotype than those with gene duplication (Ross et al., 2008). This suggests that there is a "dosage effect" whereby higher levels of a-syn, whether WT or mutant, are associated with more toxicity. (Breydo et al., 2012)

It follows that if excessively high levels of WT a-syn are toxic *per se*, then any event contributing to increases in a-syn levels – *e.g.* decreased degradation of a-syn or increased transcription/translation of *SNCA* – may be involved in PD pathogenesis. In fact, there is evidence suggesting that both of these mechanisms are involved in PD. Alpha-synuclein can be degraded through various pathways depending on its ubiquitination state (Rott et al., 2011). These include degradation via the ubiquitin-proteasome system and lysosomal degradation via chaperone-mediated autophagy, both of which appear to be dysregulated in PD and are themselves targets of a-syn toxicity (Cuervo, Stefanis, Fredenburg, Lansbury, & Sulzer, 2004; Mazzulli et al., 2011; Xilouri, Vogiatzi, Vekrellis, Park, & Stefanis, 2009). Furthermore, glucocerebrosidase (*GBA1*) – a lysosomal enzyme that causes Gaucher's disease in homozygous ($GBA^{-/-}$) individuals – has been identified as a genetic modifier of a-syn levels and toxicity in

heterozygous (*GBA*$^{+/-}$) individuals and confers an approximately 5-fold increased risk of PD (DePaolo, Goker-Alpan, Samaddar, Lopez, & Sidransky, 2009; Marder, 2010). It is thought that GBA hypofunction in *GBA*$^{+/-}$ individuals leads to decreased lysosomal degradative activity with consequent accumulation and aggregation of a-syn (Cookson, 2011; T. M. Dawson & Dawson, 2011).

Dysregulation of *SNCA* at the transcriptional level also appears to be an important factor in a-syn accumulation and toxicity in PD. Chiba-Falek and colleagues examined SNCA mRNA levels in human post-mortem tissue homogenates and found significantly increased SNCA in PD SNc as compared to control (Chiba-Falek, Lopez, & Nussbaum, 2006). Gründemann and colleagues investigated the content of SNCA mRNA specifically within nigral dopamine neurons in post-mortem human brain using laser-capture microdissection and found that SNCA mRNA is significantly increased in surviving nigral dopamine neurons in PD relative to controls (Gründemann, Schlaudraff, Haeckel, & Liss, 2008). McLean and colleagues have recently reported similar findings in PD post-mortem specimens (J. R. McLean, Hallett, Cooper, Stanley, & Isacson, 2011). Notably, some reports have failed to show increases in SNCA mRNA in PD SNc (Dacshel et al., 2007; DePaolo et al., 2009; Kingsbury et al., 2004; Marder, 2010). These discrepancies may be explained by technical issues regarding method of assessment or variability in the severity of disease in the samples.

GATA transcription factors were the first transcription factor regulators of *SNCA* to be identified (see Section 1.3.4) and it has been proposed that they may contribute to PD pathogenesis by aberrantly increasing transcription of *SNCA* (and potentially other PD-relevant genes) (M. Chesselet, 2009; Scherzer et al., 2008). Additionally, two micro-RNAs (miRNA) have been identified as post-transcriptional down-regulators of *SNCA in vitro* (Doxakis, 2010;

Junn et al., 2009). In at least two reports, the levels of specific miRNAs have been reported as decreased in the PD SN relative to controls; however, more work is needed to confirm these findings and establish their relevance to a-syn accumulation in PD (Hebert & De Strooper, 2007; Kim et al., 2007).

Cis-regulatory elements also appear to contribute to *SNCA* transcriptional dysregulation. Common sequence variants – single nucleotide polymorphisms (SNP) – in the 3' untranslated region of *SNCA* were recently associated with an increased risk of PD in two large genome-wide association studies, one examining a population of European descent and the other a Japanese population (Satake et al., 2009; Simón-Sánchez et al., 2009). Additional studies have also shown that SNPs can influence SNCA levels in certain tissues and modify age of PD onset (Cardo et al., 2011; Fuchs et al., 2007). A PD-associated dinucleotide repeat polymorphism (Rep1) has been identified 10 kb upstream of the *SNCA* transcriptional start site and leads to increased *SNCA* expression in cells (Chiba-Falek & Nussbaum, 2001; Cronin et al., 2009; Farrer et al., 2001; Maraganore et al., 2006; Pals et al., 2004). A recent epidemiological study has shown that individuals with the expanded Rep1 allele plus history of head trauma have a greater risk of developing PD than either factor alone, providing an example in support of genetic and environmental interactions contributing to PD (Goldman et al., 2012).

Alpha-synuclein accumulation may not be simply a cell-autonomous event. For example, when the brains of PD patients who had received fetal mesencephalic tissue transplants came to autopsy they showed synuclein pathology (Lewy bodies) within dopaminergic neurons of the graft (Kordower, Chu, Hauser, Freeman, & Olanow, 2008a; Kordower, Chu, Hauser, Olanow, & Freeman, 2008b; Li et al., 2008). Since the grafts were from fetal tissue – and therefore not themselves expected to develop parkinsonian pathology – it was proposed that the pathology

spread from host to graft, either through noxious stimuli that initiate a-syn aggregation such as inflammation and/or through the cellular uptake of a-syn itself in a prion-like manner. Indeed, it was shown subsequently that a-syn can enter neurons from the extracellular space and participate in 'permissive templating', leading to aggregation of endogenous a-syn *in vitro* (Desplats et al., 2009; Volpicelli-Daley et al., 2011). The stereotyped "spread" of Lewy body pathology that Braak has proposed to occur over the course of PD (Braak et al., 2003) may be mechanistically supported by these findings. (However, the affected regions are not connected mono-synaptically, and the intervening connecting neurons seem to be unaffected by a-syn pathology.) Since endogenous a-syn pathology in PD patients appears to undermine cell transplant therapies, strategies to lower a-syn levels – either at the transcriptional or post-transcriptional level – are an active area of investigation.

Leucine-rich repeat kinase 2 (LRRK2)

Gain-of-function mutations in leucine-rich repeat kinase 2 (*LRRK2*) have been identified as the most common cause of familial PD (accounting for an estimated 5-10% of cases) as well as a relatively common cause of sporadic PD (an estimated 1-5% of cases) (Paisán-Ruíz et al., 2004; Satake et al., 2009; Simón-Sánchez et al., 2009; Westerlund, Hoffer, & Olson, 2010; Zimprich et al., 2004). One of the *LRRK2* mutations, G2019S, is remarkably common in certain populations. For example, in North African and Ashkenazi Jewish populations, up to 40% of familial and sporadic PD is associated with the G2019S mutation (Lees et al., 2009; Westerlund et al., 2010). This mutation has an age-dependent and highly variable level of penetrance, however. It has been estimated that the penetrance of the G2019 mutation is 28% at age 59, 51% at age 69, and 74% at age 79 (Healy et al., 2008). Thus, this and other *LRRK2* mutations may function more as

genetic risk factors than high penetrance disease genes in many cases. If so, there must be other factors, perhaps including environmental exposures, which influence risk of disease. Clinically, patients with *LRRK2* mutations are generally difficult to distinguish from patients with sporadic PD in that they exhibit similar age of onset, similar (if not more benign) signs and symptoms, and are generally responsive to L-DOPA treatment. Rarely, *LRRK2* mutations are associated with dementia and a tauopathy that is not seen in typical PD.

LRRK2 is a large gene (51 exons) that encodes a cytosolic serine/threonine kinase that likely acts in a complex with other proteins and is often found in association with membranes (e.g. endoplasmic reticulum, endosomes, mitochondria) in neuronal cell bodies, axons, and dendrites (Biskup et al., 2006). LRRK2 is unique in that it is a multi-domain protein that contains both a kinase domain and a GTPase domain in the same open reading frame (Kumar & Cookson, 2011). How these domains interact to influence LRRK2 function under physiological and pathophysiological conditions is still under investigation. PD-causative mutations have been localized to conserved regions throughout several disparate domains, and it is possible that mutations in different functional domains dysregulate LRRK2 in different ways. This may account for the diverse neuropathological features of *LRRK2* PD discovered upon autopsy: histopathology that ranges from nigral dopamine cell loss in the absence of Lewy bodies to nigral degeneration with Lewy body pathology involving the SNc as well as other structures – and may even include the presence of tau-positive neurofibrillary tangles or TDP43 proteinopathy (Dickson et al., 2009; Zimprich et al., 2004).

The most prevalent *LRRK2* mutation, G2019S, occurs within the kinase domain and causes an increase in kinase activity (West et al., 2005). The increased kinase activity of G2019S mutants may induce pathogenic signaling cascades, however 43 *LRRK2* mutations have been

associated with PD to date (8 confirmed pathogenic) and many of these do not alter LRRK2 kinase activity. Nevertheless, kinase-activating mutations have received the most attention and there is a great amount of effort to identify substrates of LRRK2-mediated phosphorylation and determine whether dysregulation of their corresponding pathways plays any significant role in PD pathogenesis from G2019S *LRRK2* mutations. LRRK2 kinase inhibitors have been developed and show some success in ameliorating certain parkinsonian features in animal models (B. D. Lee et al., 2010; Z. Liu et al., 2011).

Parkin

Mutations in *parkin*, an E3 ubiquitin ligase, cause a recessive, early-onset, slowly progressive parkinsonism (Kitada et al., 1998). Mutations in *parkin* account for the majority (~50%) of early-onset familial PD, and more than 100 distinct mutations have been identified (Westerlund et al., 2010). Point mutations and deletions can present in a homozygous or compound heterozygous fashion, in any combination. Single heterozygous mutations have been found in some PD cases, but these are difficult to interpret in terms of causality. There are also reports that SNPs in *parkin* may contribute to some cases of sporadic PD (S. J. Chung et al., 2011). Clinically, *parkin*-associated PD typically has an early onset (30s rather than 50-60s), a good response to L-DOPA, and a benign course.

As an E3 ubiquitin ligase, parkin catazlyzes the transfer of ubiquitin to target proteins to either mark them for degradation by the ubiquitin proteasome system, or for non-degradative signaling purposes. Several putative parkin ubiquitylation substrates have been identified *in vitro*, but only some of these putative parkin targets accumulate in the brains of patients with pathogenic parkin mutations and none of these proteins is found to be significantly elevated in

parkin knockout animals, suggesting E3 ubiquitin ligase redundancy for some substrates. Though additional parkin substrates will likely be identified, so far, accumulation of particular parkin substrates does not appear to be the mechanism by which mutant parkin causes toxicity. This leaves open the possibility that *parkin* mutations cause neurodegeneration via loss of a non-degradative (signaling) function.

Parkin has been implicated in mitochondrial maintenance, where it may, under some circumstances, play a role in selectively targeting depolarized mitochondria for mitochondria-specific autophagic degradation (mitophagy) (Narendra, Tanaka, Suen, & Youle, 2008). Narendra and colleagues have shown *in vitro* that depolarization of mitochondria with an uncoupling agent results in translocation of cytosolic parkin selectively to depolarized mitochondria and that these mitochondria are subsequently removed in an autophagic process (Narendra et al., 2008). These findings have been replicated by others, and it appears that the presence of PINK1 on depolarized mitochondria is critical for proper translocation of parkin (see 1.1.3.1.4). What parkin is doing once it reaches mitochondria in order to seal their degradative fate is unresolved at this point, but may involve ubiquitylation of a key mitochondrial target protein involved in initiating mitophagy. It is important to note that although these results are intriguing, the studies have been carried out primarily in immortalized cell lines, and there is as yet little evidence that these events occur in *bona fide* neurons (Van Laar et al., 2011).

PTEN-induced putative kinase 1 (PINK1)

Mutations in *PINK1* cause a rare form of early-onset autosomal recessive parkinsonism that is both clinically and neuropathologically similar to parkinsonism due to mutations in parkin (Valente, Abou-Sleiman, Caputo, Muqit, Harvey, Gispert, Ali, Del Turco, et al., 2004a). PINK1

is a nuclear-encoded, mitochondrial protein kinase. Most mutations occur in or near the kinase domain and consequently disrupt the kinase activity of the protein (Hatano et al., 2004; Valente, Salvi, Ialongo, Marongiu, Elia, Caputo, Romito, Albanese, et al., 2004b).

PINK1 knockout flies exhibit a phenotype of mitochondrial defects that is strikingly similar to that of *parkin* knockout flies and genetic rescue experiments demonstrated that parkin over-expression can rescue the *PINK1* knockout phenotype, though the converse does not occur (Clark et al., 2006; J. Park et al., 2006). This indicates that PINK1 not only operates in the same genetic pathway as parkin, but also acts upstream of parkin. Based on this finding and the possibility that parkin plays a role in targeting dysfunctional mitochondria for mitophagy (see above), efforts have continued to delineate the role of PINK1 and parkin in this process – reviewed recently by Youle and Narendra in (Youle & Narendra, 2011). More work is required – particularly in neurons – to define the biochemical pathway responsible for PINK1/parkin-dependent mitophagy in response to mitochondrial membrane impairment, but these studies have enhanced our understanding of PINK1 and parkin dysfunction in genetic forms of PD, and have further implicated mitochondrial dysfunction in the pathogenesis of PD.

DJ-1

Homozygous or compound heterozygous mutations in *DJ-1* are an extremely rare cause of early-onset, recessive parkinsonism (Bonifati et al., 2003), the neuropathology of which is unknown. DJ-1 is a redox-active protein expressed predominantly in astrocytes (at least in humans) that senses oxidative stress through modification of a critical cysteine and is subsequently translocated to mitochondria to protect the cell against oxidative stress (Canet-Aviles et al., 2004). Mutations lead to misfolding, decreased stability, and degradation of the protein, resulting

in lower protein levels and thereby loss of function (Westerlund et al., 2010). Consensus is lacking as to how exactly DJ-1 orchestrates cytoprotection as the protein has been proposed to have a broad range of functions – *e.g.* RNA binding, stabilization of antioxidant transcription factors, and acting as a cysteine protease. A role in signaling may explain these disparate observations, but details are still lacking. Despite these uncertainties, DJ-1 represents the third 'mitochondrial' protein (after parkin and PINK1) whose loss of function results in a parkinsonian syndrome and, as such, helps reinforce the concept that mitochondrial dysfunction is central to PD pathogenesis.

1.1.3.2 Environmental Factors and PD

The term "environmental factor" refers to any influence that originates from outside the genome. Environmental factors include compounds in the air we breathe, substances we ingest, and certain metabolic changes induced by activities we perform. When considering chemical toxicants, there is a tendency to focus on man-made synthetic compounds, but humans are exposed on a daily and chronic basis to a huge number of naturally-occurring compounds in the atmosphere and in our food and water supplies. If environmental factors influence PD pathogenesis or progression, they may do so either through direct action on the cells that die in PD, or through indirect actions – for example, by altering metabolism of other substances, permeabilizing the blood-brain barrier, activating the immune system, or altering hormonal signaling.

The list of environmental factors associated with PD continues to grow in number and diversity. Unfortunately, scientific support for many of the factors posited to contribute to PD risk is sometimes limited to retrospective (case-control) studies of low sample size that might be

biased by the subjects' ability to recall past exposures or activities. These studies can, at best, uncover associations, but they cannot prove causality. Demonstration that any of these factors might be causative requires the use of *in vitro* and *in vivo* disease modeling.

1.1.3.3 Age, Gender, and Lifestyle Factors

Among the factors most commonly associated with PD are increased age and male gender. The risk for sporadic PD increases in an age-dependent manner across all populations studied to date, and the penetrance of some monogenic forms of PD has also been shown to increase with age (*e.g* the G2019S mutation in *LRRK2*). The odds ratio for risk of developing PD in men compared to women is consistently found to be approximately 1.5 – 2.0 (Elbaz et al., 2002; Elbaz & Tranchant, 2007). It is unknown whether the underpinning for this difference is biological (*e.g.* hormonal), sociological (*e.g.* occupational) or both. Lifestyle and dietary habits seem to exert an influence over one's risk of developing PD as well. There are numerous reports of an inverse association between tobacco use and PD that is dose-dependent. Whether this effect is due to a component of tobacco or a feature related to tobacco users themselves remains to be determined; however, there is evidence that nicotine alters various components of dopaminergic systems and may protect against dopaminergic cell death (Quik, O'Leary, & Tanner, 2008). Caffeine consumption—from coffee and tea alike—is also associated with a dose-dependent decrease in risk of developing PD in some studies (Ascherio et al., 2001). Lastly, environmental factors related to occupational exposures – including pesticides (see below), industrial solvents (Gash et al., 2008; M. Liu et al., 2010), metals (Guilarte, 2011), and head trauma – have been associated with the development of PD or parkinsonism (Cannon & Greenamyre, 2011).

1.1.3.4 Pesticides

Initial investigation into the relationship between pesticides and PD began after several young intravenous drug users residing in the same area of California developed acute onset of severe parkinsonism that proved to be irreversible (Langston, Ballard, Tetrud, & Irwin, 1983). It was discovered that they had mistakenly injected 1-methyl-4-phenyl-1,2,3,6-tetrahyropyridine (MPTP), a potent neurotoxin that easily crosses the blood brain barrier into the brain, where its toxic metabolite selectively poisons dopaminergic neurons. This work provided the first proof-of-principle that an 'environmental' toxin could produce parkinsonism in man. Because MPTP acts by inhibiting mitochondrial function, this work also provided the first clue that mitochondrial impairment might be important in PD pathogenesis. Furthermore, a structural similarity between MPTP and the commonly used herbicide, paraquat, was noted (although more recent studies have shown that they have different mechanisms of toxicity). Nevertheless, based in part on this structural similarity, subsequent epidemiological studies found an association between agricultural pesticide use and death from PD (Ritz & Yu, 2000).

An increasing number of studies have reported an association between pesticides and PD; however, several issues make such studies difficult to perform and hard to interpret unambiguously. First, they often rely on reports from individual study subjects on the duration, amount, and type of pesticide exposure; thus, there may be reporter (recall) bias. Second, the accuracy of clinical diagnosis of PD is variable, and depends in large part on the training and experience of the investigators. Third, professional pesticide users are often exposed to several pesticides over time rather than a single agent. Lastly, the degree of exposure can vary based on the duration of use, the concentrations handled, and the safety precautions taken by pesticide handlers. Nonetheless, meta-analyses find an increased incidence of PD among subjects who

have a history of exposure to pesticides. The odds ratio for an association between professional pesticide use and PD development was 1.9 (95% CI = 1.5-2.5) in one such meta-analysis (Priyadarshi, Khuder, Schaub, & Shrivastava, 2000). Living in rural areas and drinking water from wells have both been associated with an increased risk of PD in some studies, and these associations are thought to be due to pesticide exposure.

Identifying individual pesticides that are associated with PD has been challenging for the aforementioned reasons, but recent well-designed studies have done so (Costello, Cockburn, Bronstein, Zhang, & Ritz, 2009; Kamel et al., 2006). In a case-control study involving 368 PD cases and 341 controls from the same area, Costello and colleagues used state records of pesticide use over a 25-year period and corresponding land maps where these pesticides were used in order to remove recall bias (Costello et al., 2009). They found a 75% increased relative risk for PD (95% CI = 1.13-2.73) in subjects who were exposed to paraquat and maneb (a fungicide that is often used concomitantly with paraquat) (Costello et al., 2009). Interestingly, subjects younger than 60 years old at the time of exposure who were exposed to either of these agents alone had a significantly higher risk of PD with an odds ratio (OR) of 2.27 (95% CI = 0.91-5.70) (Costello et al., 2009). This risk was significantly elevated when these younger subjects were exposed to both paraquat and maneb (OR = 4.17, 95% CI = 1.15-15.16) (Costello et al., 2009). In another report, Kamel and colleagues used data from the Agricultural Health Study, a large self-report study examining pesticide exposure and PD, and found an increased risk of PD associated with application of four individual pesticides: dieldrin, maneb, paraquat, and rotenone (Kamel et al., 2006).

Since two prominent features of cellular and animal models of PD are mitochondrial complex I inhibition and generation of oxidative stress, Tanner and colleagues focused a recent

epidemiological study on rotenone and paraquat – two pesticides known to act via these respective mechanisms (Tanner et al., 2011). They found that pesticide applicators have an increased risk of developing PD after exposure to rotenone (OR = 2.5, 95% CI = 1.3-4.7) or paraquat (OR = 2.5, 95% CI = 1.4-4.7) (Tanner et al., 2011). These findings are significant because they strengthen the relevance of the pathogenic mechanisms (mitochondrial complex I inhibition and oxidative stress) in experimental models of PD as well as animal models of PD that use these toxicants.

1.1.3.5 The Rotenone Rat Model of PD

Sub-chronic administration of the pesticide rotenone, a lipophilic mitochondrial complex I inhibitor, to rats reproducibly induces many of the hallmark behavioral, neurochemical, and neuropathological features of PD, as well as several non-motor features of human PD, including gastrointestinal dysfunction (Betarbet et al., 2000; Greenamyre, Cannon, Drolet, & Mastroberardino, 2010). Rotenone also causes a-syn accumulation, aggregation and oxidation as well as mitochondrial translocation of DJ-1 (Betarbet et al., 2000; 2006; Cannon et al., 2009). Iron accumulation in SN is a well-recognized feature of PD and is reproduced in the rotenone rat and monkey models of PD (Mastroberardino et al., 2009). Using these models, we found that the iron import protein, transferrin receptor 2 (TfR2), is upregulated in dopaminergic neurons in the SNc, which provides a plausible mechanism for the iron accumulation observed in these cells (Mastroberardino et al., 2009). This finding was then confirmed in post-mortem human PD SNc specimens (Mastroberardino et al., 2009). Therefore, the rotenone model not only recapitulates many key features of human PD, it is also predictive of features that may be critical to pathogenic processes.

1.1.3.6 Common Mechanisms?

Although the etiology of the vast majority of PD cases remains incompletely understood, the use of cellular and animal models based on some of the monogenic forms PD and environmental toxins has elucidated several key cellular pathways that appear to be central to PD pathogenesis. Several lines of evidence support a role for mitochondrial impairment in PD: First, cells taken from patients with sporadic PD show complex I impairment relative to controls (Banerjee, Starkov, Beal, & Thomas, 2009; Barroso et al., 1993; Haas et al., 1995). Second, two of the main neurotoxic models of PD utilize complex I inhibitors – MPTP and rotenone – to produce a parkinsonian phenotype (Betarbet et al., 2000; Jackson-Lewis, Blesa, & Przedborski, 2012). Third, the three genes known to cause autosomal recessive parkinsonism – *i.e. parkin*, *PINK1*, and *DJ-1* – have been implicated in maintaining mitochondrial function in the face of cellular stress, and their mutation leads to mitochondrial dysfunction (see 1.1.3.1.3-1.1.3.1.5). Fourth, there is evidence of mitochondrial DNA damage in PD and models thereof (Arthur, Morton, Dunham, Keeney, & Bennett, 2009; Sanders et al., n.d.). Associated with the mitochondrial dysfunction in PD is oxidative stress, which is another central feature of PD (Tsang & Chung, 2009).

Disruption of protein homeostasis is another critical feature of PD supported by several findings both in PD patients and in animal and cellular models of PD. First, the neuropathological hallmark of PD is the Lewy body, which is an intracellular insoluble inclusion of aggregated proteins, including a-syn. Lewy bodies arise when accumulated proteins either outstrip the ability of the cell to degrade them and/or where the degradative machinery is dysfunctional. Second, there is evidence that the ubiquitin proteasome system and autophagy – key cellular degradative systems – are impaired in PD. Third, mutation and locus multiplication

of *SNCA*, which encodes the aggregation-prone protein a-syn, cause PD with clinical severity and onset depending on gene copy number. Taken together, these converging lines of evidence strongly support a role for dysregulation of protein homeostasis in the pathogenesis of PD.

1.1.4 PD Treatment

There is currently no treatment that successfully modifies the progression of PD. Instead, PD therapy is limited to symptomatic treatment of motor symptoms. Given that many of these motor symptoms arise due to degeneration of nigrostriatal fibers and consequent dopamine depletion in the striatum, the majority of PD therapies are intended to replenish dopamine. These drugs fall into three general categories: 1) dopamine agonists, 2) inhibitors of the dopamine-catabolizing enzymes, and 3) the dopamine precursor, L-DOPA. L-DOPA is the most effective treatment for motor symptoms, but continued use leads to loss of efficacy and the development of dyskinesias in more than 80% of patients, which can be just as debilitating for patients as the bradykinesia or tremor for which they were initially given the drug (Yacoubian & Standaert, 2009). Deep-brain stimulation (DBS) is a surgical intervention in which an electrode is introduced into a brain region (usually the subthalamic nucleus) to modulate inhibitory tone in the basal ganglia, essentially accommodating for the loss of dopaminergic neurotransmission from the SNc (Pizzolato & Mandat, 2012). It is effective in treating some motor symptoms and reducing dependence on L-DOPA, but not all patients are good surgical candidates for DBS (Pizzolato & Mandat, 2012). Since none of the therapies modifies disease course or alleviates the (often more debilitating) non-motor symptoms of PD, novel therapeutics are desperately needed.

Despite the divergent causes implicated in the development of PD, the common set of dysregulated pathways that appear to be central to PD pathogenesis (see 1.1.3.2.4) have provided a rational basis for drug development. Unfortunately, all drugs tested to date in clinical trials – including antioxidants, anti-apoptotic drugs, anti-inflammatory drugs, mitochondria-stimulating drugs – have failed to demonstrate meaningful clinical neuroprotection (*i.e.* modification of disease course) (Meissner et al., 2011). This disappointing outcome has been attributed to several potential issues. First, there is no animal model that fully and accurately recapitulates PD pathobiology. With this limitation comes poor predictive power for determining whether a therapy will fare well in human clinical trials (Yacoubian & Standaert, 2009). Second, the design of clinical trials may be limiting investigators' ability to determine whether a therapy is neuroprotective. Strong placebo effect (which is common in PD clinical trials, (Goetz et al., 2008)), small (underpowered) trials, inadequate outcome measures, short-duration studies, and symptomatic effects of the experimental therapies can all mask neuroprotection, leading to failure of the therapy in clinical trials (Meissner et al., 2011; Yacoubian & Standaert, 2009). Third, the lack of surrogate indicators of nigrostriatal system preservation (*e.g.* brain imaging) make it difficult to determine whether an experimental therapy is indeed neuroprotective or just exerting symptomatic effects. Lastly, inclusion and exclusion criteria for these clinical trials are not based on specific PD sub-types (*e.g.* the sub-types described in (van Rooden et al., 2011), so a therapy that may be neuroprotective in one population of PD patients but not another cannot be determined.

While the majority of PD drugs in the market act on the dopamine system, the majority of drugs in PD clinical trials have non-dopamine system targets (Meissner et al., 2011). Among the more exciting non-dopaminergic drugs in development at the pre-clinical trial stage, are

inhibitors of LRRK2 kinase activity (for patients with the *LRRK2* G2019S mutation), various therapies attempting to lower a-syn toxicity (*e.g.* via gene silencing, stimulation of a-syn-degrading pathways, and aggregation inhibitors), and gene therapy approaches to deliver trophic factors (see 1.2.3) (Meissner et al., 2011; Yacoubian & Standaert, 2009). This shift from dopaminergic drugs to drugs targeting specific pathways relevant to disease pathogenesis seems like an appropriate response to the unmet needs in PD therapy; however, certain measures must be taken in order to ensure their best chances of success in clinical trials. In addition to clinical trial reform and refinement of PD animal models, discovery of a biomarker (or set of biomarkers) that can diagnose PD at a pre-symptomatic stage will be a significant therapeutic advance. Other biomarkers might track with disease progression or allow an assessment of therapeutic "target engagement". Such biomarkers would allow for earlier intervention and likely a greater dynamic range for testing therapeutic benefit.

1.2 GENE THERAPY

1.2.1 Introduction

The aim of gene therapy is to modulate gene expression therapeutically *in vivo* by delivering nucleic acids to cells of interest. Gene therapy may involve delivery of genes to compensate for those that are deficient in a patient (*e.g.* loss-of-function disorders), or nucleic acids to suppress the expression of endogenous genes (*e.g.* gain-of-function disorders). Just as there is a wide range of available nucleic acids that can be used depending on the therapeutic goal, there are also

different options for delivery systems. For *in vivo* delivery to brain, viral vectors are most commonly employed. This is for several reasons, among them: 1) they have manipulable genomes that can be engineered for the expression of nucleic acids of interest, 2) they can be infused systemically or into focused regions depending on the need, 3) and they can be selected based on their natural tropism for certain cell types or engineered to preferentially target certain cell populations.

Viral vectors that are commonly used for gene therapy applications include: lentiviruses, retroviruses, adenoviruses, adeno-associated viruses (AAV; see 1.2.2), and herpes simplex viruses. They differ in their basic biology, the target cell populations that they transduce, and their packaging capacity, and are exploited for different features depending on the therapeutic requirement. Since this dissertation involves the use of AAV2, we will restrict our discussion to AAV vectors.

1.2.2 Adeno-associated Virus (AAV)

Adeno-associated viruses are members of the *Parvoviridae* family, which are small, single-stranded DNA-containing viruses that lack an envelope (Giacca, 2010). They are prevalent in various species and are not associated with any disease (Giacca, 2010). At least 80% of adults have been infected with AAV and have antibodies against it (Giacca, 2010). There are over 100 different genetic variants (serotypes, AAV1-AAVn) that differ mostly in the protein composition of their capsids. Capsid proteins are the main determinant of viral tropism since it is they that interact with cell surface antigens to mediate adsorption and internalization of viral nucleic acids. For the most part, the cell surface antigens that AAV capsid proteins recognize are ubiquitously

expressed. For example, AAV2 recognizes heparin sulfate proteoglycans as well as αvβ5 integrin, FGFR1, and HFGR co-receptors. AAVs can differ not only in the cells they infect but also in their mode of transmitting their genome into the target cells.

The AAV genome is small (~4.7 kb) and consists of two open reading frames: a *rep* gene whose products are necessary for viral replication and a *cap* gene whose proteins compose the viral capsid (Giacca, 2010). Importantly, the coding region is flanked by 145-nucleotide inverted terminal repeat (ITR) sequences, which are required for all AAV functions (Giacca, 2010). For naturally occurring AAVs to replicate they depend on either co-infection with other "helper" viruses (*e.g.* adenovirus) or treatment with chemical agents (Giacca, 2010). This fact is exploited when engineering an AAV vector for use as a gene therapy agent.

AAV vectors are made by removing the entire viral genome (*rep* and *cap* genes) – except for the required ITR sequences – and cloning into their place a transcriptional cassette of interest that is no larger than 4-4.5 kb. The transcriptional cassette may be, for example, a constitutive promoter, the coding region of a therapeutic cDNA (or short-hairpin RNA), and a poly-A termination sequence. Since the viral genome now lacks the *rep* gene (required for replication and integration into the host genome) and the *cap* gene (required to form the viral capsid), viral production requires either co-infection of cells with a virus that provides these necessary cassettes or transfection of cells with a plasmid containing the cassettes. The *cap* cassette (which determines serotype) can be chosen based on the cell population intended for therapeutic AAV transduction. The presence of these "helper" cassettes allows for productive replication of recombinant virus, but these cassettes do not enter the recombinant viral genome. Therefore, the purified recombinant vector lacks the ability to replicate and make viral capsid *per se*.

When recombinant AAV vectors infect (transduce) their target cells, the viral genome does not integrate into the host genome; instead it remains transcriptionally active as an episome. This feature is advantageous over other viral vectors (*e.g.* lentiviruses) whose genome integrates into host DNA with the associated risk of insertional mutagenesis. Another advantage of the viral genome staying in an episomal form is that the therapeutic genes are less frequently methylated (and hence suppressed) by endogenous mechanisms, allowing expression of therapeutic genes to persist for long periods of time (to at least 8 years in non-human primates, (Hadaczek, Eberling, Pivirotto, Bringas, Forsayeth, & Bankiewicz, 2009a)).

1.2.3 Gene Therapy in PD

There are three general categories of gene therapy currently being evaluated in PD clinical trials. The first, like most of the PD drugs used clinically, involves delivering genes to restore dopamine production and release in the striatum. The second involves correcting basal ganglia signaling in a manner similar to DBS. The third category of gene therapy for PD delivers a growth factor in an attempt to prevent further degeneration of dopaminergic neurons.

The rationale in delivering genes that enhance endogenous dopamine production is to decrease reliance on L-DOPA, and therefore presumably decrease the likelihood of complications that frequently occur with long-term L-DOPA use (A. Björklund & Björklund, 2011; T. Björklund & Kirik, 2009). In the first of these clinical trials, a lentivirus was used to deliver three genes involved in dopamine synthesis to the striatum of patients with advanced PD: tyrosine hydroxylase (TH, the rate-limiting enzyme), aromatic amino acid decarboxylase (AADC), and GTP cylcohydrolase-1 (which is required for the synthesis of a cofactor necessary

for TH enzymatic activity). There has also been a smaller study in which AADC alone was delivered to the striatum of patients with advanced PD using an AAV vector (Mittermeyer et al., 2012). In this case, dopamine synthesis would only occur when the L-DOPA substrate is present, thereby allowing clinicians to safely titrate dopamine production by adjusting the L-DOPA dose they administer.

The loss of dopamine release from degenerating nigrostriatal fibers in PD results in overactivity of the subthalamic nucleus (STN) and down-stream dysregulation of basal ganglia signaling. DBS improves motor features of PD by normalizing subthalamic nucleus activity electrically. STN activity may also be normalized via local production of the inhibitory neurotransmitter, GABA. A small phase II clinical trial in which the gene for the rate-limiting GABA synthesis enzyme, glutamic acid decarboxylase (GAD), was delivered to the STN of PD patients via AAV2 was recently completed (LeWitt et al., 2011). Patients who received the viral infusion showed a statistically significant improvement in motor symptoms at 6 months as compared to patients who received sham surgery (LeWitt et al., 2011).

Neurturin is a member of the transforming growth factor-β family of growth factors. Its closely-related family member, glial cell line-derived neurotrophic factor (GDNF), has been shown to be required for the survival of catecholaminergic neurons, providing a rationale for delivery of such growth factors (Pascual et al., 2008). In fact, pre-clinical trials in the non-human primate MPTP model of PD showed that delivery of neurturin is neuroprotective (Herzog et al., 2009; Kordower et al., 2006). A clinical trial was undertaken in which PD patients received intraputamenal infusions of AAV2 containing the neurturin gene (Marks et al., 2010). At 12 months, infusion of AAV2-neurturin did not yield any improvement over sham surgery on a motor rating scale, though there was some improvement in secondary measures of the study

(Marks et al., 2010). The difference in outcome between the human PD and non-human primate MPTP studies has been ascribed to poor axonal transport of the therapeutic gene from the site of infusion (putamen) to the cell bodies in the SNc, a problem that could potentially be circumvented by infusing the viral vector instead directly into the SNc (Lewis & Standaert, 2011).

Although no PD gene therapy clinical trial has shown success in conferring neuroprotection, there are many approaches that are showing promise in pre-clinical development. Particularly attractive are therapeutic approaches that target cellular pathways known to be dysfunctional in PD, such as a-syn accumulation. Recently, we used AAV2 to deliver shRNA against SNCA to the rat SNc and found robust neuroprotection against rotenone, suggesting that this may be a therapeutically beneficial approach for PD patients (Cannon et al., n.d.).

1.3 GATA TRANSCRIPTION FACTORS

1.3.1 Basic Biology

GATA transcription factors play important roles as master regulators in the development and maintenance of various organ systems. The GATA factor family comprises GATA factors 1-6, which are zinc-finger transcription factors that bear a high degree of sequence and structural homology (Bresnick, Katsumura, Lee, Johnson, & Perkins, 2012). GATA factors 1 and 2 are largely involved in hematopoiesis, though GATA2 also plays important roles in various other

tissues, including brain. GATA3 functions as a master regulator in immune system development, where it is critical to T-cell lymphopoiesis (Hosoya, Maillard, & Engel, 2010); it is also important for development of some neuronal populations (Nardelli, Thiesson, Fujiwara, Tsai, & Orkin, 1999). In contrast to these "hematopoietic" GATA factors, GATA4/5/6 are important for visceral organ development. Each GATA factor recognizes the consensus sequence, WGATAA, which is referred to as a GATA element (T. Fujiwara et al., 2009). GATA factors recognize many of the same loci, though there are some GATA elements that are recognized specifically by one GATA factor and not another – *e.g.* GATA1- or GATA2-specific GATA elements – likely due to the presence of *cis* regulatory elements and/or co-factors (T. Fujiwara et al., 2009).

Because they are short sequences, GATA elements abound in the genome (there are an estimated 7 million motifs) (Bresnick et al., 2012). However, very few GATA elements (<1%) are actually occupied by GATA factors and influence gene expression (Bresnick et al., 2012). The majority of occupied, functional GATA elements are located outside of the proximal promoter, often ≥ 1kb up- or downstream; the most frequent occupied GATA elements are in introns and distant enhancers (T. Fujiwara et al., 2009). GATA elements do not have an absolute requirement for *cis* elements in order to bind GATA factors, but *cis* elements frequently do appear to influence GATA element functionality, often from a long distance away (Pi et al., 2010). Specific chromatin signatures in the form of post-translational histone modifications mark occupied GATA elements, however it is unclear whether this epigenetic modification occurs prior to or as a result of GATA factor binding (Bresnick et al., 2012). Occupancy and function of GATA elements are also influenced by *trans*-acting factors, such as transcriptional co-activators or co-repressors, which are likely cell type-dependent and bind chromatin in multi-protein complexes containing GATA factors (T. Fujiwara et al., 2009).

To add a further level of complexity to GATA factor transcriptional regulation, individual GATA factors can reciprocally regulate each other in some contexts and can also exert qualitatively different effects at the same GATA element. These forms of regulation are exemplified by GATA "switch sites," at which a GATA factor exerting positive regulation on a gene can be displaced by another GATA factor that suppresses expression of the same gene, or *vice-versa* (Snow et al., 2011). GATA switch sites are commonly used to drive wide-ranging changes in gene expression, often at cell fate decision points, such as differentiation. A classic example of GATA factor switching occurs during erythropoiesis, where GATA2 positively regulates its own expression until GATA1 levels rise and suppress *GATA2* expression (Bresnick, Lee, Fujiwara, Johnson, & Keles, 2010). Dynamic exchange of GATA1 and GATA2 at GATA switch sites occurs at up to one third of occupied GATA elements (Dore, Chlon, Brown, White, & Crispino, 2012). The result is a major shift in transcriptional output, from GATA2-regulated gene sets to GATA1-regulated gene sets.

For the most part, regulators of GATA transcription factor activity are unknown. There are conflicting reports about the importance of post-translational modifications – *e.g.* phosphorylation, acetylation, SUMOylation – in influencing GATA factor activity (Bresnick et al., 2012; Viger, Guittot, Anttonen, Wilson, & Heikinheimo, 2008). These discrepancies may be explained by the limitation of *in vitro* methods used to study GATA factor activity. Most *in vitro* activity studies are performed using GATA element-containing fragments linked to luciferase reporters, and often in conjunction with over-expression of GATA transcription factors. Because GATA factor occupancy and function are dependent on specific chromatin modifications and distal *cis* elements, the use of naked DNA in these experiments confounds interpretation. Similarly, over-expression of GATA transcription factors *in vitro* can lead to occupancy of

GATA elements that are normally unoccupied by endogenous GATA factors, again obscuring interpretation.

Characterization of GATA factor target genes (and hence GATA factor function) has been aided by various genome-wide analyses *in vitro*, in which a given GATA factor-expressing cell type is subjected to chromatin immunoprecipitation sequencing (ChIP-seq) followed by gene expression profiling after specific silencing of a GATA factor (T. Fujiwara et al., 2009). This method allows for unbiased examination of GATA factor gene targets and furthermore determines whether the bound GATA elements are functional and what the nature of the regulation is (positive *vs.* negative). In hematopoiesis, GATA2 functions to preserve progenitor stem cells in an undifferentiated state whereas GATA1 is active in differentiation of erythrocytes, megakaryocytes, eosinophils, and mast cells (Wozniak et al., 2008). Germ-line deletion of either GATA factor results in embryonic lethality due to severe anemia (Pevny et al., 1991; Tsai et al., 1994). The sets of genes that each regulates therefore relates to the master function that each drives – *e.g.* globin chain synthesis for GATA1 as immature erythroblasts start to produce hemoglobin. So far, most examination has been performed in hematopoietic cell lines, focusing on GATA1 and GATA2 gene targets (T. Fujiwara et al., 2009). However, examination of other cell types has revealed that GATA factor gene targets appear to be cell type-dependent, along with the co-factors and co-regulators they utilize. For example, whereas GATA2 target genes in hematopoietic cell lines relate to erythropoiesis, GATA2 target genes detected in an endothelial cell line suggest that GATA2 functions in inflammatory pathways (Linnemann, O'Geen, Keles, Farnham, & Bresnick, 2011). Importantly, no such characterization has been performed *in vivo*, so it is not clear to what degree the *in vitro* findings can be generalized.

1.3.2 GATA2

Rat *GATA2* has 6 exons, the first two of which contain alternative transcriptional start sites that are used to drive tissue-specific expression: hematopoietic and neuronal expression is driven by exon IS; expression in other tissues is driven by exon IG (A. Björklund & Björklund, 2011; Shimahara, Yamakawa, Nishikata, & Morishita, 2010). Exons 4 and 5, which contain the zinc-fingers, are highly conserved across the six GATA factors. GATA2 has large untranslated regions (1967 bp of the 3,411-bp rat mRNA is non-coding), suggesting that post-transcriptional regulation may occur, however no such regulators have been convincingly identified to date. The main factor that positively regulates *GATA2* transcription (at least in hematopoietic cell lines) is *GATA2* itself, though there is dynamic interaction with GATA1, which suppresses *GATA2* (Bresnick et al., 2012). GATA2 protein is degraded by the proteasome and, *in vitro* experiments (again in hematopoietic cell lines) have shown that GATA2 half-life is approximately 1 hour, suggesting that post-translational regulation may also play a role in regulating GATA2 activity (Lurie, Boyer, Grass, & Bresnick, 2008; Minegishi, Suzuki, Kawatani, Shimizu, & Yamamoto, 2005).

Although the majority of investigations into GATA2 target genes (and hence function) have been conducted in hematopoietic systems, where GATA2 regulates gene sets related to erythroid phenotype, cell cycle, and autophagy (T. Fujiwara et al., 2009; Kang et al., 2012), GATA2 is also expressed in non-hematopoietic tissues, including: neurons (Kala et al., 2009), endothelial cells (Dorfman, Wilson, Bruns, & Orkin, 1992), placenta (Ma et al., 1997), the urogenital system (Zhou et al., 1998), and pituitary (D. F. Gordon et al., 1997). Of these additional cell types, genome-wide analysis of GATA2 target genes has only been carried out in

endothelial cells, where it was shown to regulate many genes related to endothelial cell phenotype and inflammation, in concert with the inflammatory gene regulator, AP-1 (Linnemann et al., 2011). This finding illustrates more generally that GATA transcription factors regulate target genes in a cell type-specific manner, which is likely influenced by the presence of additional regulatory factors.

Mutations in *GATA2* have been associated with various hematopoietic and non-hematopoietic diseases. Four hematopoietic syndromes can arise from germline mutations in *GATA2* and carry an increased incidence of myelodysplastic syndrome and acute myeloid leukemia, often with associated immune dysfunction (Dickinson et al., 2011; Hahn et al., 2011; Ostergaard et al., 2011). Features of these syndromes are explained by the critical and broad hematopoietic role that *GATA2* plays. GATA2 SNPs have also been associated with increased risk for familial early-onset coronary artery disease (Connelly et al., 2006). This may be explained by the role that *GATA2* has recently been shown to play in regulating inflammatory gene sets in endothelial cells since inflammation is a critical process in atherosclerosis (Linnemann et al., 2011). Lastly, high levels of (wild-type) GATA2 have been linked to increased risk of prostate cancer recurrence (Böhm, Locke, Sutherland, Kench, & Henshall, 2009), suggesting that not only mutations but also aberrant levels of wild-type GATA2 may lead to cellular dysfunction and disease.

1.3.3 GATA Factors in the Brain(T. Fujiwara et al., 2009; Lurie et al., 2008)

The only GATA transcription factors that are normally expressed in mammalian brain are *GATA2* and *GATA3*. They are expressed in regionally overlapping but not identical patterns at

embryonic and post-natal stages of development, where they generally appear to act as selectors of phenotype in post-mitotic neurons rather than as developmental drivers in neurogenesis. GATA2 and *GATA3* expression is evident in midbrain nuclei, the raphe nucleus, cranial motor neurons, the pituitary, and spinal cord interneurons (Kala et al., 2009; Nardelli et al., 1999; Nozawa et al., 2009; Richter, Meurers, Zhu, Medvedeva, & Chesselet, 2009; Tsarovina et al., 2004; Willett & Greene, 2011; Zhao et al., 2008).

Within the midbrain, *GATA2* is expressed in the superior and inferior colliculi, the substantia nigra pars reticulata (SNr), and the SNc. Nozawa and colleagues have demonstrated that two GATA elements in the *GATA2* gene are necessary for inducing and maintaining *GATA2* expression in midbrain regions, with each playing unique roles within the superior colliculus and the inferior colliculus (Nozawa et al., 2009). GATA2 and GATA3 are not co-expressed in the same cells, although they are co-expressed in some of the same brain regions (Nozawa et al., 2009). Notably, no *in vivo* reports have shown GATA factor expression in glia, though an *in vitro* report demonstrates expression of *GATA2* and *GATA3* in primary mouse cortical neurons (Wallach et al., 2009). GATA2 expression is turned on as neurons exit the cell cycle in embryonic development, but expression is also present in many nuclei in post-natal stages (Kala et al., 2009). Kala and colleagues showed that GATA2 acts as a GABAergic phenotype selector in various nuclei by regulating gene sets related to GABAergic neuronal phenotype (Kala et al., 2009). In these cells, GATA2 regulates genes involved in the GABA synthesis pathway (*e.g. GAD1* and *GAD2*) as well as other genes necessary for GABA production (Kala et al., 2009). Interestingly, conditional loss of *GATA2* in these cells results in glutamatergic phenotype selection (Kala et al., 2009). Using conditional mutagenesis, they showed that *GATA2* does not participate in progenitor cell patterning or other features of neurogenesis (Kala et al., 2009).

Therefore, it appears that the primary function of GATA2 in post-mitotic midbrain GABAergic neurons is to act as a selector gene, controlling large sets of genes related to GABAergic phenotype.

Both *GATA2* and *GATA3* are expressed within the murine substantia nigra. A study using *in situ* hybridization (ISH) in mice to map GATA factor expression showed, within the SN, that *GATA3* expression is confined to the SNr (Zhao et al., 2008). Richter and colleagues used laser-capture microdissection on rat midbrain sections combined with quantitative RT-PCR to examine globin gene expression in SNc, and found that *GATA2* is expressed in SNc dopaminergic and GABAergic cells, though this was not a focus of their study (Richter et al., 2009). The role of GATA2 in SNc dopaminergic neurons has not been investigated and remains unknown.

1.3.4 GATA Transcription Factors and PD

Many lines of evidence from human PD support a central role for alpha-synuclein in PD pathogenesis: (i) *SNCA* mutations causes a rare form of PD, (ii) multiplications of the wild-type gene causes PD with dose-dependent severity, (iii) a-syn pathology marks the pathological process anatomically in the majority of PD cases, and (iv) a-syn accumulates in degenerating neurons in the majority of PD cases (section 1.1.3.1.1). Together, these facts suggest that lowering a-syn levels in PD patients would confer neuroprotection. However, surprisingly little is known about regulation of *SNCA* expression.

Scherzer and colleagues approached this problem using an innovative approach to identify transcriptional regulators of *SNCA* (Scherzer et al., 2008). After finding that SNCA

mRNA was detectable in blood, the authors interrogated gene expression databases for over 14,000 genes across blood samples from 22 healthy humans. They performed numerous pair-wise comparisons to determine genes whose expression correlated with that of *SNCA – i.e.* genes whose expression was high when SNCA levels were high and low when SNCA levels were low. At the end of their analysis, following several validation steps with additional datasets, this group comprised 35 genes.

The authors hypothesized that a transcription factor coordinately regulates this gene block, thus accounting for the correlated expression of its genes. Three of the genes in this expression block – erythroid 5-aminolevulinate synthase 2 (*ALAS2*), biliverdin reductase B (*BLVRB*), and ferrochelatase (*FECH*) – are genes involved in heme metabolism and one of them (*ALAS2*) is known to be regulated by the transcription factor GATA1 (Surinya, Cox, & May, 1997). A transcription factor that putatively coordinately regulates *SNCA* and various genes involved in iron metabolism is intriguing given the fact that both *SNCA* (section 1.1.3.1.1) and iron pathways (Horowitz & Greenamyre, 2010b; Sian-Hülsmann, Mandel, Youdim, & Riederer, 2011) are dysregulated in PD.

GATA1 is not expressed in brain, but Scherzer and colleagues showed that GATA2 protein is detectable in homogenates from human SN and cortex, two regions affected by a-syn pathology in PD (Scherzer et al., 2008). They then showed that *GATA2* silencing in the human dopaminergic neural cell line, SH-SY5Y, leads to significant down-regulation of *SNCA* at both the mRNA and protein levels, suggesting that GATA2 positively regulates *SNCA*. The authors showed that this regulation results from GATA2 directly and specifically binding to one of the 10 GATA elements in the *SNCA* locus.

In summary, the authors used a novel approach to identify and validate *in vitro* the first transcription factor shown to regulate *SNCA* expression. The *in silico* finding that GATA transcription factors may coordinately regulate the expression of *SNCA* and iron-related genes is particularly intriguing since both a-syn and iron are known to accumulate pathologically in vulnerable SNc dopaminergic neurons in PD. However, in order for GATA2 to be relevant to PD, it remains to be shown that it is expressed in these neurons and regulates *SNCA in vivo* as it does *in vitro*.

If GATA2 coordinately and positively regulates *SNCA* and genes relevant to iron homeostasis *in vivo*, then GATA2 might be envisioned as a potential therapeutic target to ameliorate a-syn and iron pathologies simultaneously. If GATA2 is pathologically activated in PD such that it induces excessive *SNCA* and iron homeostasis gene expression, then inhibition of GATA2 may normalize levels of its downstream target genes. If GATA2 is not involved in the pathological up-regulation of these genes, GATA2 may nevertheless be a useful target for inhibition in order to attenuate the basal expression of these genes. While this connection to PD is attractive, it must first be determined whether *GATA2* is expressed in the neuronal populations that pathologically accumulate a-syn and iron and degenerate in PD.

1.4 HYPOTHESIS & EXPERIMENTAL DESIGN

In this dissertation project, we test two hypotheses. Our first hypothesis is that GATA2 regulates *SNCA* in dopaminergic SNc neurons *in vivo*. In order to test this hypothesis, we create and validate viral vectors for the delivery of shRNA against rat GATA2 and assess *SNCA* expression

under conditions of *GATA2* silencing in the rat SNc. In the Chapter 2, we confirm that GATA2 regulates *SNCA in vitro*, as described previously (Scherzer et al., 2008) and we validate rat as a reliable model system for examining GATA2 regulation of *SNCA in vivo*. The Chapter 3 describes the development and validation of the viral vector used to test our first hypothesis, which we test in the third data chapter.

Our second hypothesis is that down-regulation of *GATA2* in SNc dopaminergic neurons protects these cells from rotenone-induced degeneration in the rotenone rat model of PD. We test this hypothesis in Chapter 4, by silencing *GATA2* in SNc using the viral vector and by assessing neuropathological endpoints related to nigrostriatal integrity and animal survival.

2.0 GATA2 REGULATES SNCA IN VITRO AND IS PRESENT IN RAT AND HUMAN BRAIN

2.1 ABSTRACT

GATA2 has been shown to positively regulate *SNCA* in a human dopaminergic neural cell line and is expressed in human SN and cortex, regions that are susceptible to a-syn pathology in PD. Together, these findings prompt the idea that GATA2 may play a role in PD pathogenesis, however whether this mode of regulation occurs in brain has not been investigated. In this chapter, we confirm the finding that GATA2 positively regulates *SNCA in vitro*, and validate the rat as a suitable model for examining GATA2 regulation of *SNCA in vivo*. We find that GATA2 regulation of *SNCA* is conserved between human and rat, and we localize GATA2 expression to various regions of the adult rat midbrain, including the SNc, which we confirmed in human SNc. Together, these findings set the stage for testing the hypothesis that GATA2 positively regulates *SNCA in vivo*.

2.2 INTRODUCTION

Given the central role that elevated levels of alpha-synuclein (*SNCA*, a-syn) play in PD pathogenesis, lowering the expression of *SNCA* within dopaminergic neurons of the SNc may be a reasonable therapeutic goal in PD. The lack of knowledge about transcription factors that regulate *SNCA* has hampered progress toward modulating *SNCA* expression at the transcriptional level. Recently, GATA transcription factors were shown to regulate *SNCA* positively and directly in a mouse erythroid cell line and in a human dopaminergic neural cell line (Scherzer et al., 2008). For this mode of regulation to be relevant to PD, it must be demonstrated that GATA2 is expressed within neurons that exhibit synucleinopathy and degeneration in PD. Scherzer and colleagues have shown that GATA2 is present in human brain homogenates from SN and cortex – two regions that undergo degeneration in PD – but their methods did not allow for localization to particular cell types in these regions (Scherzer et al., 2008). To the best of our knowledge, there has been only one report of *GATA2* expression within adult rat SNc dopaminergic neurons (Richter et al., 2009), and no localization studies performed in human SNc. A comprehensive description of *GATA2* expression in adult midbrain in general and SNc dopaminergic neurons in particular is therefore necessary to lend relevance of this regulatory system to PD.

In order to establish the relevance of GATA2 in PD, we sought first to replicate the findings of Scherzer and colleagues (Scherzer et al., 2008) and then to demonstrate that GATA2 is expressed in SNc dopaminergic neurons. Our results confirm and extend their findings, by demonstrating that GATA2 positively regulates *SNCA* in the human dopaminergic cell line, SH-SY5Y, as well as in the rat dopaminergic cell line, PC12. Importantly, we show that *GATA2* is expressed in neurons within the rat midbrain, including dopaminergic neurons within the SNc; it

was also expressed in human SNc. Taken together, these data suggest that GATA2 may plausibly play a role in regulating *SNCA* expression in relevant neuronal populations in PD. These data also set the stage for *in vivo* investigations of GATA2 regulation of *SNCA* in rat.

2.3 MATERIALS & METHODS

2.3.1 Cell lines and reagents

SH-SY5Y (#CRL-2266) and PC12 (#CRL-1721) cell lines were purchased from American Type Culture Collection (ATCC). PC12 cells were grown on dishes coated with mouse collagen IV according to manufacturer's instructions (Trevigen, #3410-010-01). Dulbecco's Modified Eagle Medium (DMEM) and OptiMEM Reduced Serum Medium (#31985-070) were purchased from Gibco. Small-interfering RNA (siRNA) against human GATA2 (Stealth RNAi siRNA Duplex Oligoribonucleotides #HSS178122) and Lipofectamine RNAiMAX transfection reagent (#13778-075) were purchased from Invitrogen. The human GATA2 siRNA sequence used was: 5'-UGAAGAAGACGUCCACCUCGUCUGG-3'. (See section 3.3.2 for rat GATA2 siRNA sequence design.) Stealth RNAi Negative Control duplex siRNA was purchased from Invitrogen (#12935-300). All chemicals were purchased from Sigma, expect where noted otherwise.

The following pre-validated human and rat QPCR primer sets were purchased from Applied Biosystems: GATA2 (PPH0245A-200, PPR48683A-200), alpha-synuclein (SNCA, PPH05943E-200, PPR42596A-200), transferrin receptor 2 (TFR2, PPH05762A-200,

PPR51159A-200), neurofilament light chain (NEFL, PPH02430A-200, PPR46667A-200), GAPDH (PPH00150E-200), and beta-actin (ACTB, PPR06570B-200).

2.3.2 Transfection

SH-SY5Y cells of low passage number (4-8) were seeded onto 6-well plates at a density of 750,000 cells/well in DMEM containing 10% FBS, pencillin (100 units/mL), streptomycin (100 μg/mL) and grown overnight at 37° C in 5% CO_2. Cells were transfected the following day, at 40-60% confluency. Twenty minutes prior to transfection, media was removed and cells were gently rinsed with warm PBS. 1.5 mL warm OptiMEM was added per well and the plates returned to 37° C. Lipofectamine RNAiMAX was diluted 1:50 in OptiMEM, then combined with an equal volume of OptiMEM containing siRNA against human GATA2 and incubated at room temperature for 25 min. Solutions were then added drop-wise at 500 μL/well and returned to 37° C. OptiMEM was replaced with serum media 6-12 hours after transfection. Samples were collected 48 hours post-transfection. Transfection of PC12 cells with siRNAs against rat GATA2 was performed as described above for SH-SY5Y cells.

2.3.3 Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-QPCR)

RNA was isolated from cells and purified using an RNeasy minikit (Qiagen #74104) according to manufacturer's instructions. Only RNA of sufficient purity ($A_{260/280} \geq 2.0$) was used for analysis. Reverse transcription was performed using an RT^2 First Strand Synthesis kit (SA Biosciences, #C-03) according to manufacturer's instructions. Quantitative PCR using the SYBR

green method with dissociation curve was performed on a Stratagene MX3000P machine. Sample reactions were prepared on 96-well PCR plates in technical triplicates using: 5 µL cDNA template, custom QPCR primer sets (SA Biosciences), and SYBR Green PCR Master Mix (Applied Biosystems #4309155) per 25 µL reaction. Triplicate wells containing reactions with no template cDNA were used as negative controls to assess purity of reagents. For SH-SY5Y, GAPDH was used as a housekeeping gene for normalization. For PC12, ACTB was used as a housekeeping gene for normalization. Fold-change was calculated based on means of threshold cycle (C_t) values from technical triplicates in each independent experiment using the ΔΔCt Method (fold-change = $2^{(-\Delta\Delta Ct)}$).

2.3.4 Immunocytochemistry (ICC)

Cells on poly-D-lysine-coated coverslips were fixed in 4% PFA (EM #15710-S, Electron Microscopy Sciences) in PBS (pH 7.4) for 20 min. at room temperature, then washed 3 x 10 min. in PBS (pH 7.4). Coverslips were then blocked for 1 hour in 10% normal donkey serum (NDS) in PBS containing 0.03% Triton-X (PBST). Primary antibodies were prepared in 1% NDS in PBST as follows: goat anti-human GATA2 polyclonal antibody (R&D Systems, #AF2046), 1:2000; mouse anti-alpha-synuclein monoclonal antibody (BD Transduction Laboratories, #610787), 1:2000. Primary antibody incubation was carried out overnight at 4° C. Coverslips were washed 3 x 10 min. in PBS (pH 7.4), then incubated for 1 hour in the dark in secondary antibody solution containing 1% NDS in PBST and the following secondary antibodies: Alexa Fluor 488-conjugated donkey-anti-goat IgG antibody (Molecular Probes, #A-11055), 1:500; Cy3-conjugated donkey-anti-mouse antibody (Jackson Immunoresearch, #715165151), 1:500.

Following removal of secondary antibody solution, coverslips were incubated in bisBenzimide Hoechst 33342 1:5000 (Sigma, #B2261) in PBS (pH 7.4) for 3 min. at room temperature in the dark. Coverslips were washed 3 x 10 min. in PBS (pH 7.4) in the dark, then mounted onto Superfrost Plus (Fisher, #12-550-15) slides with aqueous mounting media and dried overnight in the dark at room temperature.

2.3.5 Confocal Microscopy & Quantification of Fluorescence Intensity

Images of the fluorescently stained cells were obtained on a laser scanning confocal microscope (Olympus, Japan) at 60X magnification. Acquisition parameters—laser intensity, pinhole diameter, detector gain, and amplifier offset—were optimized and subsequently used for acquisition of all images across all treatment conditions. Importantly, fluorescence intensity of all channels was optimized such that no saturated pixels were present in any channels.

Three coverslips per condition were imaged and analyzed; for each coverslip, at least three 60X fields were acquired, each containing approximately 10 cells. Regions of interest (ROIs) were precisely drawn around somata using the confocal microscope image analysis software, Fluoview FV1000 (Olympus, Japan). Average fluorescence intensity was measured for each ROI and averages and standard error of the mean (SEM) were calculated for each condition. An unpaired, two-tailed Student's t-test was used to compare normalized fluorescence intensity means between siNeg and siGATA2 conditions (α=0.05).

2.3.6 Animals

Three male Lewis rats aged 7-9 months were housed and treated in accordance with National Institutes of Health guidelines and University of Pittsburgh Institutional Animal Care and Use Committee (IACUC)-approved protocols. For sacrifice, animals were deeply anesthetized with sodium pentobarbital then decapitated. Brains were fixed by transcardial perfusion with 100 mL of ice-cold PBS (100 mM, pH 7.4) followed by 200-400 mL of fresh (less than one week old), ice cold 4% paraformaldehyde (PFA), pH 7.4. Brains were removed and fixed overnight at 4° C in 4% PFA, then transferred to an ice-cold PBS solution containing 30% sucrose for 5 days. Brains were cut at 35 μm on a freezing microtome and stored at -20° C in cryoprotectant solution (100 mM PBS, 25% glycerin, 30% ethylene glycol, pH 7.4).

2.3.7 *in situ* Hybridization (ISH)

A 399-bp fragment from the 3' untranslated region of rat GATA2 was PCR amplified from total rat brain RNA (Clonetech) using a Phusion High-Fidelity PCR kit (New England Biolabs) with the following primers (5'-3'): (forward) CCAGCAAATCCAAGAAGAGC, (reverse) AGGTGGCTTCAGCCAGACTA**.** The PCR product was gel purified and incubated at 70° C for 10 min. with *Taq* polymerase and PCR buffer, including dNTPs. The fragment was introduced into a pCR2.1-TOPO vector (Invitrogen) by T-A cloning and purified plasmids from resulting clones were sequenced to determine orientation of the insert. A plasmid containing the insert in the sense direction and a plasmid containing the insert in the antisense direction were used for *in vitro* synthesis of anti-sense and sense complementary RNA (cRNA) probes using a MAXIscript

T7 kit (Ambion) supplemented with digoxigenin-labeled uracil ribonucleotides (Roche). Resulting cRNA probes were resuspended in formamide buffer (50% formamide, 25% 5X SSC-DEPC, 25% H_2O-DEPC) and stored at -20° C.

ISH was performed on perfusion-fixed free-floating brain sections under RNAse-free conditions. Sections were washed in 12-well nuclease-free plates 4 x 10 min. in DEPC-treated PBS (PBS-DEPC) to remove cryoprotectant. Active DEPC treatment of (0.1%, v/v; Sigma D5758) in PBS was performed in 24-well nuclease-free plates 2 x 15 min. followed by a 15-min. incubation in 5X SSC-DEPC. Sections were re-fixed in 4% PFA-DEPC for 20 min., then washed 2 x 5 min. in PBS-DEPC. Blocking of non-specific nucleic acid interactions was performed by incubating sections in UltraHyb Ultrasensitive Hybridization Buffer (Ambion, #AM8670) containing Torula RNA at a final concentration of 1mg/mL (Sigma, #R6625) for one hour at 68° C, rocking. Antisense or sense cRNA probes for rat GATA2 were used at a concentration of 300 ng/mL in pre-warmed UltraHyb Ultrasensitive Hybridization Buffer and incubations were carried out at 68° C, wrapped in plastic to prevent evaporation, rocking overnight.

Following hybridization, sections were washed with the following buffers prepared from 20X SSC (Ambion, #AM9763) in molecular biology-grade water (Sigma): 2X SSC, 2 x 10 min. at room temperature rocking; 2X SSC, 2 x 15 min. at 68° C rocking; 1X SSC, 2 x 15 min. at 68° C rocking; 0.1X SSC, 2 x 30 min at 68° C rocking. Sections were equilibrated in a maleic acid buffer (MAB; 100 mM maleic acid, 150 mM NaCl, pH 7.5) 2 x 10 min. at room temperature, then serum-blocked (2% blocking reagent [B. Mannheim, #1 096 176], 5% lamb serum [Sigma, #S4877], in MAB buffer) for 30 min. at room temperature. Alkaline-phosphatase-conjugated sheep anti-digoxigenin Fab fragments (Roche, #11093274910) were used as secondary antibody at a concentration of 1:500 in blocking buffer. Sections were incubated in secondary antibody for

2 hours at room temperature, rocking, then washed 2 x 5 min. in PBS. Sections were incubated 2 x 5 min. in staining buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween-20, pH 9.5) at room temperature, rocking. Staining was performed by incubating sections in BM Purple (Roche, #11442074001) at room temperature in the dark rocking for 12 hours.

Following overnight staining, sections were rinsed three times in PBS then washed 3 x 10 min. in PBS. Sections were mounted onto gelatin-coated Superfrost Plus slides (Fisher) and allowed to dry overnight in the dark. Sections were dehydrated through graded ethanols, cleared in Histoclear (National Diagnostics, #HS-200), and coverslipped in Histomount (National Diagnostics, #HS-103).

2.3.8 Immunohistochemistry (IHC) on Human Substantia Nigra (SN) Tissue

Slides containing cryostat-cut, paraffin-embedded human SN sections were obtained from the University of Pittsburgh brain bank in accordance with institutional regulations. Prior to staining, sections were de-paraffinized as follows: 60°C x 30 min., 3 x 4 min. Histo-Clear (National Diagnostics, #HS-200), 2 x 4 min. 100% ethanol, 2 x 4 min. 95% ethanol, 1 x 4 min. 70% ethanol, 1 x 5 min. water. Blocking of endogenous peroxidases was performed in 3% H_2O_2 for 10 min. at room temperature, followed by a 5-min. wash in water. Heat-mediated antigen retrieval was conducted by incubating sections in a citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0) at 95-100° C for 20 min. Sections were allowed to cool at room temperature for 5 min. then washed 3-min. in water. Serum block was performed in 10% NDS in PBS containing 0.3% Triton-X (PBST) for 1 hour. Primary antibody (polyclonal goat anti-human GATA2, R&D Systems, #AF2046) was resuspended in 1% NDS in PBST at a concentration of

1:100, and sections were incubated overnight at 4° C. Primary antibody solution was then re-applied to the sections the following day for 2 hours at room temperature. After 3 x 10 min. washes in PBS, sections were incubated for 1 hour in a solution containing biotin-conjugated donkey-anti-goat secondary antibody at 1:200 (Jackson Immunoresearch, #705-065-147) in 1% NDS in PBST. After 3 x 10 min. washes in PBS, sections were incubated in ABC peroxidase kit solution (Vectastain, #PK-6100) for 1 hour then washed 3 x 10 min. washes in PBS. Chromogenic development using Vector VIP chromogen (Vector Laboratories, #SK-4600) was carried out according to manufacturer's instructions. Sections were dehydrated through graded ethanols, cleared in Histoclear (National Diagnostics, #HS-200), and coverslipped in Histomount (National Diagnostics, #HS-103).

2.4 RESULTS

2.4.1 GATA2 positively regulates *SNCA* in the human dopaminergic neural cell line SH-SY5Y

To confirm that GATA2 positively regulates *SNCA*, as described by Scherzer and colleagues (Scherzer et al., 2008), we sought first to reproduce their findings in the same human dopaminergic neural cell line they used, using the same methods. If GATA2 positively regulates *SNCA* under basal conditions, then silencing GATA2 expression should lead to consequent down-regulation of *SNCA* expression. Transfection of SH-SY5Y cells with small interfering RNA (siRNA) against human GATA2 (siGATA2) for 48 hours led to a significant and dose-

dependent decrease in GATA2 mRNA relative to a non-targeting siRNA control (siNeg), as assessed by RT-QPCR (data not shown). Based on this dose-response curve, an siRNA concentration of 120 nM was selected for subsequent experiments.

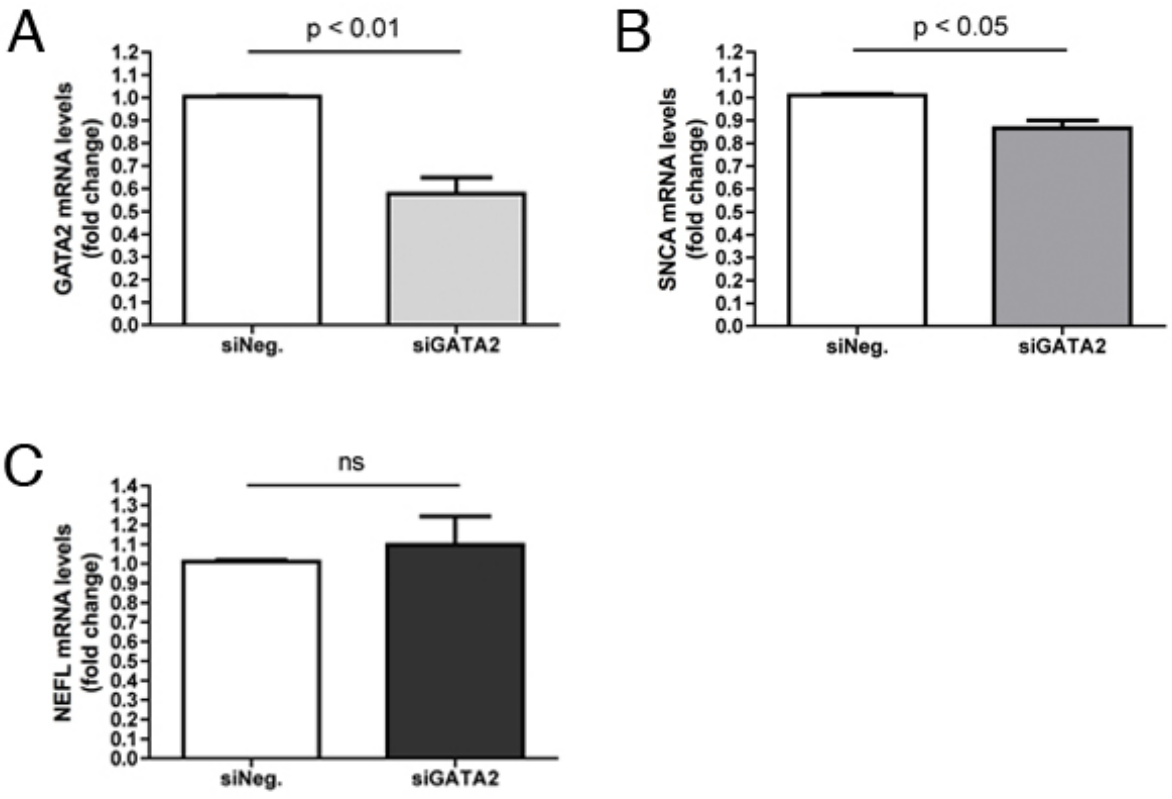

Figure 1. GATA2 positively regulates transcription of *SNCA* in SH-SY5Y cells

(A) GATA2 mRNA levels are reduced after 48-hour transfection of SH-SY5Y cells with siRNA against GATA2 as compared to negative control siRNA (siNeg) ($p < 0.01$). **(B)** SNCA mRNA levels are reduced by 14% and **(C)** NEFL mRNA levels are unchanged. *GAPDH* was used as a normalization control to calculate fold-change in mRNA levels. At least 5 independent experiments were performed. $\alpha=0.05$.

At this concentration, GATA2 message was decreased by about 50% (Figure 1a). In turn, GATA2 silencing resulted in a significant (14%) down-regulation of SNCA mRNA, which is consistent with the report by Scherzer and colleagues (Figure 1b) (Scherzer et al., 2008). Importantly, mRNA levels of neurofilament light chain (*NEFL*)—a gene that contains no GATA elements and thus serves as a negative control—were unchanged following GATA2 knock-down, indicating that the decrease in *SNCA* mRNA is due to the loss of GATA2 rather than off-target effects related to the concentration of siRNA (Figure 1c).

To determine whether the down-regulation of *SNCA* observed at the mRNA level is also present at the protein level, we performed immunocytochemistry (ICC) for GATA2 and alpha-synuclein (a-syn) and quantified fluorescence intensity by confocal microscopy. We found that GATA2 silencing led to a significant reduction in a-syn protein levels (~60%) (Figure 2). This finding is again consistent both in direction and magnitude of a-syn protein reduction with what Scherzer and colleagues found by ELISA under the same cell culture conditions (Scherzer et al., 2008).

Taken together, these data confirm the results of Scherzer and colleagues, demonstrating that GATA2 is a positive transcriptional regulator of *SNCA* in the human dopaminergic neuronal cell line, SH-SY5Y.

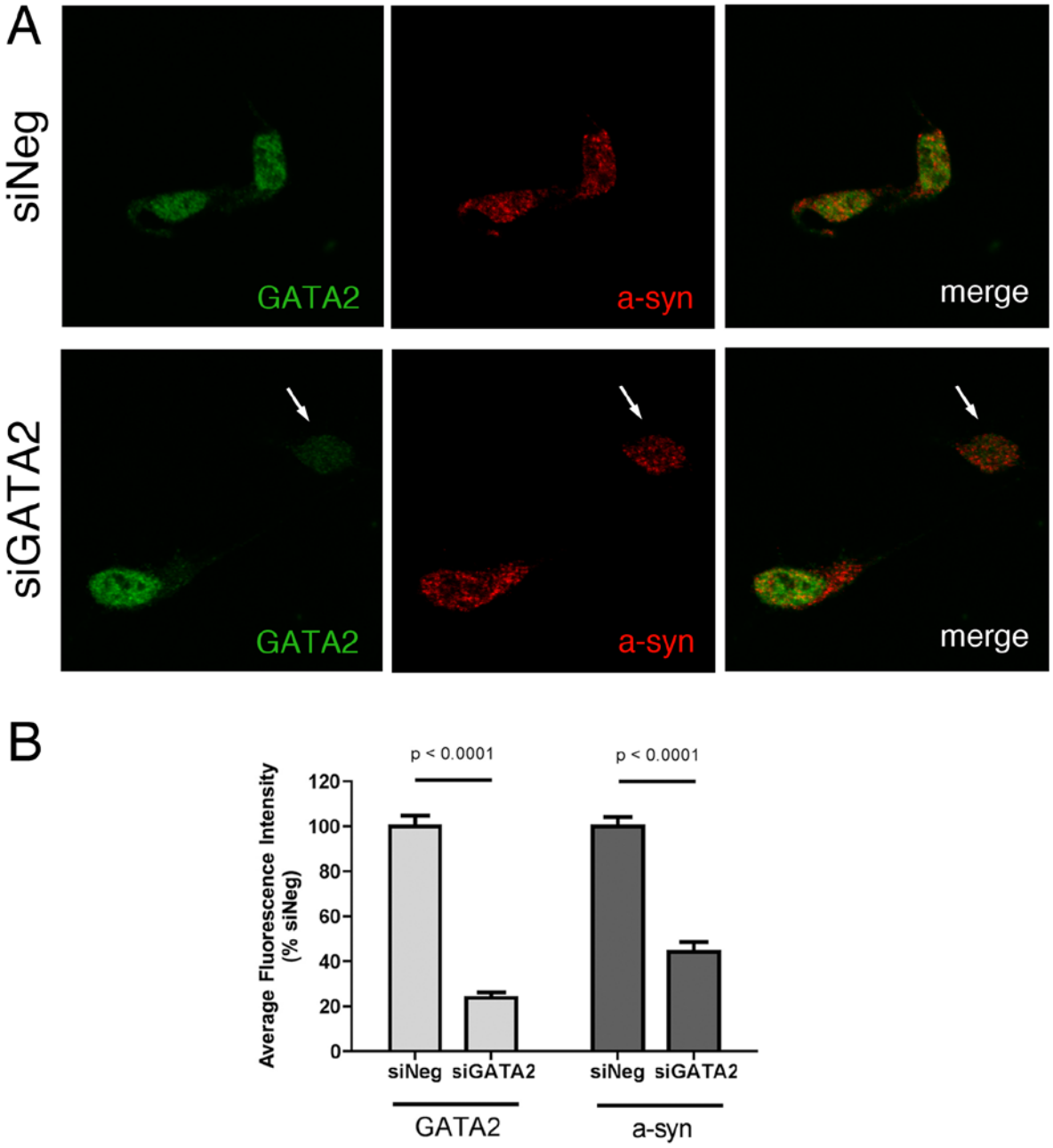

Figure 2. GATA2 silencing leads to significant down-regulation of *SNCA* at the protein level

Cells were transfected for 48 hours with either negative control siRNA (siNeg) or siRNA against human GATA2 (siGATA2). Cells were fixed, ICC was performed, and fluorescence intensity corresponding to protein levels was quantified for GATA2 and a-syn. (A) Representative confocal images of cells transfected with siNeg or siGATA2 and stained for GATA2 (green) or a-syn (red). Arrow indicates a cell presumably transfected with siGATA2 and showing lower GATA2 expression relative to non-transduced cells or cells transduced with siNeg. (B) GATA2 protein levels are reduced by 76% after 48 hours transfection with siGATA2 as compared to siNeg ($p < 0.0001$). Alpha-synuclein protein levels are reduced by 56% after 48 hours transfection with siGATA2 as compared to siNeg ($p < 0.0001$). Three coverslips from one experiment were analyzed. α=0.05.

2.4.2 GATA2 positively regulates *SNCA* in the rat dopaminergic cell line PC12

The overarching goal of this dissertation project is to test the hypothesis that GATA2 positively regulates *SNCA in vivo*, using rat as a model. Therefore, it is important to determine whether the GATA element that GATA2 employs to regulate *SNCA* in human cells is conserved in rat. Although the human *SNCA* locus contains 10 GATA elements, endogenous GATA transcription factors occupy only a single GATA element in intron 1 (Scherzer et al., 2008). Alignment of the human and rat *SNCA* intron 1 sequences reveals that this element is in fact well-conserved between these species, supporting the possibility that this mode of regulation is likewise conserved between these species (Figure 3).

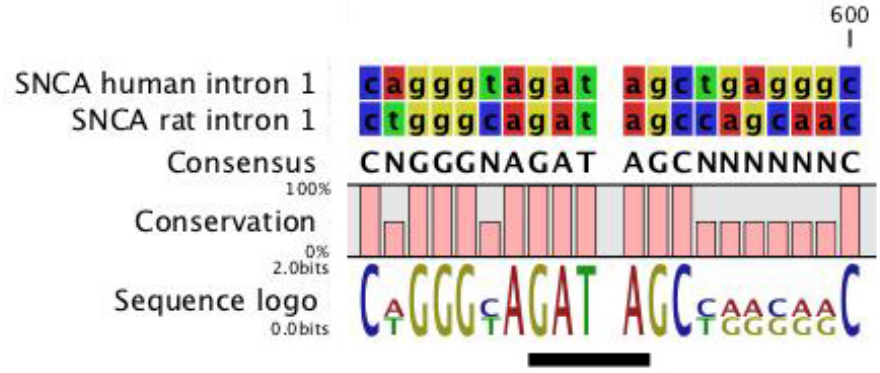

Figure 3. A GATA element in intron 1 of *SNCA* is conserved between human and rat

Human and rat *SNCA* intron 1 genomic DNA sequences were obtained from Ensembl Genome Browser (www.ensembl.org/) and aligned using CLC Main Workbench 6. A conserved GATA element was found spanning bases 588-591, as indicated by the black bar beneath the sequence logo.

In order to determine whether GATA2 also positively regulates *SNCA* in rat cells, we silenced GATA2 in the rat dopaminergic cell line, PC12, and assessed mRNA levels of *SNCA* by RT-QPCR. An siRNA was designed to recognize a unique region of GATA2 mRNA and thereby to silence *rat* GATA2 specifically (see Section 3.3.2). Transfection of PC12 cells for 48 hours with this siRNA (siGATA2) led to a significant and robust decrease in GATA2 mRNA levels relative to a non-targeting negative control siRNA (siNeg), as assessed by RT-QPCR (Figure

4a). This decrease in *GATA2* expression was accompanied by a significant down-regulation in *SNCA* (by 18%), indicating that GATA2 positively regulates *SNCA* in rat dopaminergic cells in addition to human dopaminergic cells (Figure 4b).

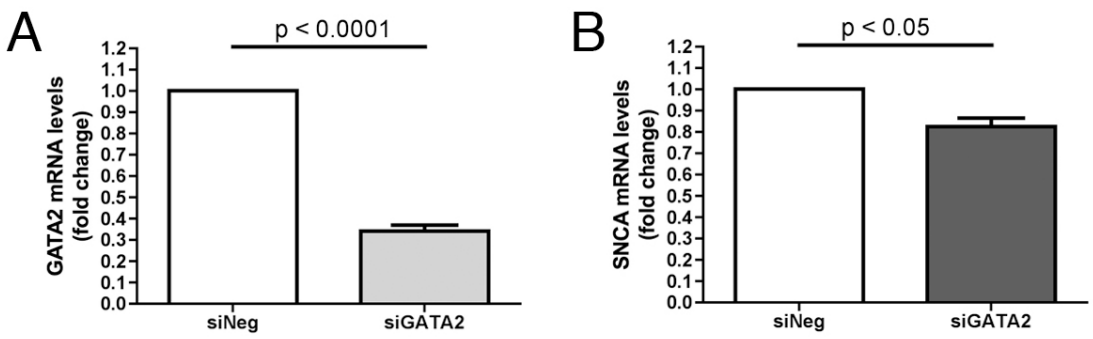

Figure 4. GATA2 silencing leads to down-regulation of SNCA mRNA in a rat dopaminergic cell line

(A) GATA2 mRNA levels are reduced by 66% in PC12 cells after 48 hours transfection with either negative control siRNA (siNeg) or siRNA against human GATA2 (siGATA2) ($p < 0.0001$). **(B)** SNCA mRNA levels are reduced by 18% in PC12 cells following 48 hours of GATA2 silencing ($p < 0.05$). Means from three independent experiments were compared by unpaired, two-tailed Student's t-test ($\alpha=0.05$).

2.4.3 GATA2 is expressed in adult rat and human substantia nigra pars compacta dopaminergic neurons

The relevance of GATA2 to PD is supported by *in vitro* data demonstrating that GATA2 regulates *SNCA* (Scherzer et al., 2008). Furthermore, it has been shown that GATA2 mRNA and protein are detectable in homogenates from human brain regions that undergo degeneration in PD, though these experiments did not localize expression to specific cell types (Scherzer et al., 2008). Work in rodents has demonstrated that GATA2 is expressed within midbrain and hindbrain neurons at embryonic and early postnatal ages (Kala et al., 2009; Nardelli et al., 1999; Nozawa et al., 2009; Willett & Greene, 2011), and one study has shown the presence of GATA2 mRNA in laser capture microdissected SNc dopaminergic neurons from adult rat (Richter et al., 2009). However, a more thorough assessment of GATA2 expression within the adult rat SNc and human SNc has not yet been performed.

We conducted a survey of GATA2 mRNA expression in adult rat midbrain using *in situ* hybridization (ISH) as a first step toward understanding whether GATA2 is expressed in PD-relevant cell populations. As shown in Figure 5, GATA2 mRNA is present in various midbrain nuclei in the adult rat, including: zona incerta (5a'), subthalamic nucleus (5a"), ventral tegmental nucleus (5b'), substantia nigra pars reticulata (5b"), periaqueductal gray (5c'), red nucleus (5c"), superior colliculus (5d'), medial geniculate nucleus (5d"). Importantly, GATA2 is expressed in SNc (Figure 6a-b).

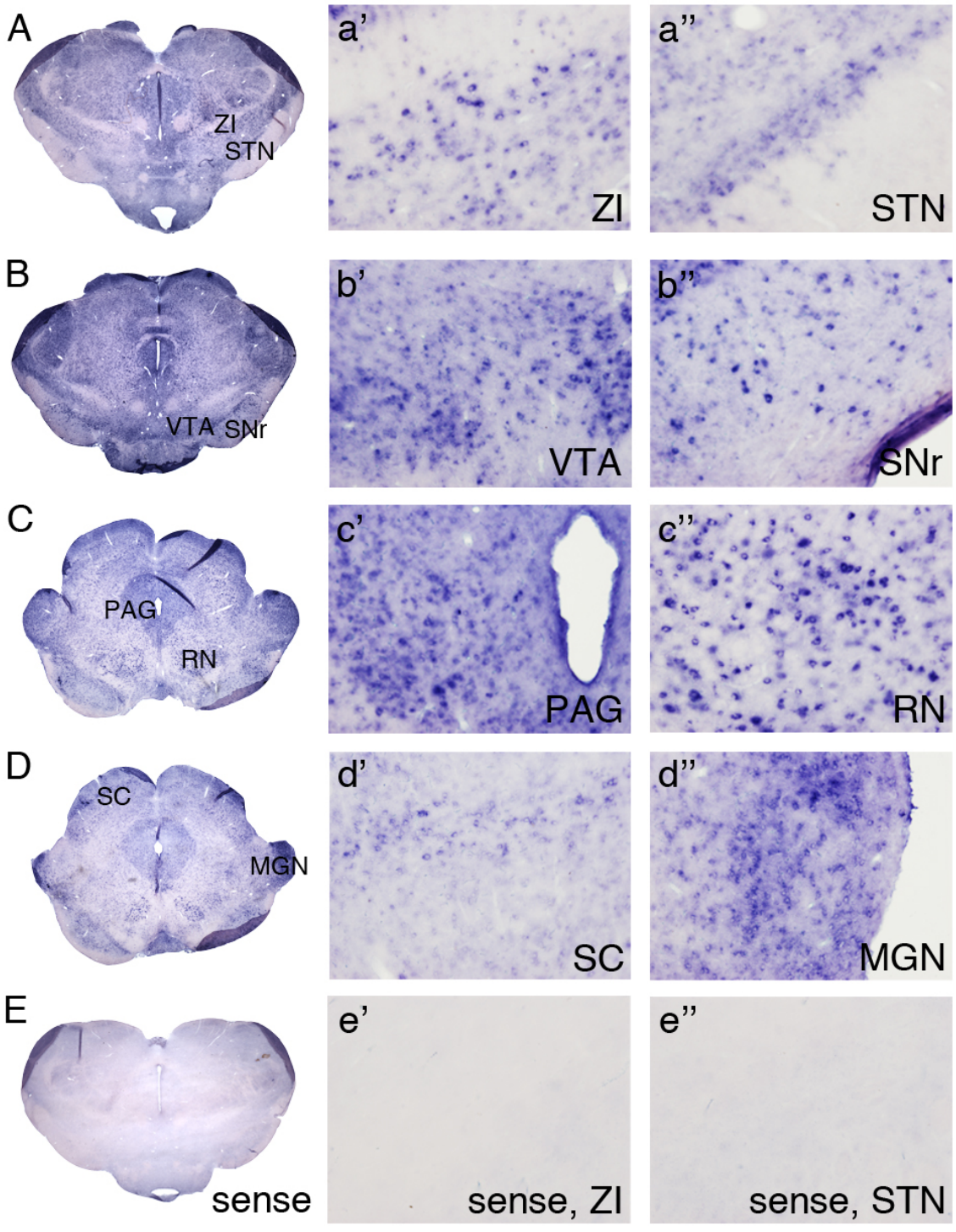

Figure 5. GATA2 mRNA is present in various midbrain nuclei in the adult rat

(A-D) ISH on coronal adult rat brain sections from approximately -4.338 mm Bregma (A) to -6.480 mm Bregma (D) demonstrates GATA2 expression in many midbrain nuclei: zona incerta (ZI), subthalamic nucleus (STN), ventral tegmental nucleus (VTA), substantia nigra pars reticulata (SNr), periaqueductal gray (PAG), red nucleus (RN), superior colliculus (SC), medial geniculate nucleus (MGN). **(E)** ISH using a sense cRNA probe produces no staining, indicating specificity of the antisense probe for GATA2 mRNA. The section used as a negative control in (E) is anatomically equivalent to (A), i.e. approximately -4.338 mm (Bregma).

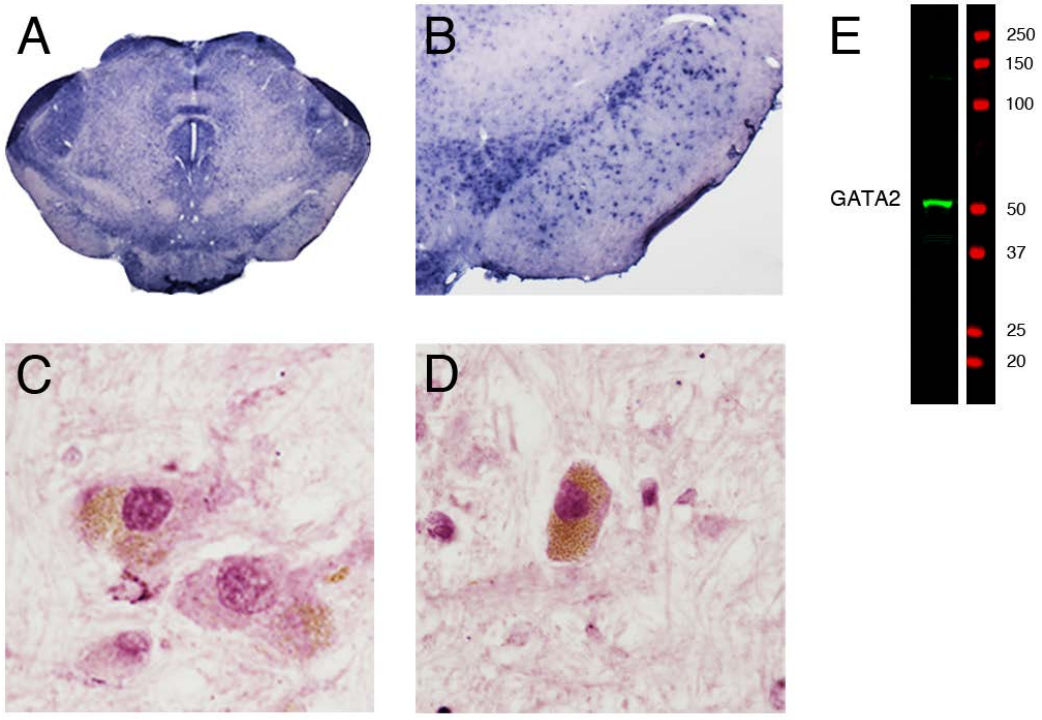

Figure 6. GATA2 is expressed in dopaminergic and non-dopaminergic neurons of the rat and human SNc

(A) In *situ* hybridization for GATA2 (purple) on adult midbrain sections demonstrates expression of GATA2 in various midbrain structures. **(B)** Higher magnification view of ISH from (A) demonstrates GATA2 expression within SNc and SNr neurons. **(C-D)** In human SN sections, IHC for GATA2 (purple) reveals GATA2 expression in both cells containing neuromelanin (i.e. dopaminergic neurons; brown) and cells without neuromelanin (i.e. non-dopaminergic neurons). **(E)** Western blot using total protein lysate from the human dopaminergic neuronal cell line SH-SY5Y shows that the GATA2 antibody used in (C) and (D) specifically recognizes a single band at the expected migration of GATA2.

Staining of human SN with a specific antibody that was raised against full-length recombinant human GATA2 protein revealed GATA2 expression in neuronal profiles containing neuromelanin as well as neuronal profiles without neuromelanin (Figure 6c-d). This finding indicates that the same cellular distribution of GATA2 expression within the SN is found in rats and humans. Importantly, the antibody used recognizes a single band at the predicted molecular weight of GATA2 (Figure 6e).

In summary, GATA2 mRNA is expressed in many nuclei of the adult rat midbrain, including the SNc, which is susceptible to degeneration in PD. Within the human SNc, GATA2 is expressed in both neuromelanin-containing (i.e. dopaminergic) and neuromelanin-lacking (i.e. non-dopaminergic) cells. Taken together, these results strengthen the idea that GATA2 may play a role in regulating *SNCA* within PD-relevant cell populations and validate the rat as a suitable model of *in vivo* investigations into GATA2 function in the SNc.

2.5 DISCUSSION

A potential role for GATA transcription factors in PD was suggested by Scherzer and colleagues, who performed *in vitro* studies demonstrating the ability of GATA2 to regulate *SNCA* expression in a human dopaminergic neuronal cell line (Scherzer et al., 2008). In this chapter, we have confirmed the results of Scherzer and colleagues, showing that GATA2 positively regulates *SNCA* in the human dopaminergic neuronal cell line, SH-SY5Y (Figures 1-2**)**. The over-arching goal of this dissertation project is to test the hypothesis that regulation of *SNCA* by GATA2 occurs *in vivo* in relevant neuronal populations using the adult rat as a mammalian model system. Therefore, we first sought to determine whether GATA2 positively regulates *SNCA* in a rat cell line. Given that only one of the 10 GATA elements present in the human *SNCA* locus is functional under basal conditions in SH-SY5Y, it was important to determine whether this GATA element is conserved in rat *SNCA*. The chromatin immunoprecipitation approach that Scherzer and colleagues used to determine GATA factor occupancy at human *SNCA* localized the GATA element within intron 1 (Scherzer et al., 2008). By aligning human and rat *SNCA* intron 1 sequences, we found complete conservation of a GATA element at position 588 (relative to human *SNCA*) in intron 1 (Figure 3**)**. Furthermore, we found that silencing GATA2 in the rat dopaminergic cell line PC12 led to the expected down-regulation of *SNCA*, indicating that the positive regulation that GATA2 exerts on SNCA expression is conserved between rat and human. This finding not only strengthens the idea that regulation of *SNCA* by GATA2 is biologically relevant – *i.e.* it is a mode of regulation worthy of evolutionary conservation – but also provides a rationale for using the adult rat as a model system for further studies *in vivo*.

In an attempt to examine the relevance of GATA2 to PD, we asked whether GATA2 is expressed in neurons in the SNc, a brain region that pathologically accumulates a-syn and undergoes degeneration in PD. Initial attempts to localize GATA2 protein to rat SNc and cortical neurons were fraught with antibody non-specificity issues, rendering the resulting staining uninterpretable (data not shown; see section 4.5.3). To circumvent this antibody issue, we used *in situ* hybridization (ISH) to evaluate the expression pattern of GATA2 mRNA. We found that GATA2 is expressed in various adult rat midbrain nuclei, including the SNc (Figure 5, 6a-b). These findings are consistent with reports showing *GATA2* expression in embryonic and early post-natal midbrain structures, wherein GATA2 appears to act as a selector gene for GABAergic phenotype in post-mitotic neurons within the ventral midbrain (Kala et al., 2009) and neuronal migration and maturation in the superior colliculus (Willett & Greene, 2011).

For GATA2 to be most directly relevant to nigrostriatal degeneration in PD, it is necessary to show that it is expressed in SNc dopaminergic neurons. Immunohistochemistry (IHC) on human (non-PD) SNc sections revealed that GATA2 is expressed in neurons containing neuromelanin (a surrogate marker for dopaminergic neurons). Notably, GATA2 is also expressed in non-dopaminergic neurons, likely GABAergic neurons; this finding is consistent with previous reports in early post-natal animals (Kala et al., 2009; Willett & Greene, 2011).

What role GATA2 may be playing in SNc dopaminergic neurons is unclear. Genome-wide analysis of GATA2-regulated genes has been undertaken in erythroid cells and epithelial cells *in vitro* (T. Fujiwara et al., 2009; Kang et al., 2012; Linnemann et al., 2011). When the authors compared GATA2 chromatin occupancy in primary human umbilical vein endothelial cells (HUVEC) versus the human erythroleukemia K562 cell line, they found that only 11-15%

of the occupied GATA elements overlapped between the cell types (Linnemann et al., 2011). This striking divergence in the majority of presumed GATA2 target genes suggests that the role that GATA2 plays can vary markedly by cell type. Therefore, it is difficult to predict what the function of GATA2 is in SNc dopaminergic neurons. To examine this issue, the *in vivo* experiments described in Chapter 4 assess whether GATA2 regulates transcription of *SNCA* in nigral neurons of the intact rat.

3.0 DESIGN, CLONING & CHARACTERIZATION OF VIRAL VECTORS FOR SILENCING GATA2 *IN VIVO*

3.1 ABSTRACT

In Chapter 2, we demonstrated that GATA2 positively regulates *SNCA in vitro* by silencing *GATA2* and assessing SNCA levels. Silencing a given gene in brain poses delivery issues that are not encountered *in vitro*, such as blood-brain barrier impermeability and the many systems that can degrade exogenous nucleic acids. Viral-mediated gene delivery overcomes these issues and is therefore a useful means of modulating gene expression *in vivo*. In this chapter, we describe the design, cloning, and *in vitro* validation of viral vectors for the specific silencing of rat *GATA2* in rat SNc dopaminergic neurons. These vectors are used in Chapter 4 to test the two hypotheses of this dissertation project *in vivo* (section 1.4).

3.2 INTRODUCTION

One of the over-arching goals of this dissertation project is to test the hypothesis that GATA2 regulates *SNCA in vivo*. Based on the results of Chapter 2, the adult rat is an appropriate model for *in vivo* investigations into GATA2 function in brain since GATA2 regulation of *SNCA* in cell

lines and cellular distribution of *GATA2* expression are conserved in rat (Figures 4,6). In order to test the hypothesis that GATA2 regulates *SNCA* in rat SNc, we decided to use viral-mediated gene delivery of short-hairpin RNA (shRNA) to silence *GATA2* expression within the rat SNc, then assess whether *SNCA* expression is consequently down-regulated.

Experiments in Chapter 2 utilized small-interfering RNA (siRNA) to show that GATA2 positively regulates *SNCA in vitro*. siRNA is a convenient tool for modulating expression of specific genes *in vitro*, but its utility for *in vivo* application is limited because of its propensity to degrade (Dykxhoorn, Novina, & Sharp, 2003). siRNA is generated intracellularly from shRNA upon enzymatic cleavage of the characteristic shRNA hairpin (Dykxhoorn et al., 2003). Subsequent incorporation of the siRNA into the RNA-inducing silencing complex (RISC) allows for targeted degradation of mRNA. shRNA is more suitable for *in vivo* applications since it can be expressed from plasmids and become siRNA intracellularly; these plasmids can be packaged into viral vectors and thereby protected from degradation *en route* to their cellular site of action. An additional advantage of plasmid-based RNA interference is that other cassettes – *e.g.* one expressing a GFP reporter gene under the control of a CMV promoter – can be introduced into the plasmid and co-expressed with the shRNA. Figure 7 provides an overview of our experimental approach in developing a viral vector for silencing GATA2 in rat SNc.

This chapter shows the rational design and *in vitro* validation of siRNAs for silencing *rat GATA2*, and hence describes the production of the viral vectors that will be used for the *in vivo* experiments in Chapter 4. Our results from this chapter set the stage for *in vivo* modulation of rat *GATA2* expression by demonstrating that the viral vector we generated robustly and specifically silences rat *GATA2 in vitro* and expresses the reporter gene GFP, which will aid in assessing transduction efficiency *in vivo*.

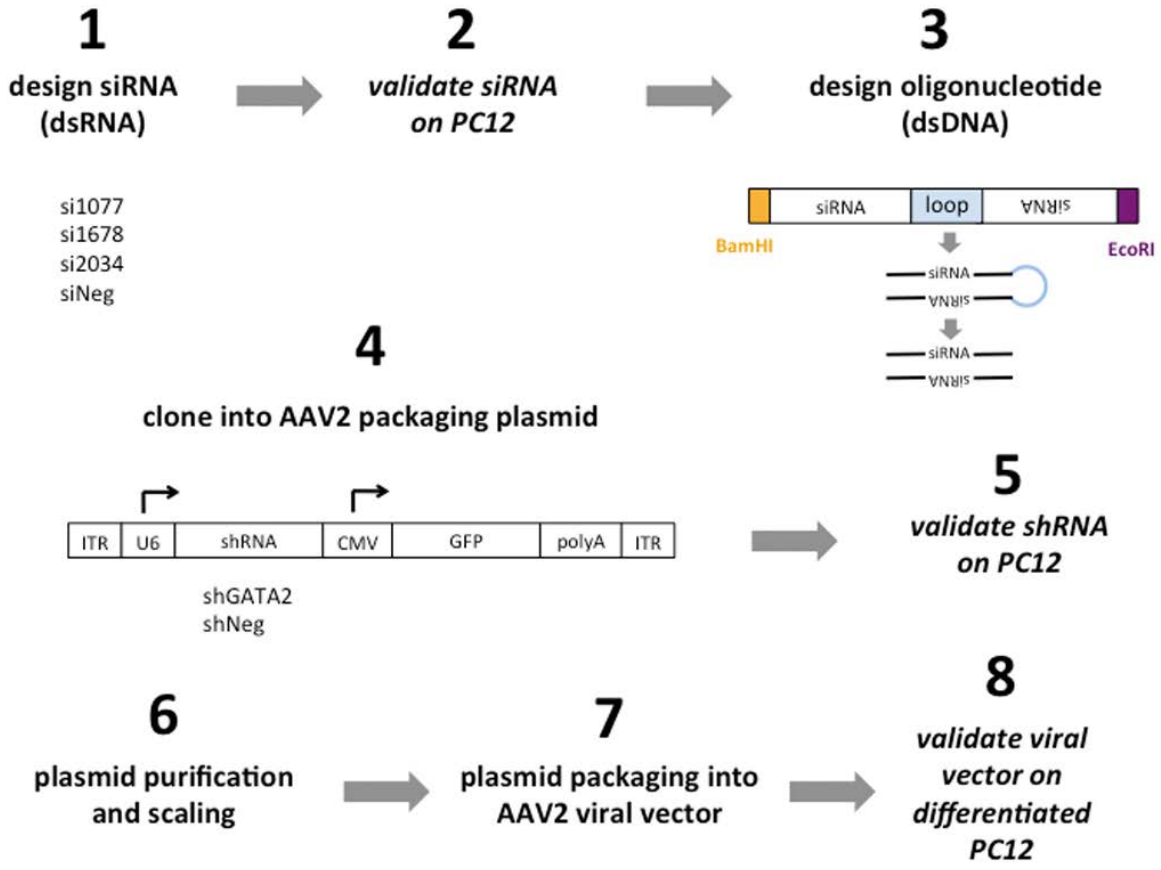

Figure 7. Schematic for design, cloning, and viral packaging of plasmids expressing shRNA against rat GATA2 or negative control shRNA

This process involved: (1) designing custom siRNAs to unique regions of rat *GATA2* transcript **(**see also Figure 8**)**, (2) characterizing their ability to silence rat *GATA2 in vitro* both robustly and specifically, (3) designing a double-stranded oligodeoxyribonucleotide for eventual expression of shRNA based on the sequence of the siRNA sequence that showed strong and specific silencing of rat *GATA2*, (4) cloning the double-stranded oligodeoxyribonucleotide into an adeno-associated virus serotype 2 (AAV2) packaging plasmid, (5) characterizing the ability of the plasmid-expressed shRNA to silence rat *GATA2* robustly and specifically *in vitro*, (6) scaling up plasmid production, (7) packaging the purified plasmid into AAV2, and lastly (8) characterizing the ability of the viral vector to silence rat *GATA2* robustly and specifically *in vitro*. A viral vector containing a plasmid that expresses a non-targeting (universal negative control) shRNA was engineered using the same methods. ITR, inverted terminal repeat.

3.3 MATERIALS & METHODS

3.3.1 Cell culture

Cell culture reagents were purchased from the companies listed in 2.3.1. Undifferentiated PC12 cells (passage #8-12; ATCC, #CRL-1721) were grown on dishes coated with mouse collagen IV (Trevigen, #3410-010-01), and cultured in DMEM growth media containing 5% FBS, 5% horse

serum, pencillin (100 units/mL), and streptomycin (100 µg/mL). Cells were grown at 37° C in 5% CO_2 and growth media was replaced every three days. For *in vitro* viral transduction experiments, PC12 cells were differentiated with a 6-day treatment of 100 µg/mL nerve growth factor (NGF, BD Biosciences) in DMEM medium containing 1% FBS, 1% horse serum, pencillin (100 units/mL), and streptomycin (100 µg/mL). Media changes were performed every three days.

3.3.2 siRNA design, cloning & viral packaging

Design of siRNA was based on rat GATA2 mRNA sequence (NM_033442.1). This sequence was imputed into siRNA design software on the Ambion website **(**http://www.invitrogen.com/site/us/en/home/brands/ambion.html**)** and a BLAST search was used with candidate siRNA sequences to assess alignment to the rat GATA2 mRNA sequence as well as homology to other mRNA in the rat transcriptome. Table 1 contains the sequences of the three custom rat GATA2 Block-iT 21-mer siRNA duplexes and the negative control siRNA (siNeg) that were ordered from Invitrogen, as well as the custom double-stranded oligodeoxyribonucleotides that were subsequently ordered from Invitrogen (Table 1).

Table 1. Sequences of custom siRNA and shRNA oligodeoxyribonucleotides (5'-3').

siRNA duplexes	Sequence
si1077	AGACAGUGACACUUGAUACUU
	GUAUCAAGUGUCACUGUCUUU
si1678	UGCUUUGAGGAGCUAUCCAUU
	UGGAUAGCUCCUCAAAGCAUU
si2034	UCCUCCAAGGAGAGGUGGCUU
	GCCACCUCUCCUUGGAGGAUU
siNeg	AAUUCUCCGAACGUGUCACGU
	CGUGACACGUUCGGAGAAUUU
shRNA oligodeoxyribonucleotides	**Sequence**
sh1678	GCGGATCCAAAAAATGCTTTGAGGAGCTATCC AATCTCTTGAATTGGATAGCTCCTCAAAGCATTGAAT TCGC
shNeg	GCGAATTCAAAAAATTCTCCGAACGTGTCACG TTCTCTTGAAACGTGACACGTTCGGAGAATTGGGGAT CCGC

The AAV2 packaging plasmid was obtained from Bing Wang (University of Pittsburgh) (see Appendix for vector map). For directional cloning of the double-stranded oligodeoxyribonucleotide into the AAV2 packaging plasmid, *BamHI* and *EcoRI* restriction enzymes were used according to manufacturer's instructions (New England Biolabs). Following transformation of DH5-alpha chemically-competent *E. coli* (Invitrogen) with the recombinant plasmid, plasmids were purified using a QIAprep MiniPrep kit (Qiagen) and sequenced to confirm presence and fidelity of all cassette elements. Scaling up of purified plasmid and further purification was performed using an endotoxin-free QIAprep MegaPrep kit (Qiagen). Packaging of purified plasmid into AAV2 was performed and quality-controlled by Penn Vector Core (Philadelphia, PA). Viruses were diluted to 2 x 10^{12} GC/mL in sterile PBS and stored at -80° C until use.

3.3.3 Transfection & Transduction *in vitro*

Transfection of undifferentiated PC12 cells with custom siRNAs was performed as described in 2.3.2. Transfection efficiency for siRNA experiments was estimated by transfecting cells with a Block-iT fluorescent control probe (Invitrogen) and counting the average percentage of fluorescent cells per 20X field using a total of 4 fields per condition. Transfection of undifferentiated PC12 cells with shRNA plasmids was done using an Amaxa cell line nucleofector kit V (Lonza).

For viral transduction experiments, undifferentiated PC12 cells (passage #8-12) were seeded onto 6-well plates at a cell density of 1.2 x 10^6 cells/well. The following day, cells were differentiated with NGF for 6 days, as described in 3.3.1. Multiplicity of infection (MOI)

calculations for viral transduction experiments were made assuming a 1% infectivity of AAV2 for PC12. Cell media was removed and replaced with warm media containing either AAV2 expressing shRNA against GATA2 (AAV2.shGATA2.GFP) or AAV2 expressing negative control shRNA (AAV2.shNeg.GFP) at the indicated MOI. After three days, media was removed and replaced with maintenance media (no virus) for four more days.

3.3.4 Western blot analysis

Cells were trypsinized and gently pelleted, then lysed in ice-cold RIPA buffer (Sigma) containing a protease inhibitor cocktail (Sigma). Lysates were incubated in lysis buffer on ice for 10 min., then spun at 10,000 x g for 15 min. at 4° C. A DC protein assay (BioRad) was performed on the supernatant to determine protein concentration. Twenty μg protein were resuspended in NuPAGE LDS sample loading buffer (Invitrogen) in the presence of NuPAGE sample reducing agent (Invitrogen) and heated to 100° C for 3 min. Samples were iced and spun briefly, then loaded in onto 4-12% bis-tris polyacrylamide gels (Invitrogen) and run in MOPS running buffer (Invitrogen) for 50 min. at 200 V (constant). Gels were transferred to primed Immbobilon PVDF-FL membrane (Millipore) at 25 mA (constant) overnight in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol). Following transfer, the membrane was blocked by brief incubations in 100% methanol, water, and then Odyssey blocking buffer (LI-COR) for 1.5 hours. Primary antibody incubations were carried out in Odyssey blocking buffer at 4° C overnight using the following primary antibodies: goat anti-human GATA2 polyclonal antibody (R&D Systems, #AF2046; 1:2000), rabbit anti-human GATA3 polyclonal antibody (Abcam, #ab32858; 1:500), mouse anti-actin monoclonal antibody

(Millipore, #MAB1501; 1:20,000), mouse anti-GFP monoclonal antibody (Millipore, #MAB3580; 1:3,000). Following primary antibody incubation, membranes were washed 3 x 10 min. in PBS, then incubated for 45 min. in Odyssey blocking buffer (LI-COR) containing the corresponding infrared fluorophore-conjugated secondary antibody at a concentration of 1:10,000 in the dark. The following secondary antibodies (all from LI-COR) were used: IRDye 680-conjugated donkey anti-goat IgG, IRDye 800-conjugated donkey anti-rabbit IgG, IRDye 800-conjugated donkey anti-mouse IgG. Membranes were washed 3 x 10 min. in PBS in the dark, then imaged on an Odyssey scanner (LI-COR).

For each experiment, conditions were performed in triplicate wells. Band fluorimetry was performed using Odyssesy software (LI-COR), and GATA2 or GATA3 bands were normalized to their respective beta-actin loading controls. The average of the three triplicate wells was calculated and considered an experimental n of one. Depending on the conditions being compared, statistical significance was determined by performing either a one-way ANOVA with Dunnet *post hoc* test or an unpaired, two-tail Student's t-test. α=0.05.

3.4 RESULTS

3.4.1 Design and *in vitro* validation of siRNA for specific silencing of rat GATA2

The ideal siRNA for silencing rat *GATA2* would be one that exclusively recognizes the rat GATA2 transcript and nothing else in the rat transcriptome, thereby limiting off-target effects. Alignment of rat GATA transcription factor sequences using CLC Workshop 6 software revealed

that the highest degree of homology among the GATA transcription factors is found within the coding region of these genes, particularly in classical domains such as the two zinc-finger domains (Figure 8a-b). Greater sequence divergence among these genes can be found in the 5' and 3' untranslated regions (UTR). We designed 21-mer siRNAs against three unique regions of rat GATA2 mRNA that share little homology with the other five rat GATA transcription factors (Figure 8c). Two of these siRNAs (designated si1077 and si1678 for the base pair position where they were designed to bind) target sequences within the coding region and one siRNA (si2034) targets a sequence in the 3' UTR. For a negative control siRNA, we used a sequence that has been validated through microarray studies not to alter expression of any genes within the rat genome (Invitrogen).

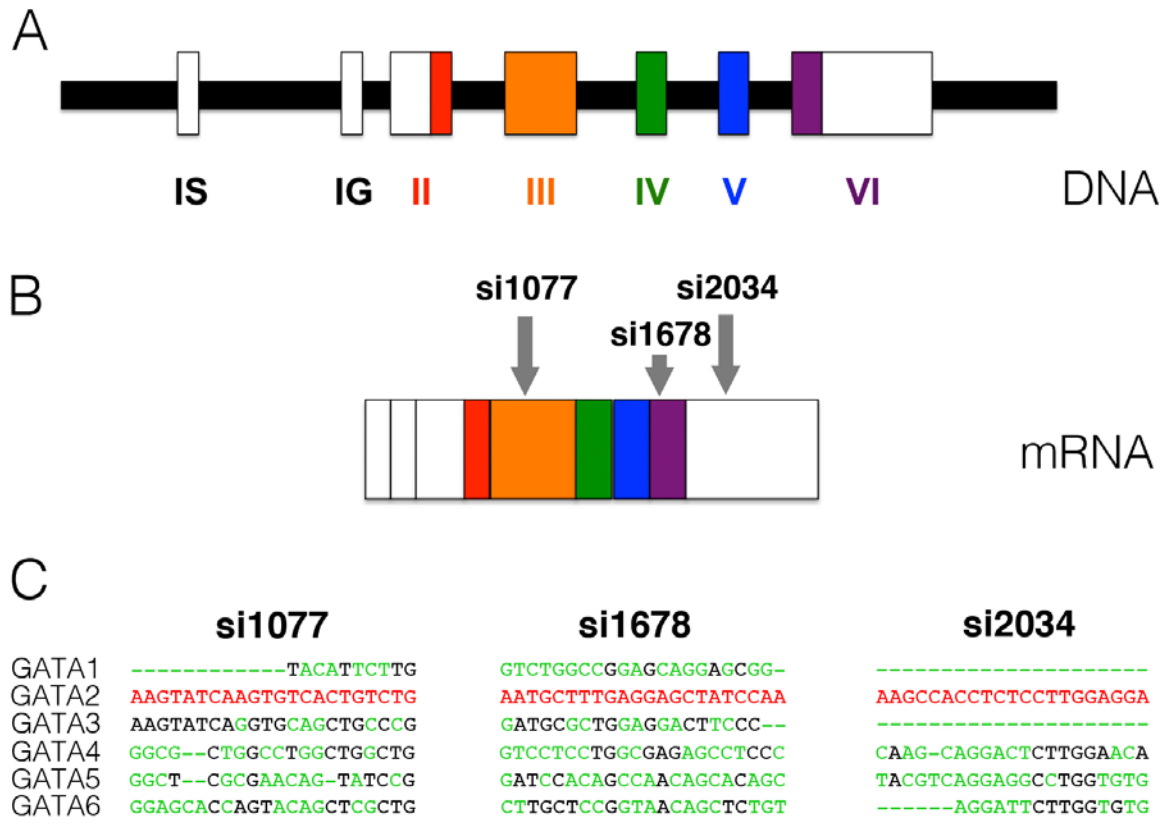

Figure 8. Rational design of siRNAs for specific silencing of rat *GATA2*

(A) Genomic DNA map of rat GATA2. Vertical boxes represent exons and are denoted by the Roman numerals below. Black bars represent intergenic sequences. Colored regions indicate coding sequence. (B) mRNA map of rat GATA2. Boxes represent exons and correspond to those in (A). White boxes on left and right represent 5' UTR and 3' UTR, respectively. Gray arrows indicate sites of homology for each of the three siRNAs. (C) siRNA sequences are homologous to rat GATA2 mRNA (red sequence) and show sequence divergence from other rat GATA transcription factors. Green nucleotides represent mismatches relative to the siRNA sequence.

To validate the siRNAs for their ability to silence rat *GATA2* robustly and specifically, we transiently transfected undifferentiated PC12 cells with each of the siRNAs for 48 hours and analyzed GATA2 protein levels by western blot (Figure 9). Relative to siNeg, we found that each of the siRNAs achieved significant dose-dependent silencing of *GATA2* (Figure 9a-b). Transfection of cells with a fluorophore-conjugated 21-mer double-stranded RNA probe under the same transfection conditions demonstrated a transfection efficiency of 70-80% (data not shown). Since si1678 achieved 70-80% *GATA2* silencing by western blot, it can be concluded that si1678 is maximally efficacious in silencing *GATA2*. In addition to *GATA2*, PC12 cells also express *GATA3*, allowing us to assess whether the strong silencing we observed is specific for *GATA2*. Western blots using the same 80 nM siRNA-treated cell lysates as in Figure 9a-b showed no significant difference in GATA3 protein levels suggesting that the silencing effect of each siRNA is specific to GATA2 (Figure 9c). Since si1678 achieved maximal silencing at lower concentrations than si1077 and si2034 and was equally specific for GATA2 (Figure 9b-c), the

si1678 sequence was selected as the basis for the subsequent design and cloning of an shRNA-expressing plasmid (sh1678).

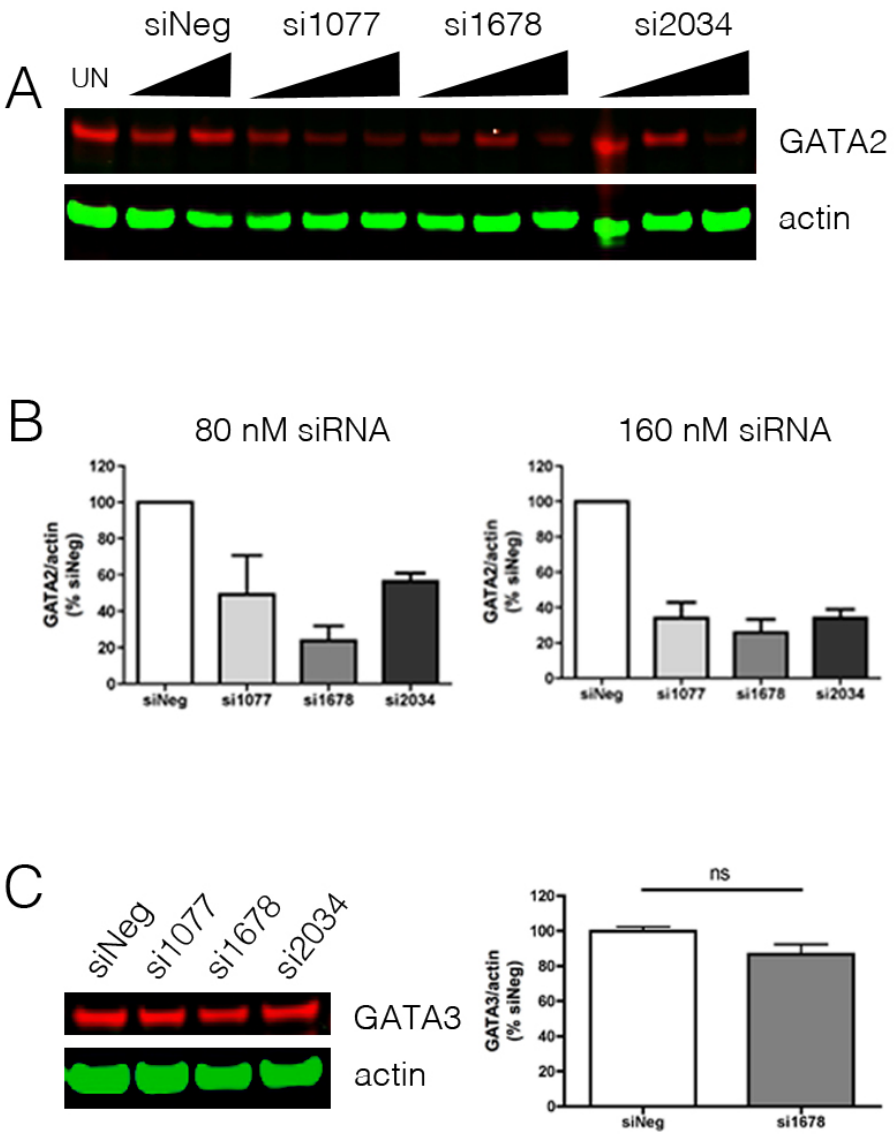

Figure 9. siRNAs robustly and specifically silence rat *GATA2* in PC12 cells

(A) A representative western blot of PC12 cell lysates following 48-hour transfection with siRNA against GATA2 shows dose-dependent GATA2 silencing for each of the siRNAs relative to siNeg. **(B)** Quantification of GATA2 silencing from three independent experiments demonstrates significant silencing for si1678 at 80 nM and for each of the siRNAs at 160nM. Means of si1077, si1678, and si2034 were compared to that of siNeg by one-way ANOVA with Dunnet *post hoc* test ($p<0.05$). **(C)** A representative western blot (left) on the 80 nM cell lysates used in (A). Quantification (right) of mean GATA3 protein levels from three independent experiments shows no significant change in GATA3 protein levels. $\alpha = 0.05$.

3.4.2 Design and *in vitro* validation of shRNA for specific silencing of rat *GATA2*

In order to develop an shRNA-expression vector for *in vivo* use, it was necessary to construct a double-stranded oligodeoxyribonucleotide (oligonucleotide) based on the validated si1678 sequence that could express shRNA after cloning it into an AAV packaging plasmid. We made an oligonucleotide sequence that contained (5'-3'): a *BamHI* restriction site, the sense si1678 sequence, a flexible "loop" sequence, the antisense si1678 sequence, and an *EcoRI* restriction site (Figure 7). The rationale behind arranging the sense and antisense si1678 sequences separated by a "loop" is to create an shRNA artificially, with the "loop" acting as the hairpin (Figure 7). Entering the oligonucleotide sequence into a secondary structure prediction program (RNAFold) yielded a prediction of very strong secondary structure, consistent with the formation of a hairpin structure (data not shown). The restriction sites flanking the shRNA sequence were

chosen so that the oligonucleotide would be in appropriate orientation relative to the U6 promoter when cloned into the AAV packaging plasmid (see Appendix, 6.0, for vector map). Oligonucleotides based on the siNeg sequence were constructed and cloned in the same manner to generate a negative control shRNA-expressing plasmid (shNeg).

Following cloning of the oligonucleotide into the AAV packaging plasmid and plasmid purification, we used western blot analysis to test whether sh1678 was able to silence *GATA2* robustly and specifically, similar to what we observed for si1678. Transfection of undifferentiated PC12 cells with sh1678 or shNeg for 48 hours resulted in approximately 60% transfection efficiency, as detected by expression of the GFP reporter gene from the plasmid (Figure 10a-b). Furthermore, significant GATA2 silencing was observed relative to transfection with shNeg (Figure 10c-d). Similar to our findings with si1678, no change in GATA3 levels was detected (Figure 10e). Taken together, these data indicate that the sh1678 plasmid robustly and specifically silences *GATA2* and that both sh1678 and shNeg express the GFP reporter gene.

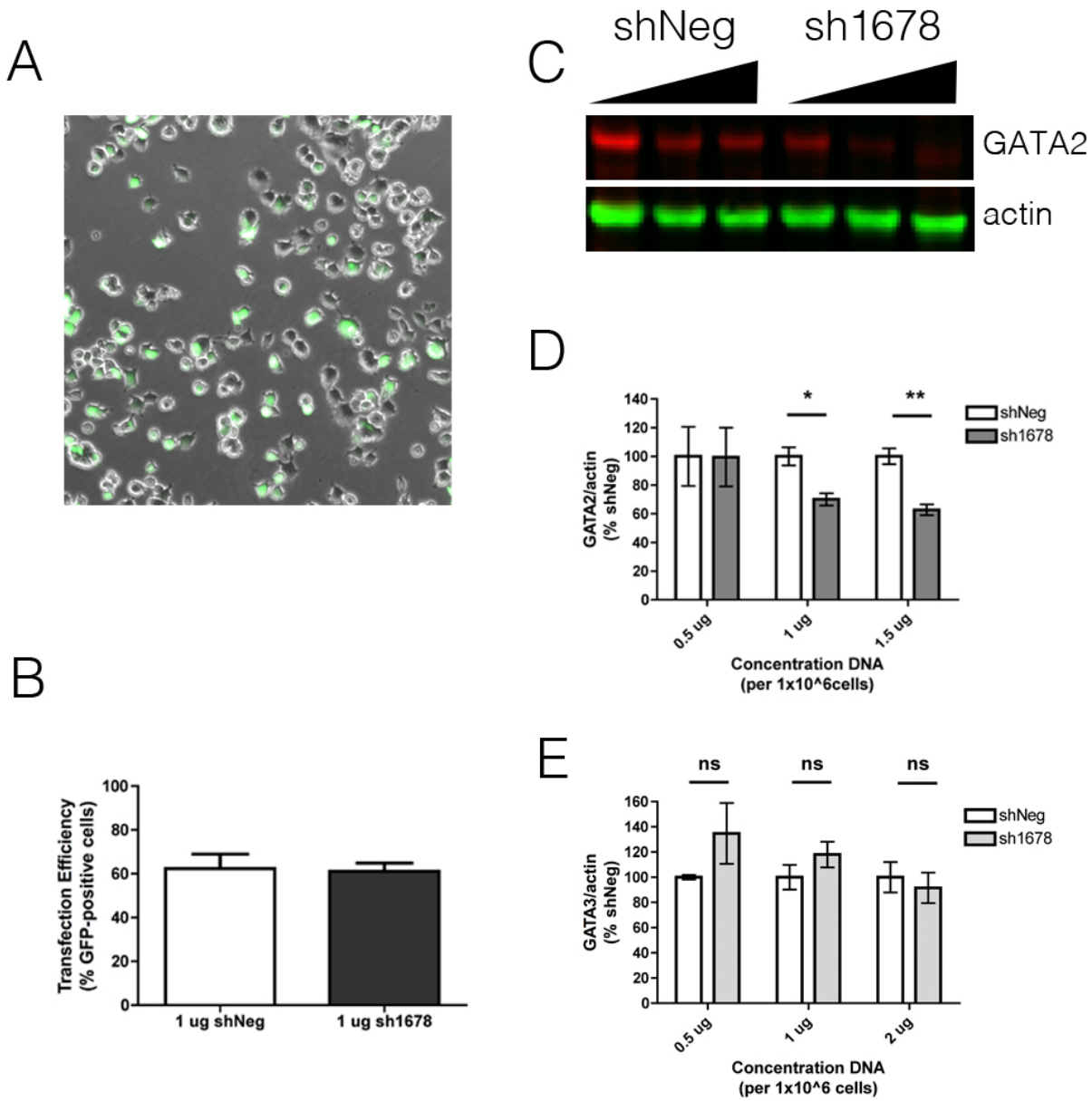

Figure 10. sh1678 robustly and specifically silences rat *GATA2* in PC12 cells

(A-B) 48-hour transfection of undifferentiated PC12 cells with sh1678 or shNeg results in 60% transfection efficiency (B), as indicated by expression of the reporter gene GFP in green (A). **(C)** Western blot on total cell lysates from undifferentiated PC12 transfected for 48 hours with sh1678 or shNeg shows dose-dependent silencing of GATA2 with sh1678 and no significant change with shNeg. A representative blot is shown. **(D)** Quantification of changes in GATA2 protein levels from three independent experiments using individual Student t-tests to compare sh1678 to shNeg at each concentration. **(E)** Qantification of western blots probed for GATA3 using the same cell lysates from PC12 transfectants shows no change in GATA3 protein levels (n = 3). $\alpha = 0.05$.

3.4.3 In *vitro* validation of viral vectors packaged with shRNA against GATA2

We selected non-pseudotyped AAV2 as a viral vector for delivery of the sh1678 and shNeg plasmids into rat SNc because it was found *in vivo* to have a strong tropism for rat SNc dopaminergic neurons (Cannon et al., n.d.). After scaling up plasmid production and purification, we had the sh1678 and shNeg plasmids packaged into AAV2 (hereafter referred to as AAV2.sh1678 and AAV2.shNeg).

As a final step in our *in vitro* validation, we sought to test whether the AAV2.sh1678 was effective in silencing *GATA2*, similar to si1678 and sh1678. Following viral transduction, it typically takes days to weeks to achieve maximal expression levels of transgenes (Dykxhoorn et

al., 2003). Since undifferentiated PC12 cells actively divide, a one-week transduction with our AAV2 vectors would allow for multiple rounds of cell division to occur, at which point the majority of the cells would likely not contain high levels of viral plasmid, thereby underestimating silencing effects. To slow cell divison rate, we differentiated PC12 cells for 6 days with NGF. Six-day differentiation in NGF resulted in extension of processes and a slowing of division rate (data not shown). We transduced the differentiated PC12 cells for one week with a range of viral titers and found by western blot that both vectors exhibited a dose-dependent increase in GFP expression, again indicating that the reporter gene cassette is functional (Figure 11). Furthermore, we found that transduction of cells with AAV2.sh1678 led to a dose-dependent reduction in GATA2 protein levels relative to AAV2.shNeg (Figure 11). Levels of GATA2 protein did not change across titres of AAV2.shNeg (Figure 11). In summary, both of the viral vectors that we generated express the GFP reporter gene – and AAV2.sh1678 strongly silences *GATA2*, whereas AAV2.shNeg has no effect on GATA2 levels.

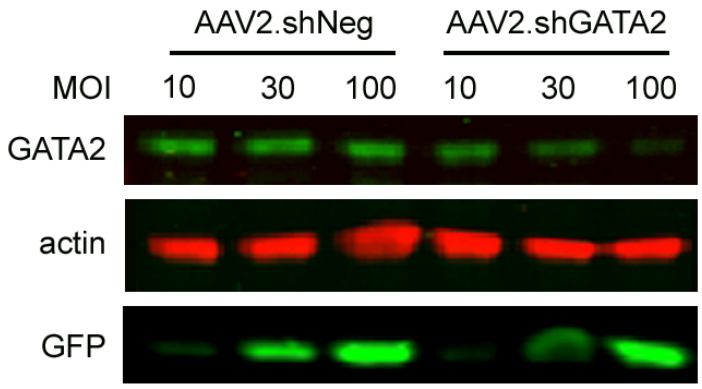

Figure 11. In *vitro* validation of viral vectors

Transfection of differentiated cells for 1 week with AAV2.sh1678 robustly silences GATA2. Both vectors express the reporter gene GFP.

3.5 DISCUSSION

Modulation of gene expression *in vivo* via RNA interference poses several challenges that are not encountered when modulating gene expression *in vitro*. Among these challenges is effective targeting of the intact interfering RNA to the cells of interest; this matter is further complicated when the cells of interest reside in the brain, which is separated from the systemic vasculature by the relatively impermeable blood-brain barrier. *In vitro* transfection of cells with RNA can be accomplished by encasing the RNA in liposomes and adding the RNA-liposome complexes to cells (lipofection). Depending on the cell type, the RNA-liposome complexes are often readily taken up by the cells. Delivery of RNA to cells *in vivo* requires first bypassing the many systems in place to degrade foreign particles like RNA and liposomes. A common approach to circumvent these issues is to perform the gene delivery with a viral vector that preferentially infects certain cell types. These vectors can be packaged with the desired gene in the form of a plasmid. These vectors still face the challenge of gaining entrance to the brain if infused systemically, so the usual approach is to infuse the viral vectors directly into the desired brain region.

In order to test our hypothesis that GATA2 regulates *SNCA in vivo*, we decided to create a viral vector containing a plasmid that expresses shRNA against rat GATA2 that we could infuse directly into the rat brain to silence *GATA2* within SNc dopaminergic neurons. We started the process of creating this viral vector by selecting unique regions of rat GATA2 mRNA against which to design siRNAs. The more specific these regions are to GATA2, the more specific the siRNA should be in silencing GATA2, thereby leaving levels of other transcripts – particularly the other closely related GATA factors – unchanged. We screened the siRNAs in PC12 cells, which express *GATA3* in addition to *GATA2*, and found that they robustly and specifically silenced *GATA2* while leaving GATA3 levels unaltered (Figure 9). As expected, the shRNA-expressing plasmid (sh1678) that we developed based on one of the siRNAs (si1678) was similarly effective and specific in silencing *GATA2* (Figure 10). Additionally, sh1678 and its corresponding negative control, shNeg, expressed the GFP reporter gene, which is important for localizing transduced neurons *in vivo* in Chapter 4. Our final validation of the AAV2 virus that was packaged with our plasmids demonstrated that these vectors are likewise effective in silencing *GATA2 in vitro* (Figure 11).

While our data in this chapter validate the viral vectors we have generated for *in vivo* use, it is nonetheless important to note some limitations of our validation studies. We chose to examine levels of GATA3 in our validation studies as an indication of specificity. Our rationale for this is that *GATA3* shares much sequence homology with *GATA2* and is therefore more likely to be targeted by siRNAs weakly specific for GATA2. Furthermore, *GATA3* is the only other GATA transcription factor known to be expressed in healthy, post-natal rodent brain. While we believe these reasons make it a suitable protein to examine for off-target effects of the

siRNA/shRNA/AAV2.shRNA, it is possible (but unlikely) that other transcripts, unrelated to *GATA2* and *GATA3* expression, are influenced by the siRNA.

It might be suggested that microarray experiments to profile expression changes in large numbers of genes following *GATA2* silencing in PC12 cells may be a better way to track off-target effects. However, this approach is confounded by the fact that GATA2 is a known master regulator of gene expression; it is expected that modulation of *GATA2* expression would bring about changes in the expression of many genes. GATA2 has not been shown to directly regulate *GATA3*, so the fact that we are not detecting changes in GATA3 levels upon *GATA2* silencing supports a lack of siRNA off-target effects.

Taken together, the data from the rigorous, step-wise characterization of our RNA interference reagents in this chapter demonstrate that our viral vectors are appropriate for testing the hypothesis *in vivo* that GATA2 positively regulates *SNCA* in SNc dopaminergic neurons.

4.0 GATA2 SILENCING IN VIVO: CHARACTERIZATION OF EFFECTS ON ALPHA-SYNUCLEIN EXPRESSION AND ASSESSMENT OF NEUROPROTECTION IN THE ROTENONE RAT MODEL OF PD

4.1 ABSTRACT

In this chapter, we directly test the two hypotheses of this dissertation project using the viral vectors that we generated in Chapter 3. In order to validate the vectors *in vivo*, we histologically examine their ability to transduce the nigrostriatal system following stereotaxic infusion without toxicity *per se* and silence *GATA2*. Following validation, we address the first hypothesis – that GATA2 regulates *SNCA in vivo* – by assessing changes in *SNCA* expression. We address the second hypothesis – that silencing *GATA2* is protective in the rotenone rat model of PD – by delivering the vectors to rats then administering the parkinsonian neurotoxin, rotenone. We find that, although GATA2 positively regulates *SNCA* in SNc neurons, silencing *GATA2* does not confer neuroprotection against rotenone.

4.2 INTRODUCTION

In this chapter, we validate *in vivo* the viral vectors that we generated in the previous chapter and we use these reagents to test two hypotheses: 1) GATA2 positively regulates *SNCA* under basal conditions in SNc dopaminergic neurons *in vivo*, and 2) silencing *GATA2* expression in SNc dopaminergic neurons *in vivo* is neuroprotective in the rotenone rat model of PD. Since testing both of these hypotheses requires modulation of *GATA2* expression in a specific subset of neurons (SNc dopaminergic neurons), it is important first to ensure that delivery of the viral vectors is anatomically accurate, that transgene expression is detectable, and that the AAV2.shGATA2 vector silences *GATA2* without the viral vectors *per se* having deleterious effects on the nigrostriatal system. Inaccurate delivery or incomplete transgene expression (especially from the shRNA cassette) would confound results from experiments assessing *SNCA* expression or neuroprotection since they depend on strong down-regulation of *GATA2* expression in the correct cell population. If the viral vectors *per se* are harmful the nigrostriatal system—e.g. by inducing a dramatic inflammatory response—then they will not warrant further testing as potential neurotherapeutic agents.

4.3 MATERIALS & METHODS

4.3.1 Animals

Twenty-two 7-month old male Lewis rats were purchased from Hilltop Lab Animals, Inc. and housed and treated in accordance with National Institutes of Health guidelines and University of Pittsburgh Institutional Animal Care and Use Committee (IACUC)-approved protocols. Rats were sacrificed and their brains processed for immunohistochemical analysis as described in 2.3.6.

4.3.2 Viruses & Stereotaxic surgeries

shRNA expression cassettes were cloned into an AAV gene transfer plasmid (see Appendix, 6.0, for vector map) and were purified, and packaged into AAV2 by Penn Vector Core (Philadelphia, PA), as described in 3.3.2. Upon receipt on dry ice, viruses were briefly thawed and diluted in sterile PBS to 2 x 10^{12} GC/mL, which was then quickly prepared into 10 μL aliquots and stored at -80° C until use. All viral work was performed in a BSL2+ facility, in accordance with approved institutional recombinant DNA and IACUC- protocols. Animals were anesthetized by inhalation of 3% isoflurane until unresponsive to painful stimuli, then mounted onto a stereotaxic frame and maintained on 1.5 L of oxygen with 2% - 3% isoflurane through a frame-mounted nasal cannula. For each infusion, an aliquot of virus was briefly thawed and drawn up in a Hamilton syringe (#7635-01) and custom needle (#7803-07; 30-gauge, 1.25-in., 45° bevel), placed on the stereotaxic frame and Bregma was measured. For each infusion, 2 μL of virus (at a

concentration of 2 x 10^{12} GC/mL) was delivered over 10 min. (200 nL/min.), and 5 min. were allowed to pass before slowly retracting the needle. AAV2.shGATA2 was infused first (-5.5 mm A/P, -2.0 mm R/L, -7.5 mm V to bregma), followed by AAV2.shNeg (: -5.5 mm A/P, +2.0 mm R/L , -7.5 mm V to bregma). Buprenorphine (0.05 mg/kg, i.p) was administered as a post-operative analgesic once animals were ambulatory without evidence of labored breathing. Animals were monitored daily and buprenorphine was administered at this dose twice per day for the first two post-operative days.

4.3.3 Chromogenic immunohistochemistry (IHC)

For chromogenic IHC, free-floating sections were removed from cryoprotectant and washed 6 x 10 min. in phosphate-buffered saline (PBS, pH 7.4). Sections were incubated for 10 min. in 3% H_2O_2 in PBS, then washed 3 x 10 min. in PBS. Sections were blocked in 10% normal donkey serum (NDS) in PBS containing 0.3% Triton-X (PBST) for 1 hour. Primary antibody solutions were prepared by resuspending the following antibodies (individually) in PBST containing 1% NDS: monoclonal mouse anti-GFP antibody (1:4000, Millipore, #MAB3580), polyclonal mouse anti-rat CD11b (OX42; 1:150, AbD Serotec, #MCA275G), mouse anti-tyrosine hydroxylase (TH; 1:2000; Millipore, #MAB318) Sections were incubated in this solution for 48 hours at 4° C. After 3 x 10 min. washes in PBS, sections were incubated for 1 hour in a secondary antibody solution containing biotin-conjugated donkey-anti-goat at 1:200 (Jackson Immunoresearch, #705-065-147) in 1% NDS in PBST. After 3 x 10 min. washes in PBS, sections were incubated in ABC peroxidase kit solution (Vectastain, #PK-6100) for 1 hour then washed 3 x 10 min. washes in PBS. Peroxidase development with the DAB chromogen was performed according to

manufacturer's instructions (Vector Laboratories, #SK-4100) then sections were washed 6 x 10 min. in PBS. Sections were mounted onto Superfrost slides (Fisher) and allowed to dry overnight. Lastly, sections were then dehydrated through graded ethanols, cleared in Histoclear (National Diagnostics, #HS-200), and coverslipped in Histomount (National Diagnostics, #HS-103).

4.3.4 Fluorescent IHC

Free-floating sections were removed from cryoprotectant and washed 6 x 10 min. in PBS (pH 7.4). Sections were blocked in 10% NDS in PBST for 1 hour, then incubated in 1% NDS in PBST containing one or more of the following primary antibodies: polyclonal goat anti-human GATA2 (1:500, R&D Systems, #AF2046); monoclonal mouse anti-GFP antibody (1:4000, Millipore, #MAB3580); polyclonal rabbit anti-TH (1:3000, Millipore, #AB152). Primary antibody incubation was carried out at 4° C for 48 h. Sections were then washed 3 x 10 min. in PBS before incubation in the following secondary antibodies in PBST containing 1% NDS: Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:500; Invitrogen), Cy3-conjugated donkey anti-rabbit IgG (1:500; JacksonImmuno), Alexa Fluor 647-conjugated donkey anti-goat IgG (1:500; Invitrogen). Secondary antibody incubation was carried out at room temperature for 1 h. in the dark. Sections were washed in PBS 3 x 10 min. in the dark, mounted onto Superfrost slides (Fisher), coverslipped in gelvatol aqueous mounting medium (200 mM Tris-HCl, glycerol, polyvinyl alcohol, DABCO, pH 8.2), and dried overnight in the dark.

4.3.5 Confocal microscopy & Quantitative fluorescent IHC

Confocal microscopy and quantification of fluorescent IHC was performed as described in 2.3.5 with the following modifications. Anatomically-matched SN sections were imaged at 40X using the same confocal settings and ROIs were precisely drawn around somata using the confocal microscope image analysis software, Fluoview FV1000 (Olympus, Japan). Average fluorescence intensity was measured for each ROI and averages and standard errors of the mean (SEM) were calculated for each condition. Approximately 100 ROIs were quantified per animal. A paired Student's t-test was used to compare normalized fluorescence intensity means (α=0.05).

4.3.6 Quantification of striatal TH fluorescence intensity

Rat striata were immunohistochemically stained for TH using a polyclonal sheep anti-tyrosine hydroxylase (TH) antibody (1:2000, Millipore, #AB1542) as described in 4.3.4, except IRDye 800-conjugated donkey anti-sheep IgG (LI-COR) was used as a secondary antibody at a concentration of 1:500 in 1% NDS in PBST. Slides containing stained striata were imaged at high resolution on an Odyssey scanner (LI-COR). ROIs were precisely drawn around the TH-immunoreactive area of the striatum, dorsal to the anterior commissure. Quantification of mean fluorescence intensity was performed using Odyssey software (LI-COR) and means were compared statistically using a paired Student's t-test (α=0.05).

4.3.7 Western blot analysis

See section 3.3.4.

4.3.8 *in situ* hybridization

See section 2.3.7 for cloning of GATA2 cRNA probes and ISH. The SNCA cRNA probes were cloned as described in (Cannon et al., n.d.).

4.3.9 Northern blot analysis

A 7-month old male Lewis rat was deeply anesthetized with CO_2 and decapitated in accordance with IACUC-approved protocol. The brain was quickly removed and a 15-mg piece of frontal cortex was dissected. Total RNA was extracted using an RNeasy mini kit (Qiagen) according to manufacturer's instructions. RNA was checked for sufficient abundance and quality ($A_{260/280} \geq$ 2.00) using a Nanodrop ND-1000 spectrophotometer. Northern blot was carried out under RNAse-free conditions. RNA samples were prepared in formaldehyde loading buffer (Ambion) with ethidium bromide (0.33 µg/µL/sample), heated at 65° C x 15 min, then loaded onto a formaldehyde/MOPS/agarose (1%) gel and run at 60V in NorthernMax denaturing gel buffer (Ambion). Running buffer was gently redistributed from cathode to anode every 40 min. Transfer to Nytran-N (+) nylon membrane (Schleicher and Schuell BioScience) was performed in NorthernMax Transfer Buffer (Ambion) over three hours. The membrane was briefly UV cross-linked, then incubated in pre-hybridization buffer (UltraHyb buffer [Ambion], 1 mg/mL

Torula RNA [Sigma]). SNCA antisense cRNA probe (see 4.3.8) was added to the pre-hybridization buffer at a final concentration of 20 ng/mL and incubated overnight at 65° C. The membrane was washed serially in 2X SSC, 0.1% SDS then 0.1X SSC, 0.1% SDS and incubated in a maleic acid-buffered solution containing alkaline phosphatase-conjugated sheep anti-digoxigenin secondary antibody Fab fragments (Roche) and blocking reagent. Following additional washes, signal was detected by chemiluminescence.

4.3.10 Rotenone rat study

Rotenone treatments were performed in accordance with an IACUC-approved protocol. Seven-month old male Lewis rats were infused with viral vectors as described in 4.3.2. Rotenone treatment was performed as described previously (Cannon et al., 2009). Briefly, animals were administered rotenone via daily i.p. injection (3.0 mg/kg/d in vehicle containing medium chain fatty acids [Miglyol]) until they reached behavioral phenotypic endpoint, at which time they were sacrificed as described in 2.3.6.

4.4 RESULTS

4.4.1 Stereotactic delivery of AAV2.shGATA2 to rat SN results in strong transduction of the nigrostriatal system

Adeno-associated virus 2 (AAV2) is an attractive vector for *in vivo* viral gene delivery based on its relative tropism for neurons, the duration of its transgene expression, its relative lack of

inflammatory response, and its general lack of toxicity (Bowers, Breakefield, & Sena-Esteves, 2011; Giacca, 2010; Hadaczek, Forsayeth, Mirek, Munson, Bringas, Pivirotto, McBride, Davidson, et al., 2009b). AAV2 delivery conditions for transduction of rat SNc dopaminergic neurons were optimized previously in our lab (data not shown). Using these conditions, we stereotactically infused one SNc with AAV2.shNeg and the other SNc with an equal viral titer of AAV2.shGATA2 and waited three or six weeks post-infusion for analysis of transduction. We found that a single viral infusion resulted in robust transduction of the SNc, as shown immunohistochemically by the presence of the GFP reporter gene throughout much of the anterior-posterior axis of the SNc (Figure 12). Transduction was evident in SNc neurons spanning from ventromedial SNc to dorsolateral SNc and was approximately equal between the side that received AAV2.shGATA2 and the side that received AAV2.shNeg (Figure 12). This finding is consistent with the similar transduction efficiency and similar levels of GFP expression that we observed with the viruses *in vitro* (Figures 10-11).

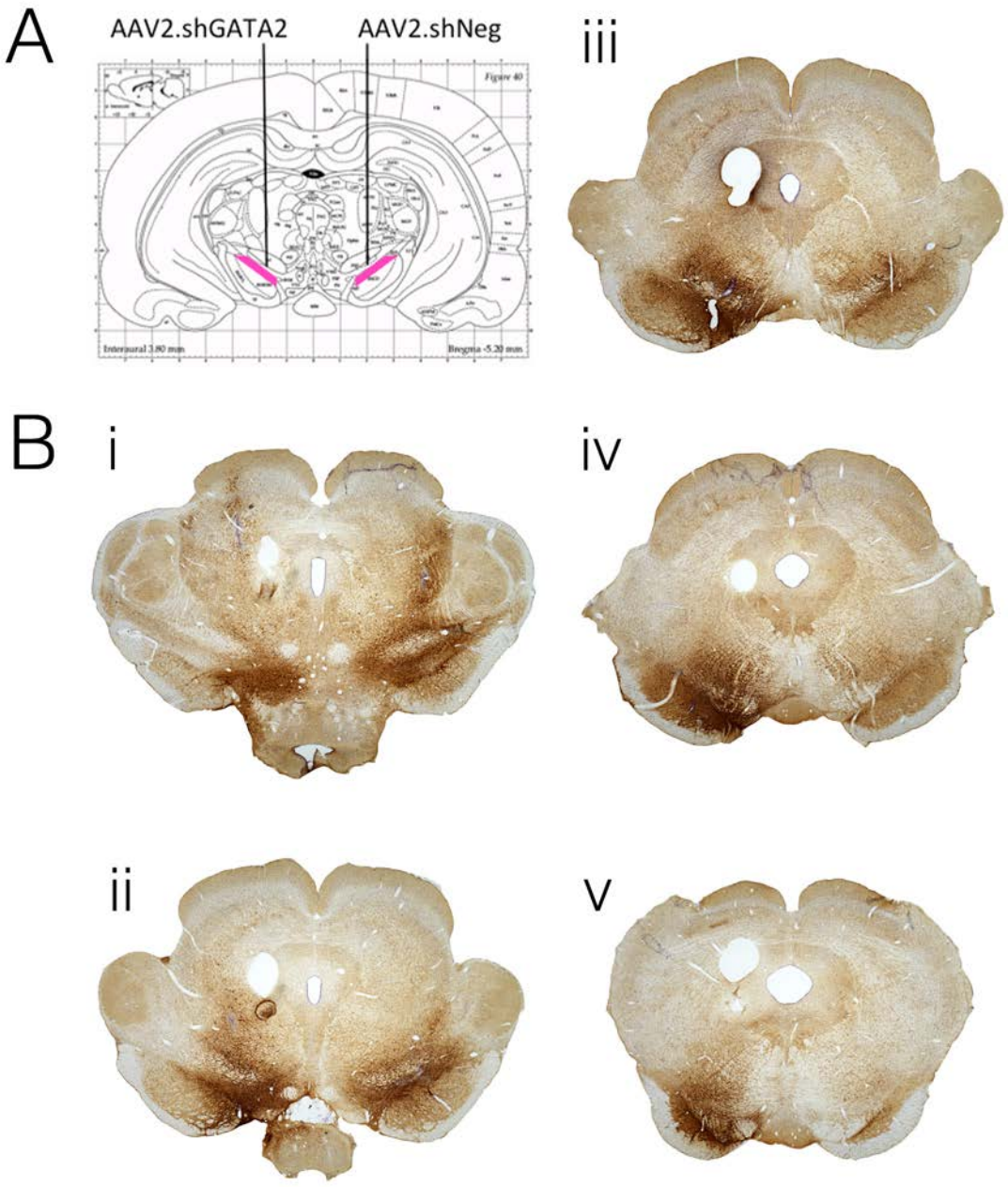

Figure 12. GFP staining indicates strong transduction of nigrostriatal cell bodies three weeks after stereotaxic delivery of AAV2.shGATA2 and AAV2.shNeg to rat SN

(A) Schematic showing anatomical sites of stereotaxic AAV2 infusion. **(B)** Midbrain sections from a representative animal collected three weeks post-infusion and stained immunohistochemically for GFP. Sections are aligned anterior to posterior (*i*-*v*). Punch holes in the dorsal midbrain indicate the SNc that received AAV2.shGATA2. The contralateral SNc received an equal titer infusion of AAV2.shNeg. (n=5)

In order to confirm that the transduced cells are nigrostriatal neurons, we assessed whether expression of the reporter gene was also present in terminals within the striatum. As shown in Figure 13, GFP expression is detected in terminals throughout the striatum in the same animals, indicating anterograde transport of the protein from the site of transduction in the SNc. This degree of transduction is consistent with our findings in the SNc **(**Figure 12).

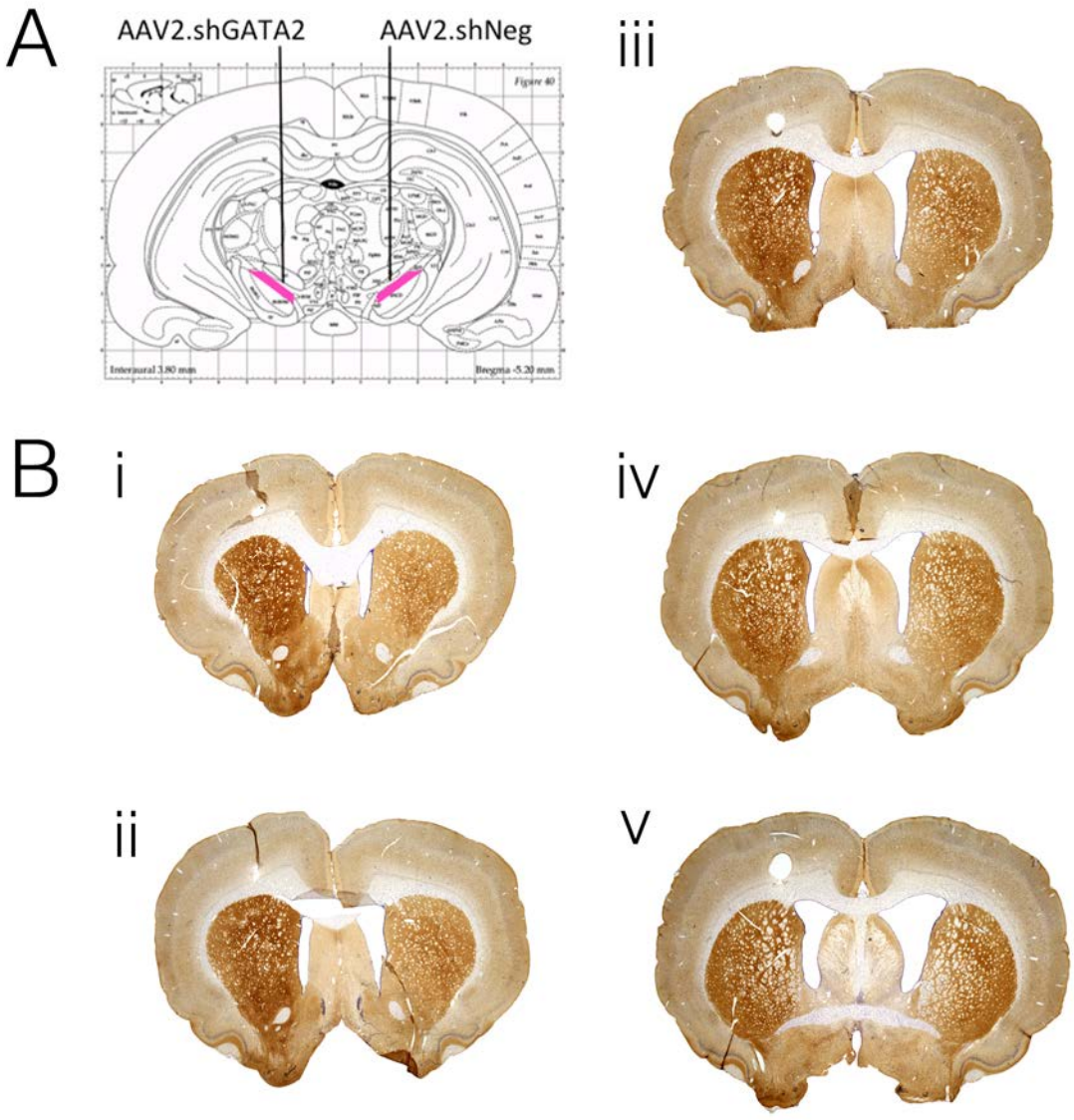

Figure 13. GFP staining of nigrostriatal terminals indicates strong transduction three weeks after stereotaxic delivery of AAV2.shGATA2 and AAV2.shNeg to rat SN

(A) Schematic showing anatomical sites of stereotaxic AAV2 infusion. **(B)** Striatal sections from a representative animal collected three weeks post-infusion and immunohistochemically stained for GFP. Sections are aligned anterior to posterior (*i*-*v*). Punch holes in the overlying cortex indicate the hemisphere that received AAV2.shGATA2 infusion into SNc. The contralateral SNc received an equal titer infusion of AAV2.shNeg. (n=5)

Because we will eventually test whether GATA2 silencing in SNc dopaminergic neurons is neuroprotective in the rotenone rat model of PD, it is important to confirm that transgene expression will persist throughout the duration of the rotenone treatment, which is typically one to two weeks long for 7-month old rats under this dosing regimen (Cannon et al., 2009). Therefore, we performed the same stereotaxic delivery of AAV2.shGATA2 and AAV2.shNeg and assessed GFP expression 6 weeks after viral infusion (3 weeks to allow for maximal transgene expression and an additional three weeks to extend beyond the longest expected rotenone treatment). Consistent with results from our lab and others' (Cannon et al., n.d.; Cederfjäll, Sahin, Kirik, & Björklund, 2012), GFP expression was still present at 6 weeks and was overall similar in degree relative to the 3-week animals (Figure 14). GFP expression was likewise detected in the striata of these animals, again demonstrating proper transduction of nigrostriatal neurons (Figure 15).

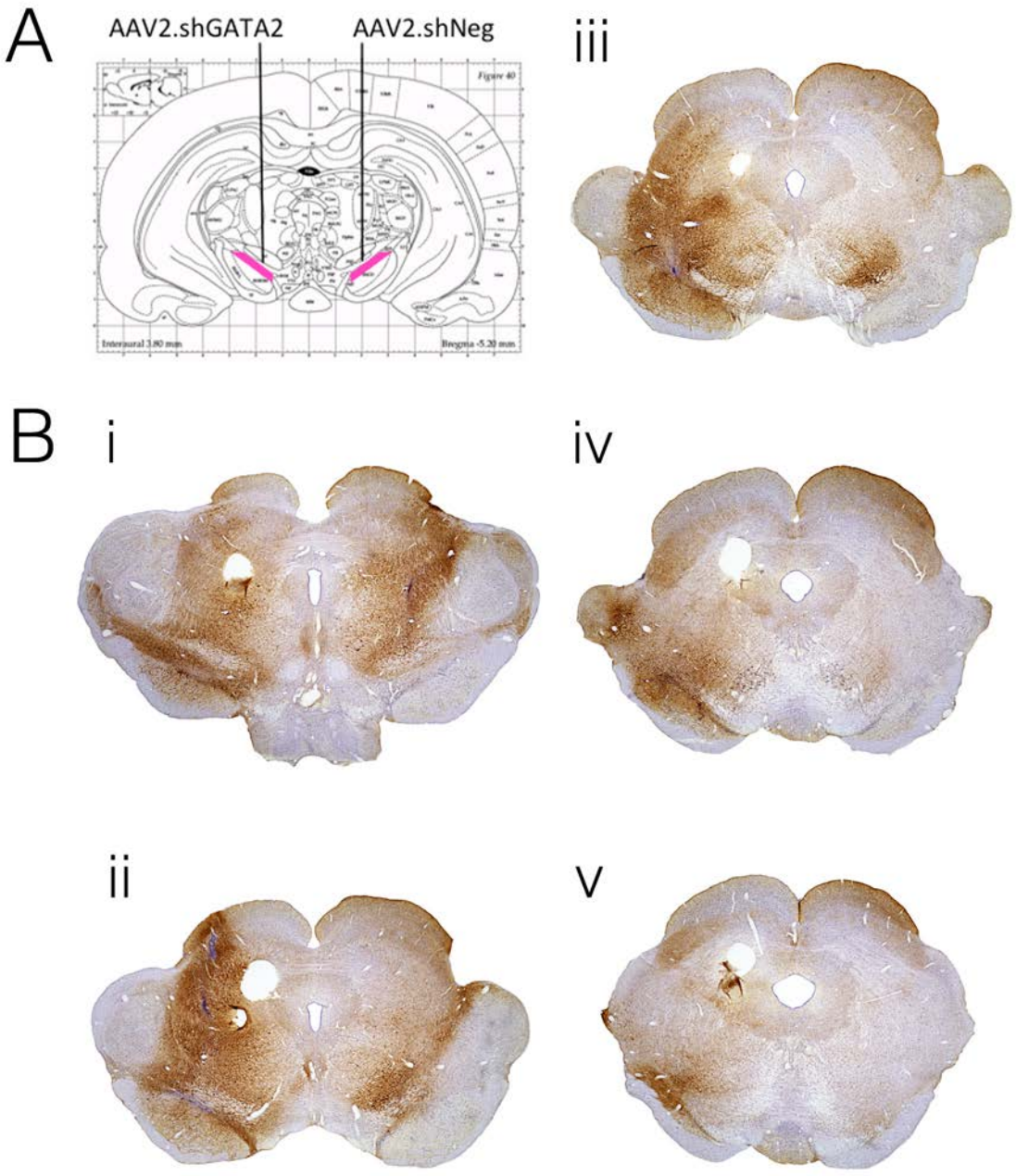

Figure 14. Strong transgene expression in nigrostriatal cell bodies persists six weeks after stereotaxic delivery of AAV2.shGATA2 and AAV2.shNeg to rat SN

(A) Schematic showing anatomical sites of stereotaxic AAV2 infusion. **(B)** Midbrain sections from a representative animal collected six weeks post-infusion and immunohistochemically stained for GFP. Sections are aligned anterior to posterior (*i*-*v*). Punch holes in the dorsal midbrain indicate the SNc that received AAV2.shGATA2. The contralateral SNc received an equal titer infusion of AAV2.shNeg. (n=6)

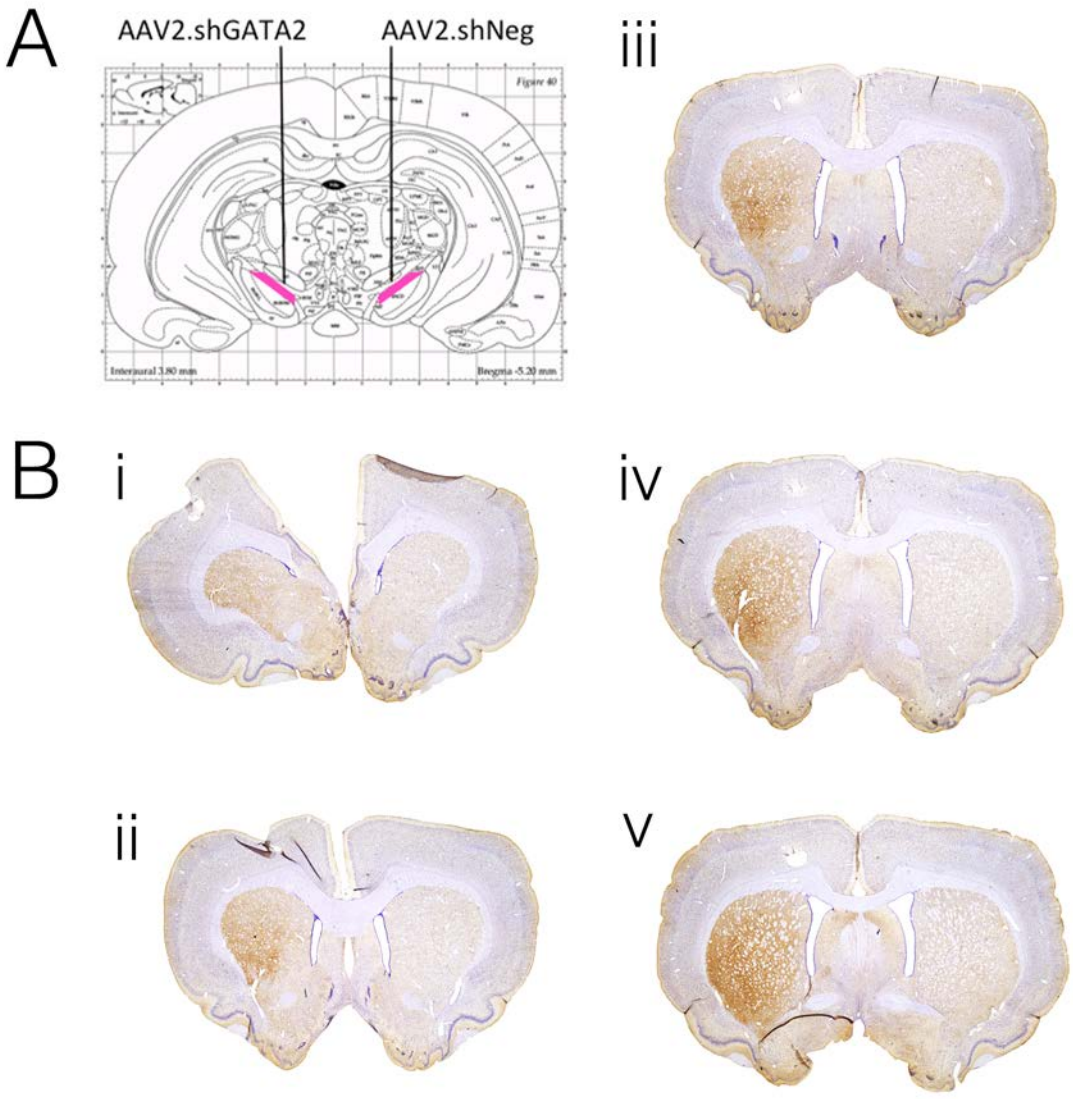

Figure 15. Strong transduction in nigrostriatal terminals six weeks after stereotaxic delivery of AAV2.shGATA2 and AAV2.shNeg to rat SN

(A) Schematic showing anatomical sites of stereotaxic AAV2 infusion. **(B)** Striatal sections from a representative animal collected six weeks post-infusion and immunohistochemically stained for GFP. Sections are aligned anterior to posterior (*i*-*v*). Punch holes in the overlying cortex indicate the hemisphere that received AAV2.shGATA2 infusion into SNc. The contralateral SNc received an equal titer infusion of AAV2.shNeg. (n=6)

Taken together, these data demonstrate that stereotaxic delivery of the viral vectors that were created and validated in Chapter 3 leads to robust transduction of the rat nigrostriatal system and that expression of the transgene persists for at least six weeks.

4.4.2 Stereotaxic delivery of AAV2.shGATA2 to rat SN produces moderate, transient inflammation that does not damage the nigrostriatal system

One of the limitations of some viral vectors for use in mammalian systems *in vivo* is their propensity to cause an inflammatory response (Giacca, 2010). This response can damage the tissue that the vectors are intended to preserve. Although the emergence of AAV as the primary type of viral vectors used in many gene therapy trials for neurological disorders is due largely to its relatively benign profile with respect to producing inflammatory responses, we nonetheless investigated whether AAV2.shGATA2 and AAV2.shNeg produce substantial inflammation. To assess inflammation, we stained sections from the animals that were sacrificed at three weeks or six weeks post-infusion for the inflammatory marker, OX42 (known alternatively as integrin

alpha M [ITGAM], macrophage-1 antigen [Mac-1], complement receptor 3 [CR3], or cluster of differentiation 11b [CD11b]). Within the brain, OX42 is expressed selectively on microglia and is up-regulated during microglial activation in the context of inflammation (Reid, Perry, Andersson, & Gordon, 1993).

To understand whether stereotaxic delivery *per se* causes inflammation, we infused PBS (equal in volume to our viral deliveries) into one SNc and qualitatively assessed for differences between ipsilateral SNc (PBS) and contralateral (no infusion) SNc with respect to OX42 staining. Ipsilateral OX42 staining was minimal and confined strictly to the needle track (Figure 16). Otherwise there was no discernable difference in staining between ipsilateral and contralateral hemispheres. Furthermore, there was no appreciable difference between the 3-week and 6-week cohorts of animals. Taken together, these data indicate that stereotaxic infusion *per se* does not induce inflammation in 7-month old rats at relevant time points for our eventual neuroprotection study.

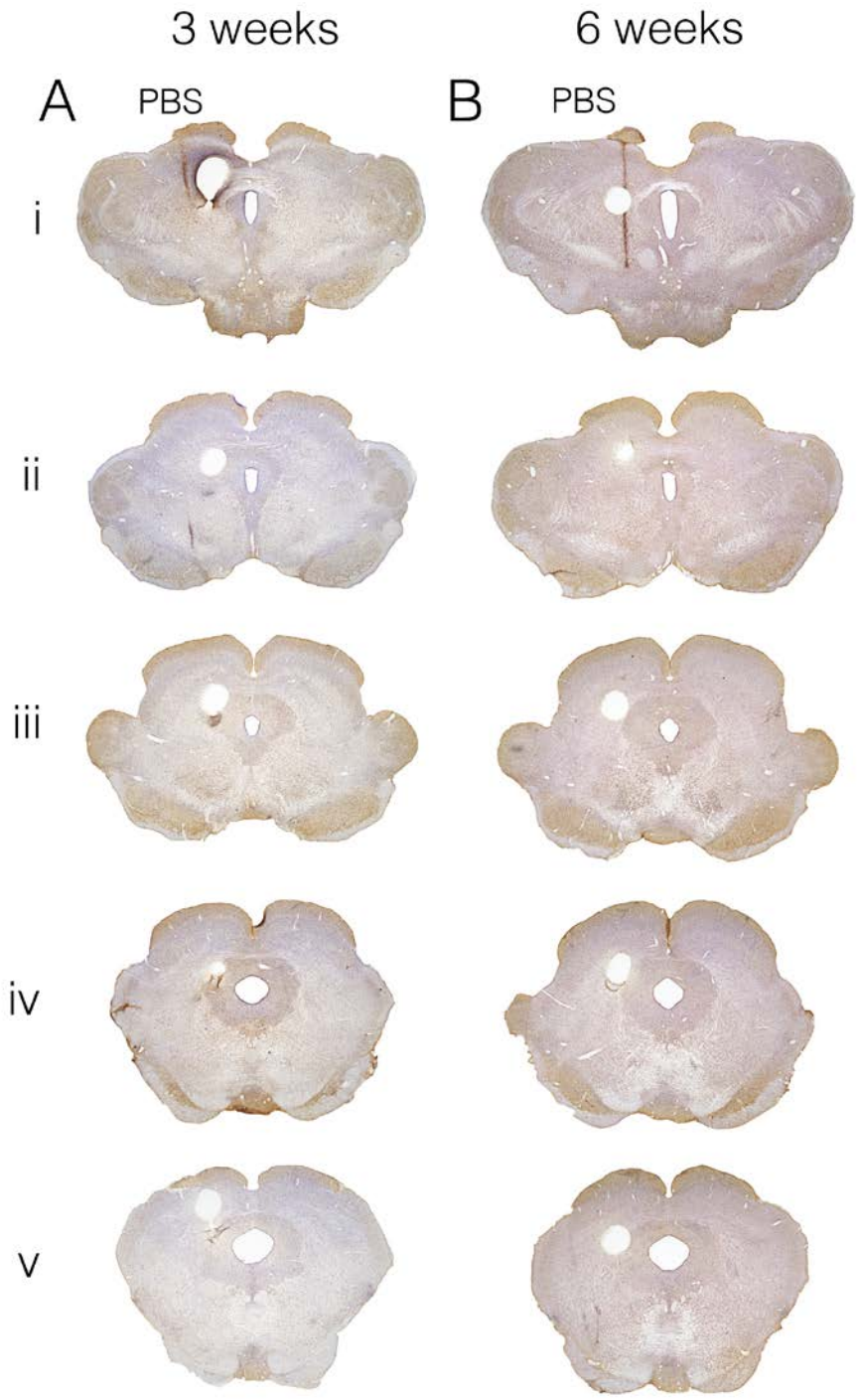

Figure 16. Unilateral PBS infusion to rat SN results in little inflammation

(A) OX42 immunohistochemical staining on rat midbrain sections (aligned anterior-to-posterior, *i*-*v*) three weeks after unilateral stereotaxic infusion of PBS demonstrates slight inflammation only along the needle track (sections i and ii). **(B)** OX42 immunohistochemical staining of rat midbrain sections (aligned anterior-to-posterior, *i*-*v*) six weeks after unilateral stereotaxic infusion of PBS demonstrates slight inflammation only along the needle track (section i), similar to what was observed in the 3-week cohort. Punch holes in the dorsal midbrain indicate the SN that received AAV2.shGATA2. The contralateral SN received no infusion. (3-week cohort, n=2; 6-week-cohort, n=2.)

We used the OX42 staining in the PBS-infused animals as a baseline to assess whether infusion of the viral vectors induced inflammation. When we stained midbrain sections from animals three weeks after infusion of virus, we found a moderate degree of inflammation relative to the 3-week animals that received a unilateral infusion of PBS (Figure 17A). This inflammation extends beyond the needle track and appears roughly equal when the ipsilateral (AAV2.shGATA2) and contralateral (AAV2.shNeg) hemispheres are compared (Figure 17A). Notably, the majority of inflammation is dorsal to the SNc even though the SNc of this representative animal was well transduced (Figure 12). Also, we did not detect in any of our animals frank necrotic lesions, suggesting the absence of severe inflammation.

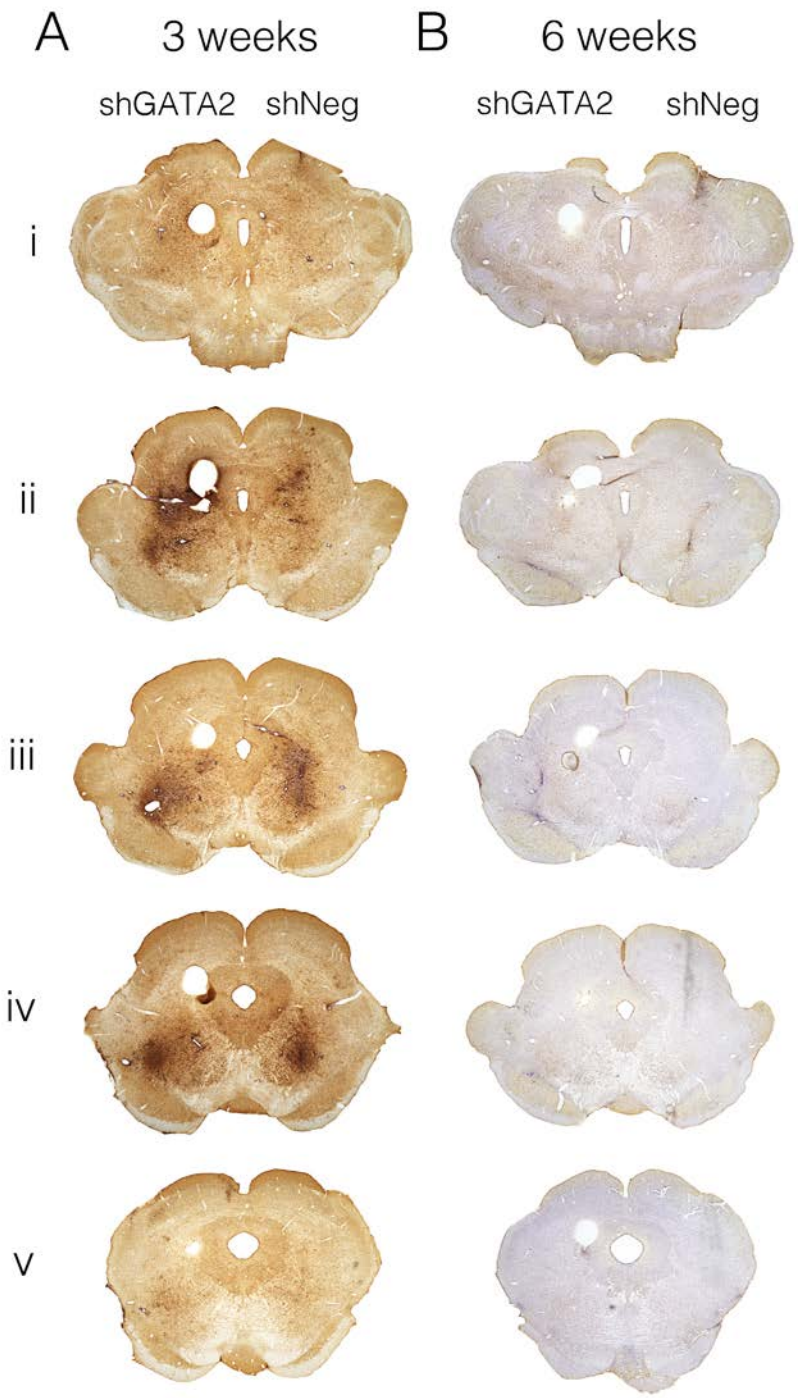

Figure 17. OX42 staining of rat midbrain sections three weeks and six weeks after AAV2 infusion to SN shows moderate but transient inflammation

(A) OX42 immunohistochemical staining on rat midbrain sections (aligned anterior-to-posterior, *i*-*v*) three weeks after bilateral stereotaxic infusion into SN of AAV2.shGATA2 (indicated by punch hole in dorsal midbrain) or AAV2.shNeg. Note similar inflammatory response for AAV2.shGATA2 and AAV2.shNeg. Also, OX42 staining is stronger and more extensive than that which was observed following unilateral PBS infusion to SN (Figure 16A). **(B)** OX42 immunohistochemical staining of rat midbrain sections (aligned anterior-to-posterior, *i*-*v*) six weeks after bilateral stereotaxic infusion into SN of AAV2.shGATA2. Note that OX42 staining is barely detectable as compared to OX42 staining at three weeks post-infusion (A). OX42 staining intensity six weeks post-infusion is instead similar to that which was observed following unilateral PBS infusion to SN (Figure 16a-b). (3-week cohort, n=5; 6-week-cohort, n=6.)

This inflammation appears to resolve by six weeks post-infusion, resulting in a pattern of OX42 staining that is similar to that observed in the animals that received unilateral infusion of PBS (Figure 17B). Nonetheless, it is important to understand what impact, if any, the observed moderate, transient inflammation induced by the viral vectors has on the integrity of the nigrostriatal system – *i.e.* the intended target for neuroprotection in our subsequent studies. To this end, we examined whether there was any preferential loss of nigrostriatal terminals in the AAV2.shGATA2 versus AAV2.shNeg hemispheres by staining sections for the dopaminergic marker, TH. Such loss of terminals is a sensitive marker for damage to the nigrostriatal system and is commonly used in parkinsonian models to detect such damage (Cannon et al., 2009; Cannon, Sew, Montero, Burton, & Greenamyre, 2011). We did not observe at either time point

(three weeks or six weeks post-infusion) a focal lesion in dopaminergic nigrostriatal terminals, suggesting that neither vector is toxic (Figure 18 a,c). When we quantitatively compared TH fluorescence intensity between the ipsilateral (AAV2.shGATA2) and contralateral (AAV2.shNeg) striata and we found no significant difference for either time point cohort, suggesting no preferential toxicity of one vector over the other (Figure 18 b,d**)**. Taken together, these data suggest that neither vector is toxic to the nigrostriatal system.

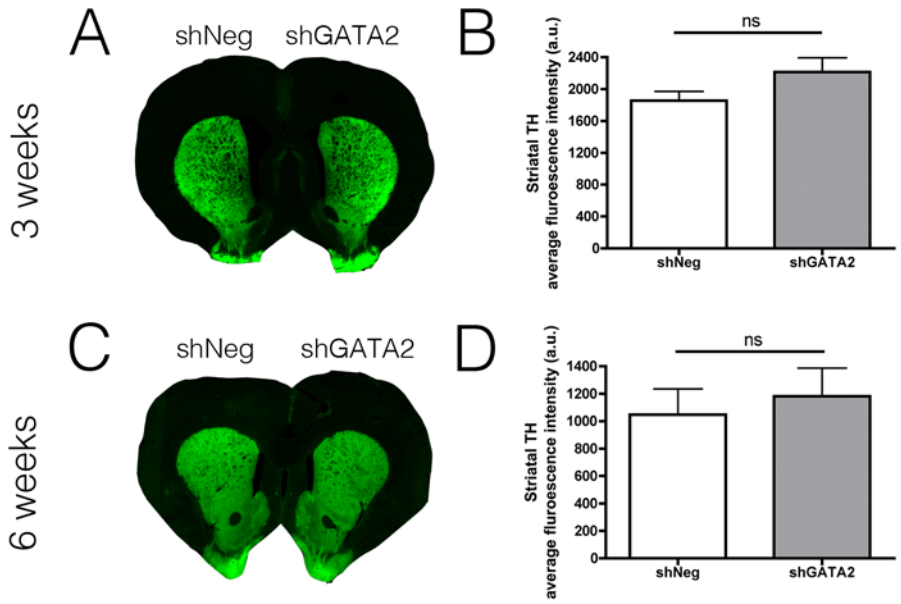

Figure 18. Moderate, transient inflammation induced by viral vectors does not lead to nigrostriatal damage

(A) Delivery of AAV2 vectors to SN does not lead to loss of nigrostriatal terminals, as indicated by the lack of focal lesion observed upon immunohistochemically staining striatal sections for TH three or six weeks post-infusion. **(B)** AAV2.shGATA2 does not alter striatal TH levels three or six weeks after infusion into rat SN, as compared to AAV2.shNeg. Statistical comparisons of mean striatal TH fluorescence intensity were performed using a paired Student's t-test. α=0.05.

4.4.3 GATA2 silencing in rat SN is not detectable by quantitative fluorescent IHC

We have shown that the viral vectors that were validated *in vitro* in Chapter 3 yield robust, persistent transduction of the rat nigrostriatal system when stereotactically delivered to the SN and that delivery of these viral vectors is not associated with chronic inflammation or damage to the nigrostriatal system. To confirm that the shRNA cassette in our viral plasmid is functional *in vivo – i.e.*, able to silence *GATA2* in SNc dopaminergic neurons – we assessed GATA2 protein levels by confocal microscopy and fluorescent immunohistochemistry quantification following viral transduction. For this purpose, we utilized the same polyclonal antibody that we used for our *in vitro* studies in Chapter 2. This antibody was raised against full-length recombinant human GATA2 protein, but given the 93% protein sequence identity between human and rat GATA2 and the fact that the antibody is polyclonal, it was predicted that the antibody would cross-react with rat GATA2 protein.

In animals that were sacrificed three weeks after bilateral infusion of AAV2.shGATA2 and AAV2.shNeg, we found widespread transduction of SNc dopaminergic neurons, as shown in the representative images in Figure 19 by the colocalization of TH and GFP (Figure 19a). Surprisingly, confocal quantification of immunohistochemical fluorescence intensity corresponding to GATA2 protein within TH-positive regions of interest (ROIs) failed to show any difference between AAV2.shGATA2 and AAV2.shNeg (Figure 19b**)**. Comparing GATA2 levels in SNc dopaminergic neurons that had been transduced with AAV2.shGATA2 with GATA2 levels in neighboring untransduced dopaminergic neurons within the ipsilateral SNc also failed to demonstrate the expected GATA2 silencing (Figure 19c).

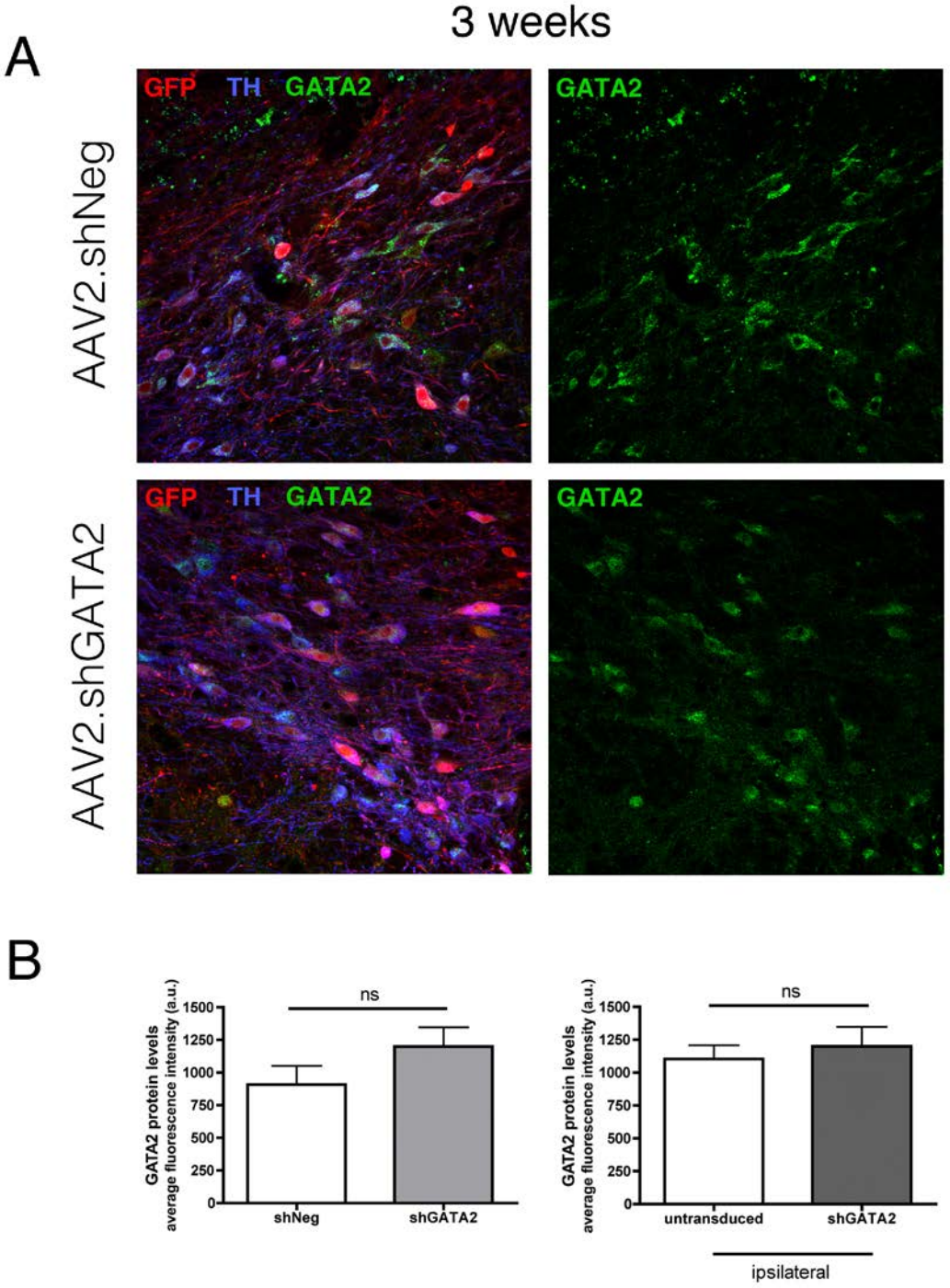

Figure 19. Confocal imaging and quantification of immunohistochemically stained midbrain sections three weeks after AAV2 infusion show no reduction in GATA2 protein levels

(A) Representative confocal images of SNc stained for GFP (green), TH (blue), and GATA2 (red) show a similar degree of transduction between AAV2.Neg-infused SN (top panel) and AAV2.shGATA2-infused SN (bottom panel). **(B)** No significant difference in GATA2 mean fluorescence intensity was observed between TH neurons transduced with AAV2.shGATA2 and TH neurons transduced with AAV2.shNeg (left). Nor was any significant difference in GATA2 mean fluorescence intensity observed when comparing TH neurons transduced with AAV2.shGATA2 and ipsilateral untransduced TH neurons (right). Statistical comparisons of mean GATA2 fluorescence intensity were made using a paired Student's t-test. α=0.05. (n=5)

Examination of GATA2 protein levels in the 6-week cohort of animals showed a very modest decrease in GATA2 protein levels in the dopaminergic neurons transduced with AAV2.shGATA2 versus AAV2.shNeg that reached significance using a paired t-test (normalized difference of means = 18%; p = 0.0217) (Figure 20c, left). The magnitude of this effect is in striking contrast to the maximal silencing that we had observed *in vitro* (Figures 9-11). Furthermore, we were unable to detect any significant difference in GATA2 protein levels when GATA2 mean fluorescence intensity in SNc dopaminergic ROIs was compared to untransduced dopaminergic ROIs in the ipsilateral SNc (Figure 20c, right).

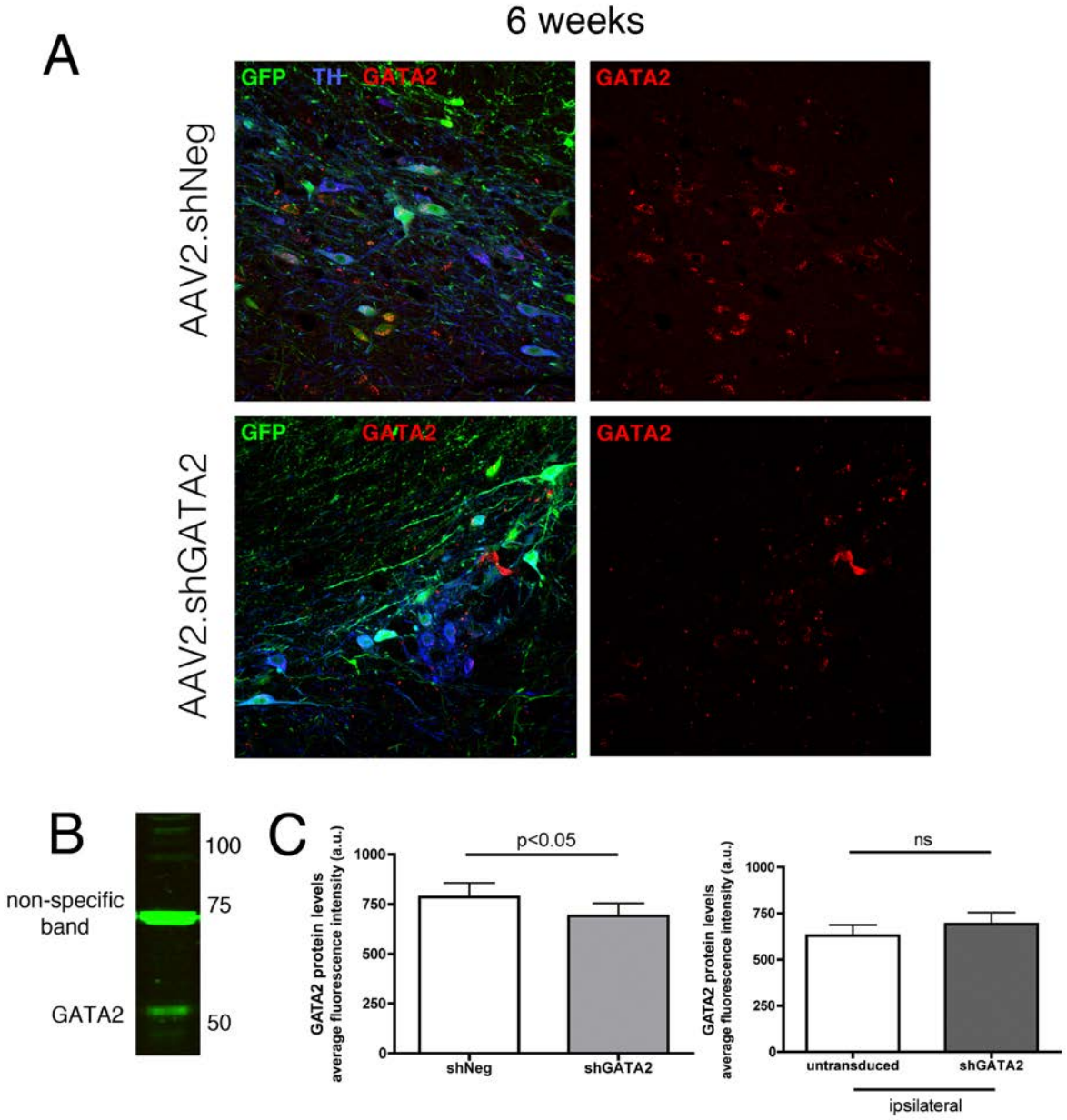

Figure 20. Confocal imaging and quantification of immunohistochemically stained midbrain sections six weeks after AAV2 infusion show no robust reduction in GATA2 protein levels

(A) Representative confocal images of SNc stained for GFP (green), TH (blue), and GATA2 (red). A similar degree of transduction is observed between SN infused with AAV2.Neg (top panel) and SN infused with AAV2.shGATA2 (bottom panel). **(B)** Western blot using the anti-GATA2 that was used for IHC reveals the presence of a non-specific band using PC12 cell lysates. (**C)** A modest decrease in GATA2 mean fluorescence intensity was detected when comparing TH-positive neurons transduced with AAV2.shGATA2 and TH-positive neurons transduced with AAV2.shNeg (left). No significant difference in GATA2 mean fluorescence intensity when comparing TH neurons transduced with AAV2.shGATA2 and ipsilateral untransduced TH neurons (right). Statistical comparisons of mean GATA2 fluorescence intensity were made using a paired Student's t-test. α=0.05. (n=6)

These immunohistochemical results suggested that the GATA2 antibody might lack specificity in rat tissue. Western blot analysis using the same polyclonal anti-human GATA2 antibody on rat PC12 cell total lysates revealed the presence of a slower-migrating (and more immunoreactive) band in addition to the GATA2 band (Figure 20b). This higher molecular weight band does not diminish when GATA2 is silenced *in vitro*, demonstrating that it is not related to GATA2 and is therefore non-specific (data not shown). Thus, the failure to detect the expected reduction in GATA2 protein levels in SNc dopaminergic neurons by confocal analysis is likely because the antibody cross-reacts with another antigen that is present in these cells and whose levels are not altered by shGATA2. In support of this hypothesis, the observed staining

pattern for this transcription factor was, for the most part, diffusely cytosolic rather than nuclear (Figures 19-20).

Taken together, these data indicate that *GATA2* silencing cannot be detected in transduced SNc dopaminergic neurons by quantitative confocal analysis of fluorescent immunohistochemistry, likely due to antibody non-specificity issues.

4.4.4 In *situ* hybridization is a sensitive technique to detect gene silencing *in vivo*

We ascribe our inability to detect robust *GATA2* silencing by quantitative fluorescent immunohistochemistry analysis to antibody non-specificity. Screening of other commercially available antibodies as well as two custom antibodies that were raised against peptide sequences of rat GATA2 showed these antibodies to be similarly inadequate (data not shown). In an attempt to circumvent these antibody issues, we asked whether GATA2 silencing could be detected at the mRNA level by *in situ* hybridization (ISH).

We developed a complementary RNA (cRNA) probe against the 422-bp coding sequence of rat a-syn (SNCA) mRNA (antisense probe) as well as a reverse-complement sequence control cRNA probe (sense probe) (Figure 21a). Northern blot analysis on total RNA isolated from rat cortex showed a single band corresponding to the migration of SNCA mRNA, indicating that the probe is specific for SNCA mRNA (Figure 21b). ISH on midbrain sections from rats that received SN infusion of AAV2.shSNCA showed effective silencing of SNCA as compared to the contralateral SNc that received an equal-titer infusion of AAV2.shNeg (Figure 21c). As expected, no staining was observed with the negative control sense cRNA probe, providing further evidence that the antisense probe specifically recognizes SNCA mRNA. These data

clearly demonstrate that ISH is a sensitive technique to assess gene silencing *in vivo*. Therefore, we applied a similar strategy to determine whether AAV2.shGATA2 infusion knocks down GATA2 message at 3 and 6 weeks post-infusion.

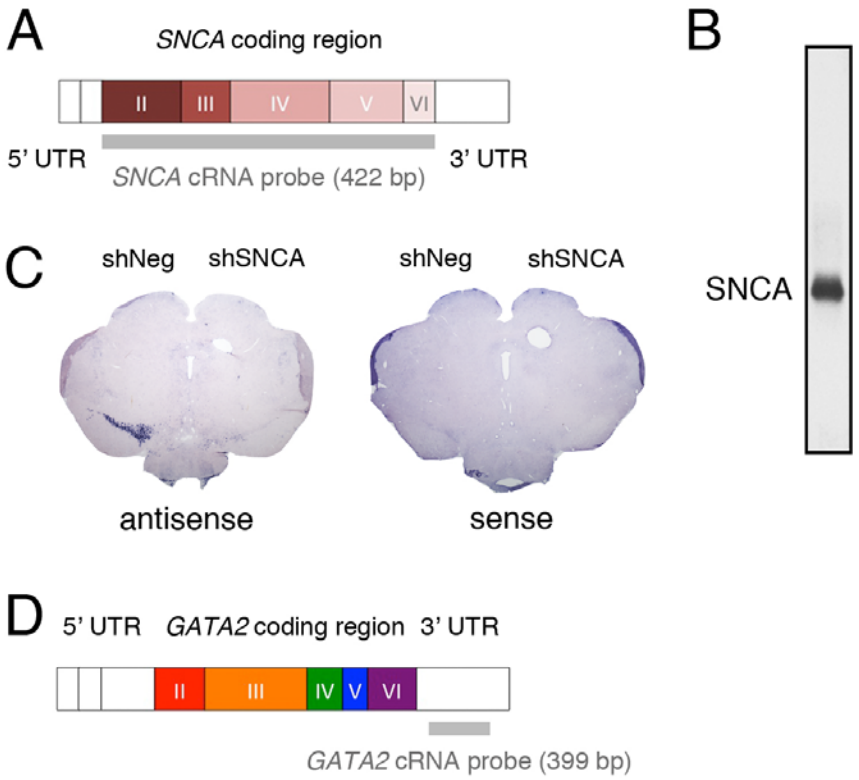

Figure 21. Validation of *in situ* hybridization as a sensitive method for detecting gene silencing *in vivo*

(A) Schematic showing SNCA mRNA and region to which the cRNA probes (gray bar) were designed for specific detection of SNCA mRNA. **(B)** Northern blot on total RNA extracted from adult rat cortex shows specific detection of SNCA mRNA. **(C)** ISH with the antisense SNCA cRNA probe performed on midbrain sections from rats three weeks after infusion of viral vector engineered to silence *SNCA* ("shSNCA") in SN shows robust silencing of SNCA mRNA in SNc as compared to the contralateral SN, which received non-targeting negative control ("shNeg") vector. Negative control ISH staining using sense SNCA cRNA probe on adjacent midbrain section shows no staining. Images in (C) are representative from three rats that received bilateral infusion of AAV2.shSNCA and AAV2.shNeg. **(D)** Schematic showing GATA2 mRNA and region to which the cRNA probes (gray bar) were designed for specific detection of GATA2 mRNA. Note: SNCA and GATA2 mRNA sizes are not drawn to same scale.

4.4.5 GATA2 silencing is detectable by *in situ* hybridization

For GATA2 ISH we designed antisense and sense cRNA probes against a 399-bp sequence in the 3' UTR, where homology among the six GATA transcription factors (as well as other rat transcripts) is lowest (Figure 21d). In contrast to our results with IHC, ISH on midbrain sections showed effective *GATA2* silencing in the SNc in both the 3-week and 6-week AAV2-infused cohorts (Figure 22a). The observed decrease in GATA2 mRNA was not due to cell loss since TH

staining was similar in the AAV2.shGATA2- and AAV2.shNeg-infused hemispheres (Figure 22b).

4.4.6 GATA2 positively regulates *SNCA in vivo*

If GATA2 regulates *SNCA* under basal conditions *in vivo* as it does *in vitro*, then the silencing of GATA2 should lead to consequent down-regulation of *SNCA*. We used the SNCA cRNA probes that we generated and validated previously (Figure 21a-c) to determine whether SNCA mRNA levels were indeed decreased following *GATA2* silencing in SN. At both 3 and 6 weeks post-transduction with AAV2.shGATA2, there was a striking reduction in SNCA mRNA levels in SNc relative to the contralateral SNc, which received AAV2.shNeg (Figure 22c). These data therefore demonstrate that GATA2 regulates *SNCA* in SNc under basal conditions in rat.

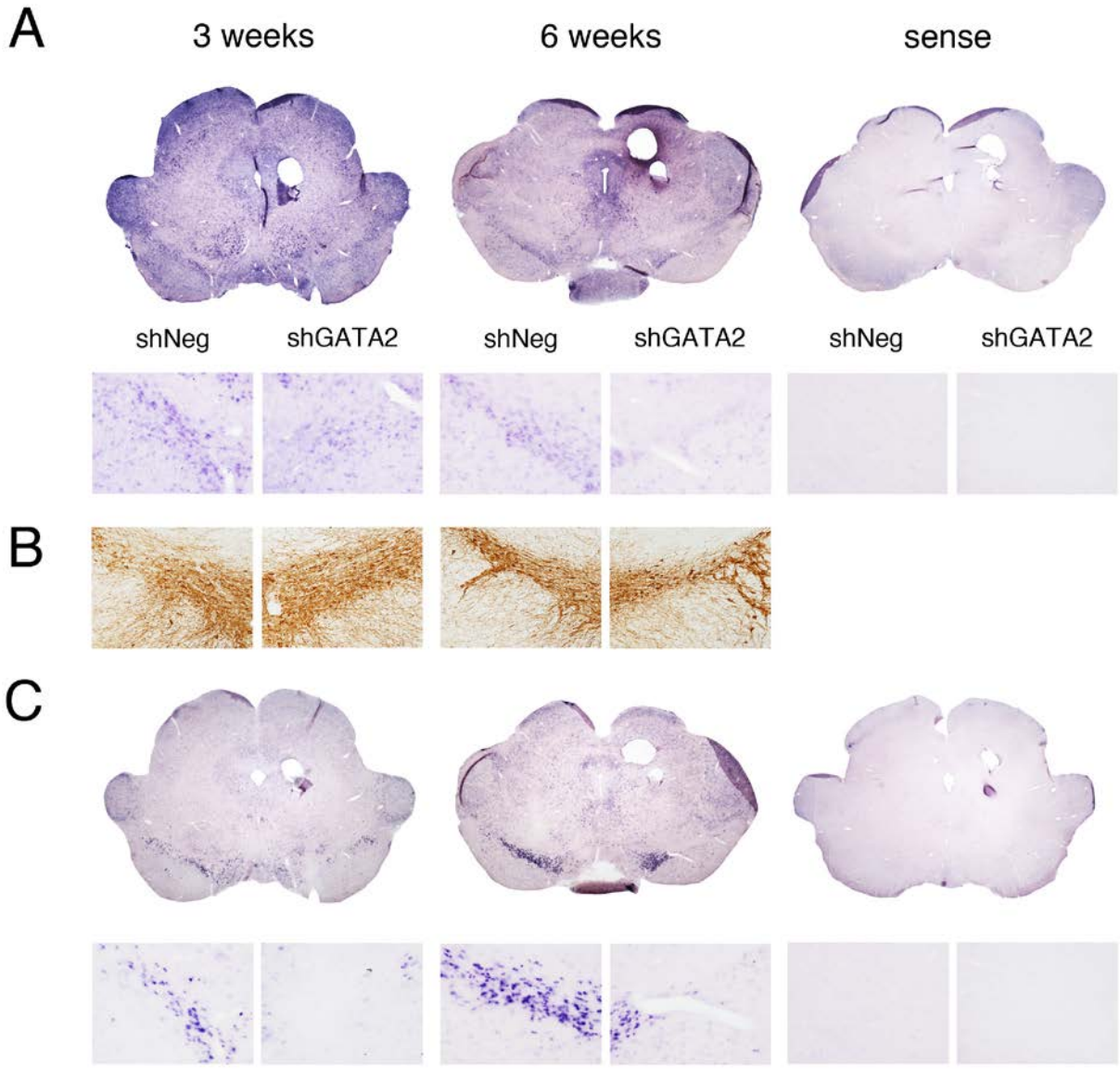

Figure 22. GATA2 positively regulates *SNCA* in rat SNc

(A) ISH performed using antisense cRNA probe for GATA2 on midbrain sections from rats three weeks (left) or six weeks (center) post-infusion of AAV2. No staining is observed when ISH is performed on midbrain sections using the corresponding sense cRNA probe (right). Higher magnification (10X) images show decrease in GATA2 mRNA in the SNc that received AAV2.shGATA2 as compared to the contralateral SNc that received AAV2.shNeg. **(B)** Staining of midbrain sections from these animals for TH shows that decrease in mRNA is not due to loss of dopaminergic neurons (10X magnification). **(C)** ISH performed using the antisense cRNA probe for SNCA mRNA on midbrain sections from rats three weeks (left) or six weeks (center) after viral infusion shows down-regulation of *SNCA* in SNc where GATA2 has been silenced relative to the contralateral (shNeg) SNc. No staining is observed when ISH is performed on midbrain sections using the corresponding sense cRNA probe (right). Higher magnification (10X) images show decrease in SNCA mRNA in SNc that received AAV2.shGATA2 as compared to contralateral SNc that received AAV2.shNeg. (3-week cohort, n=1; 6-week cohort, n=1)

4.4.7 GATA2 silencing is not neuroprotective in the rotenone rat model of Parkinson's disease

Systemic rotenone administration to rats produces a behavioral phenotype characterized by progressive bradykinesia as well as a pathological phenotype characterized by nigrostriatal

degeneration, formation of intracellular proteinaceous aggregates that contain a-syn, and various other cellular features similar to PD (Betarbet et al., 2000; Cannon et al., 2009). Importantly, the dopaminergic lesion produced by rotenone is bilateral and symmetric (data not shown). In the striatum, loss of nigrostriatal terminals can be visualized by immunohistochemical staining for TH. Therefore, effects of putative neuroprotective interventions (*e.g.* viral gene delivery) can be compared across hemispheres for their ability to preserve nigrostriatal terminals in rotenone-treated animals. We utilized this fact in designing an experiment to test the hypothesis that *GATA2* silencing protects SNc dopaminergic neurons against the cytotoxic effects of rotenone (Figure 23).

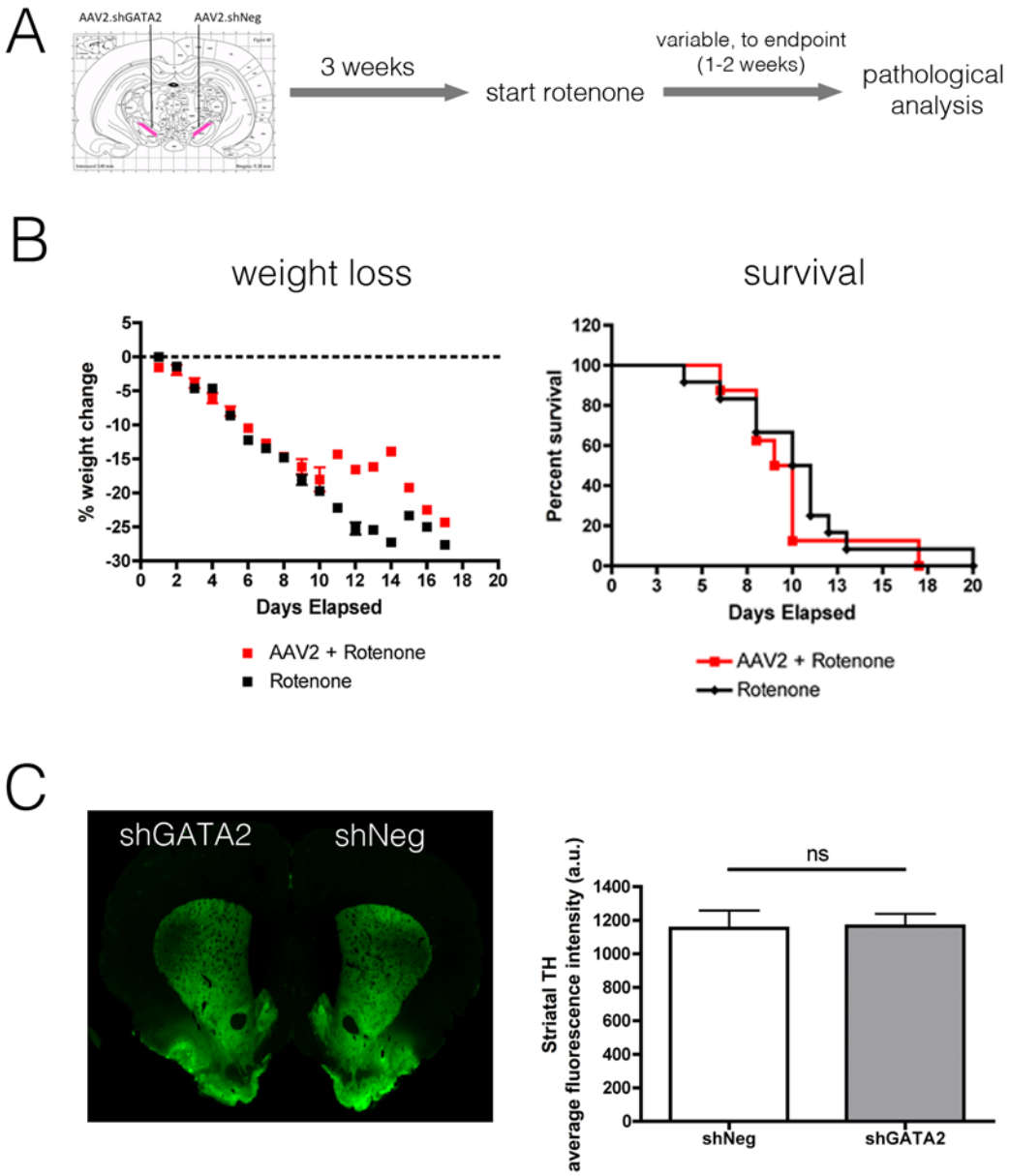

Figure 23. Silencing of *GATA2* in SNc dopaminergic neurons is not neuroprotective in the rotenone rat model of PD.

(A) Schematic showing treatment timeline. **(B, left)** Weight loss after commencement of daily rotenone injections was recorded daily as percentage of initial weight. Animals that received AAV2 viral infusions followed by rotenone treatment (AAV2 + Rotenone, n=5) show no obvious difference in rate of weight loss as compared to a cohort of animals that received rotenone and no viral infusion (Rotenone, n=12). **(B, right)** Animals in the AAV2 + Rotenone cohort show no significant difference in survival as compared to animals in the Rotenone cohort (Logrank test, χ^2=0.7180, p=0.3968, α=0.05). **(C, left)** Striatal TH staining on a representative animal from the AAV2 + Rotenone cohort shows equal nigrostriatal terminal loss between hemispheres, with focal lesions visible in dorsolateral striatum bilaterally. **(C, right)** Quantification of mean striatal TH fluorescence intensity shows no significant difference between AAV2.shGATA2 and AAV2.shNeg hemsipheres (paired Student's t-test, α=0.05).

Rats received bilateral infusion of viral vectors as before, then were given daily i.p. injections of rotenone starting three weeks after infusion until they reached behavioral phenotypic endpoint (Figure 23a). There was no significant difference in rate of weight loss or survival between the animals that received AAV2 infusions plus rotenone ("AAV2 + Rotenone") and the animals that received rotenone alone ("Rotenone") (Figure 23b). Comparison of striatal TH intensity within the AAV2 + Rotenone cohort revealed no significant side-to-side differences (Figure 23c). These findings indicate that *GATA2* silencing in SNc dopaminergic neurons does not protect against rotenone-induced nigrostriatal degeneration.

4.5 DISCUSSION

The goals of this data chapter were to validate *in vivo* the viral vectors generated in Chapter 3 and to determine: (1) whether GATA2 positively regulates *SNCA* under basal conditions in rat SNc dopaminergic neurons, and (2) whether silencing *GATA2* in these cells confers protection against rotenone, a neurotoxin that models PD in rat. Validation of the viral vectors required demonstrating that, when delivered to the rat SNc, they extensively transduce the nigrostriatal system and silence *GATA2* within SNc dopaminergic neurons without causing damage. Testing the two hypotheses required, respectively, silencing *GATA2* in rat SNc dopaminergic neurons and examining whether SNCA levels are consequently reduced and combining *GATA2* silencing with rotenone treatment followed by assessment of nigrostriatal pathology.

4.5.1 Viral transduction of rat SNc is robust

We demonstrated that transduction of the rat nigrostriatal system with our viral vectors is robust and extensive, and results in long-lasting transgene expression (to at least six weeks post-infusion, which was the latest time point we examined) (Figures 11-15). As expected for this neurotropic virus, the vast majority of transduced cells were neurons; within the SN, the majority of these neurons were dopaminergic (Figures 19-20). Based on these findings, it can be concluded that the delivery of the viral vectors was accurate and likely extensive enough to have transduced the majority of SNc dopaminergic neurons, which was a critical prerequisite for adequately testing our hypotheses.

4.5.2 Delivery of viral vectors causes moderate but transient inflammation in the midbrain

Inflammation is a potentially protective, physiological response to invading pathogens such as viruses. However, in the context of gene therapy – where viral vectors are used to deliver therapeutic genes – such a reaction can limit the utility of these reagents. Viral-mediated induction of inflammation in brain parenchyma is especially important to assess when validating gene therapy vectors for PD, where inflammation is thought to exacerbate PD and, possibly, contribute to the pathogenesis of disease (Block, Zecca, & Hong, 2007; Glass, Saijo, Winner, Marchetto, & Gage, 2010). Some viral vectors are more prone than others to inducing inflammation (e.g., adenoviruses, herpes simplex viruses); AAV is among the least immunogenic viral vectors (Giacca, 2010).

In this study, we found that stereotaxic delivery of PBS (the vehicle for the viral vectors) did not cause appreciable inflammation (Figure 16); however, we did note moderate – yet transient – inflammation in ventral midbrain after delivery of either viral vector (Figure 17). Resolution of transient inflammation has been described previously for various viral vectors, including AAV2, and is therefore not unexpected. The bilateral experimental design that we employed for our *in vivo* studies – in which each animal serves as its own control by receiving infusion of AAV2.shGATA2 into the ipsilateral SNc and AAV2.shNeg into the contralateral SNc – allowed us to conclude that the vectors are equivalent in the degree of inflammation they instigate.

Although inflammation can lead to neuronal dysfunction and death, there was no evidence of necrotic lesions, which are typical when there is a severe inflammatory response.

Nonetheless, we took a further step to ensure that the viral vectors are not harmful to the nigrostriatal system by examining whether their delivery to SNc resulted in loss of dopaminergic nigrostriatal terminals. We found no focal lesion in either hemisphere, suggesting that neither viral vector is toxic to the nigrostriatal system (Figure 18).

Without comparing striatal TH fluorescence intensity levels in these virus-infused brains to uninfused or PBS-infused brains, we cannot conclude that the delivery of these viruses has no effect at all on nigrostriatal integrity. From our data, we can only conclude that the AAV2.shGATA2 vector does not exert any preferential toxicity as compared to the AAV2.shNeg vector. This finding is by itself interesting in that it suggests that GATA2 does not regulate genes that are vital to survival of adult SNc neurons.

Taken together, these findings suggest that the vectors *per se* are not toxic to the nigrostriatal system, and are therefore suitable for testing as potential neurotherapeutic agents in the rotenone rat model of PD.

4.5.3 Detection of GATA2 silencing *in vivo*

We were surprised that we were unable to detect *GATA2* silencing reliably at the protein level by quantitative fluorescent IHC. In the course of troubleshooting this issue, we considered four possible confounders: (1) GATA2 antibody non-specificity (most likely), (2) AAV2.shGATA2 vector does not work *in vivo*, (3) *in vivo* turnover of GATA2 protein is very slow, and (4) methodological issues with confocal imaging and quantification.

Validating antibody specificity is critical to ensuring that immunohistochemical experiments are of high quality, yet antibodies can be difficult to generate and properly validate

for a multitude of reasons. Under conditions of antibody cross-reactivity, reduced levels of GATA2 would be "masked" by an extraneous signal from an irrelevant antigen expressed in SNc dopaminergic neurons, and would thereby evade detection. The odd sub-cellular staining pattern we observed for GATA2 in rat SNc – *i.e.* a mixture of diffuse and punctate cytoplasmic staining with diffuse nuclear staining – was our first indication that our failure to detect *GATA2* silencing may be confounded by antibody non-specificity. While it is possible for a transcription factor to be present in the cytosol under basal conditions and recruited to the nucleus under certain circumstances, this phenomenon has not been reported for GATA2.

The antibody we used for quantifying *GATA2* silencing *in vivo* is an affinity-purified polyclonal antibody raised against recombinant, full-length *human* GATA2 protein. There is 93% identity between human and rat GATA2 protein sequences so cross-reactivity with the rat antigen is probable, but recognition of additional, unintended antigens (non-specificity) based on structural or sequence similarity remains a hazard nonetheless. Proteins that share much sequence homology are therefore at risk of being co-detected by the same antibody. Such a situation can usually be avoided by evaluating the protein sequence against which the antibody was raised to all rat proteins for homology with other sequences in the rat proteome using a BLAST search. Using only antibodies that meet this criterion of *in silico* specificity increase the likelihood that the antibody will specifically recognize only the intended antigen. As our GATA2 antibody was raised against the full-length protein, we could not meaningfully use a BLAST search to identify proteins likely to cross-react with the antibody. The fact that our GATA2 antibody is polyclonal also increases the likelihood of cross-reactivity since it is necessarily composed of a mix of antibodies that identify various epitopes on the inoculated (full-length human GATA2) protein.

The gold standard control experiment to demonstrate antibody specificity is to compare the observed staining with staining under identical conditions on the same tissue from a knock-out animal of the same age. Given the general scarcity of knock-out rats and the fact that *GATA2* knock-out mice die at embryonic day 10.5 (Tsai et al., 1994), it was not possible for us to validate our GATA2 antibody properly.

To help test the specificity of our GATA2 antibody *in rat*, we determined whether the antibody recognizes a single band corresponding to the expected migration of GATA2 by western blot using rat cell lysates. Antibody cross-reactivity is noted when multiple bands (unrelated to the protein of interest) appear. Although the conditions for western blot (*i.e.* linearized proteins coated in SDS) and IHC (*i.e.* PFA-fixed proteins encased in permeabilized cells *in situ*) differ considerably, observing additional bands by western blot often portends antibody non-specificity by IHC. We observed an additional band that was in fact more immunoreactive than the GATA2 band by western blot using PC12 cell lysates from our *in vitro* viral transduction experiments (Figure 20b). This band does not decrease when *GATA2* is silenced, which confirms that it is a non-specific band (data not shown). Therefore, we have multiple lines of evidence supporting that antibody non-specificity may be responsible for our inability to detect *GATA2* silencing at the protein level *in vivo*.

A second (less likely) possible explanation for our inability to detect *GATA2* silencing at the protein level *in vivo* may be that the shRNA cassette in the plasmid that the viral vector delivers does not function properly. Although theoretically possible, this would be surprising since the cassette functions optimally in our *in vitro* validation experiments (Figures 9-11). To rule out this possibility, we re-sequenced the plasmid from virus and found that the sequence was correct (data not shown).

A third (and similarly unlikely) possibility is that there is a marked difference in the kinetics of GATA2 protein turnover *in vivo* as compared to *in vitro*. In *vitro* we detect reduction in GATA2 protein levels by 48 hours post-transfection (Figure 2), which is consistent with the short half-life that has been reported for GATA2 (approximately 1.5 hours) *in vitro* in a hematopoietic cell line (Minegishi et al., 2005). Differences in half-life may be cell type dependent and may indeed differ *in vivo*, but there is no easy way to explore this possibility experimentally.

The final possibility we considered that could explain our inability to detect robust *GATA2* silencing at the protein level *in vivo* is that quantitative fluorescent IHC by confocal microscopy is insufficiently sensitive to detect knock-down of GATA2 protein. However, this method successfully detected robust silencing in our *in vitro* studies in Chapter 2 (Figure 2) and we have used this technique to show effective silencing of *SNCA* in rat SNc (Cannon et al., n.d.).

In conclusion, it is most likely that the GATA2 antibody we used for *in vivo* detection of *GATA2* silencing is non-specific. Since our attempts to validate other GATA2 antibodies – both commercially available as well as custom antibodies – were unsuccessful, we decided to abandon IHC altogether as a method for demonstrating *GATA2* silencing *in vivo*.

4.5.4 In *situ* hybridization (ISH) is a sensitive method to detect gene silencing *in vivo*

We performed ISH on midbrain tissue from animals transduced with AAV2.shSNCA as a proof of principle experiment to show that ISH is a sensitive technique for detection of gene silencing *in vivo* (Figure 21). We applied this technique to tissue from our 3-week and 6-week GATA2 silencing cohorts and showed robust silencing of *GATA2* (Figure 22a). This finding validates that

our viral vectors work *in vivo* and are suitable for testing the two hypotheses that we set out to address in this dissertation project. This finding also confirms our suspicion that our inability to detect *GATA2* silencing at the protein level was due to antibody non-specificity.

Although no formal behavioral tests were conducted beyond simple observation, it is noteworthy that the animals in which *GATA2* was unilaterally silenced in SNc dopaminergic neurons displayed no gross behavioral phenotype – *e.g.* unilateral turning or unilateral abnormal movements. Consistent with the lack of nigrostriatal pathology that we detected in these animals (Figure 18), this finding suggests that *GATA2* is not essential to the survival of these cells.

ISH detection of SNCA mRNA in midbrain sections from the 3- and 6-week *GATA2* silencing cohorts clearly showed that *SNCA* expression is decreased following *GATA2* silencing. This demonstrates that GATA2 positively regulates *SNCA* in SNc dopaminergic neurons *in vivo*, and thereby confirms our first hypothesis. This finding is significant for several reasons. First, it is the only *in vivo* validation to date of a specific *trans*-acting regulator of *SNCA*. Second, it extends the findings of Scherzer and colleagues (Scherzer et al., 2008) and validates their innovative *in silico* approach to identify putative transcriptional regulators through analysis of gene expression databases. Third, the *in vivo* confirmation that GATA2 regulates *SNCA* in SNc dopaminergic neurons allows for examination of other putative target genes using a similar approach on the same tissue – *e.g.*, the heme-metabolism genes that were identified in the *in silico* studies of Scherzer and colleagues (Scherzer et al., 2008).

4.5.5 Why isn't *GATA2* silencing neuroprotective in the rotenone rat model of PD?

Since we have shown that GATA2 regulates *SNCA in vivo* in SNc dopaminergic neurons, GATA2 could theoretically contribute to PD pathogenesis by: (1) being aberrantly up-regulated or active such that it induces toxic levels of expression of target genes like *SNCA*, and/or (2) contributing to basal expression of *SNCA* (and possibly other genes) in a cellular context already burdened by excess a-syn. If either case were true, then silencing *GATA2* could theoretically be therapeutic in PD. To test this hypothesis, we conducted a neuroprotection study in which rats received bilateral infusion of viral vectors followed by treatment with rotenone until they reached behavioral phenotypic endpoint. We assessed neuroprotection based on the following endpoints: rate of weight loss (an indicator of rate of decline), survival (latency to phenotypic endpoint), and loss of dopaminergic nigrostriatal terminals (a sensitive indicator of nigrostriatal damage). As compared to rats treated with rotenone alone, rats that received the viral vectors followed by rotenone did not show any differences in rate of weight loss, survival, or dopaminergic nigrostriatal terminal loss (Figure 23b-c). Though there are additional neuroprotection endpoints that can be assessed (*e.g.*, stereological cell counting), all three of our chosen endpoints demonstrate that silencing *GATA2* in SNc dopaminergic neurons is not protective in the rotenone rat model of PD, leading us to reject our second hypothesis.

There are several potential reasons for why *GATA2* silencing is not neuroprotective in the rotenone rat model of PD. First, it is extremely probable that silencing a gene that is well known to function as a master regulator of transcription in other systems will have wide-ranging effects on gene expression (beyond *SNCA*). Unless GATA2 levels are aberrantly elevated with rotenone treatment – in which case silencing *GATA2* expression may act to normalize GATA2 activity on

its target genes – the overall effects of *GATA2* silencing would depend on the identity of its (likely numerous) target genes. Indirect down-regulation of some of these genes via *GATA2* silencing may have both deleterious and beneficial consequences for cells in the presence of rotenone. If this is the case, then a more targeted approach in which shRNA is directed against specific transcripts known to be dysregulated in PD (*e.g.*, *SNCA*, *TFR2*, *etc.*) may confer neuroprotection against rotenone. We recently showed partial neuroprotection against rotenone when this approach is used to silence *SNCA* specifically in rat (Cannon et al., n.d.).

A second potential reason for why *GATA2* silencing failed to protect SNc dopaminergic neurons from rotenone toxicity relates to the treatment paradigm that we used. Using a "within" comparison paradigm – in which each animal serves as its own control since each hemisphere is exposed to a different experimental condition (AAV2.shGATA2 versus AAV2.shNeg) – has advantages and disadvantages. One advantage is that more powerful statistical tests can be applied since comparisons are paired under these conditions. But a disadvantage to this paradigm is that it is unclear when the largest protective effect might be detected. For example, it is possible in our neuroprotection study that *GATA2* silencing conferred some protection against rotenone mid-way through treatment, but by continuing rotenone administration until the animals reached phenotypic endpoint, evidence of the neuroprotection was lost and the pathology of each hemisphere therefore looked similar. Even though the goal is robust neuroprotection, there is useful information to be gleaned from partial protection, which may have been observable prior to symptomatic endpoint. If one cohort of animals were bilaterally infused with AAV2.shGATA2 and another bilaterally infused with AAV2.shNeg, then it would have been possible to compare survival between conditions; this is not possible when using our bilateral infusion paradigm.

Although viral-mediated silencing of *GATA2* in SNc does not appear to be an efficacious strategy for neuroprotection in PD, there are nevertheless many interesting biological questions regarding the role of GATA2 in SNc dopaminergic neurons in adult brain that can be addressed with the viral vectors we have created and validated in this dissertation project. It is likely that GATA2 serves as a master regulator of gene transcription in these cells, but what pathways may be GATA2-dependent remains unanswered. Chromatin immunoprecipitation sequencing (ChIP-seq) on SNc dopaminergic neurons isolated and purified by FACS or laser capture microdissection in combination with gene expression analysis on these cells following *in vivo* transduction with AAV2.shGATA2 would provide information on GATA2 target genes. Knowing the target genes would allow us to infer GATA2 function in SNc and confirm GATA2 gene targets with additional *in vivo* experiments.

In summary, the experiments reported here provide the first *in vivo* demonstration that *SNCA* is transcriptionally regulated by GATA2 in nigrostriatal dopamine neurons. Although reduced levels of a-syn are protective in some circumstances (Cannon et al., n.d.), *GATA2* gene targeting does not appear to be beneficial therapeutically – most likely because GATA2 regulates other genes that counterbalance the positive effects exerted by *SNCA* down-regulation.

5.0 GENERAL DISCUSSION

5.1 SUMMARY AND SIGNIFICANCE OF FINDINGS FROM THIS DISSERTATION PROJECT

The over-arching goals of this dissertation project were to test two hypotheses: 1) GATA2 positively regulates *SNCA* in SNc dopaminergic neurons *in vivo*, and 2) silencing *GATA2* in SNc dopaminergic neurons protects them against rotenone-induced degeneration. We first confirmed that GATA2 regulates *SNCA in vitro*, as reported by others (Scherzer et al., 2008). Next, we assessed whether rat would be a suitable mammalian model system for these experiments (Chapter 1). Based on the fact that rat *SNCA* contains a conserved GATA element in the same region as the functional element in human *SNCA* (Figure 3), the fact that GATA2 regulates *SNCA* in a dopaminergic rat cell line (Figure 4), and the fact that *GATA2* is expressed in SNc dopaminergic neurons in human and in rat (Figure 6), we concluded that rat is an appropriate model in which to test our two hypotheses.

Modulation of gene expression in the brain requires overcoming several anatomical and biochemical challenges. The brain is encased in skull and barricaded on the cellular level by the relatively impermeable blood-brain barrier, making therapeutic delivery difficult. Delivery of genes poses an additional challenge since cells are equipped with ways of degrading foreign nucleic acids. We chose to use viral-mediated gene delivery to brain parenchyma as a means of

overcoming these challenges and testing our two hypotheses. In Chapter 3, in order to create these viral vectors, we designed and validated *in vitro* siRNAs to silence rat *GATA2* specifically (Figure 8-9), then used these sequences as a basis for creating shRNA expression cassettes, which were cloned into viral expression plasmids (Figure 7) and re-evaluated for their ability to silence rat *GATA2* specifically (Figure 10). After packaging the plasmids into a viral vector known for its strong tropism for SNc neurons, we again validated these reagents *in vitro* (Figure 11) and concluded that they are optimal for testing our two hypotheses *in vivo*.

In Chapter 4, we demonstrated that delivery of the vectors to rat SNc resulted in strong, persistent transduction of the nigrostriatal system (Figures 12-15) but caused a modest and transient inflammation in the midbrain (Figures 17) that did not result in damage to the nigrostriatal system (Figures 18). We encountered problems in detecting *GATA2* silencing at the protein level *in vivo* due to antibody specificity issues that were circumvented by using *in situ* hybridization (ISH) to detect GATA2 mRNA. We found that the viral vector expressing shRNA against GATA2 successfully silenced *GATA2* within the SNc (Figure 22). We further found that silencing *GATA2* in SNc neurons led to down-regulation of *SNCA* expression, consistent with GATA2 positively regulating *SNCA in vivo*, which confirmed our first hypothesis (Figure 22). We then conducted a neuroprotection study in the rotenone rat model of PD to test our second hypothesis, and found that *GATA2* silencing was not neuroprotective against rotenone toxicity in the SNc, which disproved our hypothesis (Figure 23).

Overall, this dissertation project contributes to PD research by providing the first *in vivo* demonstration of a transcription factor (GATA2) that regulates *SNCA*. This project also took the first step toward examining GATA2 function in adult rat SNc dopaminergic neurons. Based on our *in vivo* experiments in Chapter 4, we conclude that in adult SNc neurons *GATA2* is neither a

critical selector gene for dopaminergic cell phenotype nor a critical regulator of cell survival. The fact that GATA2 regulates *SNCA in vivo* validates two approaches as useful in identifying transcription factors that regulate genes of interest *in vivo*: the innovative *in silico* approach used by Scherzer and colleagues to discover GATA transcription factors as regulators of *SNCA* (Scherzer et al., 2008), and the viral-mediated gene delivery approach we used to confirm that this mode of regulation occurs *in vivo*. Lastly, this dissertation project has demonstrated that GATA2 would not be an appropriate therapeutic target for neuroprotection in PD. While this molecular target joins the list of many others that fail as therapeutics in pre-clinical stages of evaluation, the process by which this was determined has provided useful insights into GATA transcription factor biology in the adult mammalian brain.

5.1.1 The role of GATA2 in SNc dopaminergic neurons: an hypothesis

To test the hypothesis that GATA2 positively regulates *SNCA in vivo*, we silenced *GATA2* in rat SNc using the shRNA-expressing AAV vector we developed in Chapter 3 and assessed SNCA mRNA levels using ISH in Chapter 4. We found that silencing *GATA2* in SNc led to a robust down-regulation of *SNCA*, indicating that GATA2 positively regulates *SNCA* in this brain region under basal conditions, and thus confirming our hypothesis (Figure 22). This is the first *in vivo* demonstration of a transcription factor that regulates *SNCA*.

What purpose does positive regulation of *SNCA* by GATA2 serve in SNc neurons? Based on what is known about the role of GATA2 in other midbrain cell types, it is likely that GATA2 functions as a master regulator of transcription in SNc dopaminergic neurons as well. GATA2 operates in midbrain post-mitotic GABAergic neurons to select for GABAergic phenotype by

coordinately regulating many genes critical to GABA synthesis (Kala et al., 2009). Could GATA2 be involved in specifying dopaminergic phenotype in SNc dopaminergic neurons? This seems unlikely since TH – the enzyme that is rate-limiting in dopamine biosynthesis and thus critical for establishing the dopaminergic phenotype – has not been shown to be a direct target gene of GATA2. Also going against this hypothesized role for GATA2 is the fact that silencing of *GATA2 per se* did not result in any change in TH levels in the nigrostriatal system (Figure 18).

If instead we examine the putative transcriptional block of genes whose expression correlates with that of *SNCA* that Scherzer and colleagues described to be regulated by GATA1 in human blood cells, then we might entertain a different hypothesis about GATA2 function in SNc dopaminergic neurons (Scherzer et al., 2008). A salient feature of this list of genes is that three are involved in intracellular iron metabolism – *ALAS2*, *FECH*, and *BLVRB* (J. Chung, Chen, & Paw, 2012; Scherzer et al., 2008). We were intrigued by this finding since synuclein pathology and iron pathology are features of degenerating SNc dopaminergic neurons in PD (Horowitz & Greenamyre, 2010b). If we indulge in speculation, we might propose a role for GATA2 in stimulating iron import into mitochondria in SNc dopaminergic neurons for use in the synthesis of heme and iron-sulfur cluster prosthetic groups just as GATA1 is critical to these processes in nascent erythroblasts.

Erythroblasts have a high demand for iron since the main function of erythrocytes – the fully-differentiated cell of their hematopoietic lineage – is gas exchange via hemoglobin, a multi-subunit protein composed of globin chains that contain iron in the form of heme prosthetic groups. Utilizing a transcriptional program in which one transcription factor coordinately up-regulates many enzymes and transporters involved in this process is a powerful and parsimonious means of rapidly meeting the high iron demands of the cell. Indeed, it has been

shown that GATA1 – the GATA transcription factor that is active and required during this step in erythroid differentiation – induces expression of *ALAS2*, *ALAD*, *PBGD* (involved in heme synthesis) (R. Ferreira, Ohneda, Yamamoto, & Philipsen, 2005; Kramer, Gunaratne, & Ferreira, 2000); erythropoietin receptor (*EpoR*) (Chiba, Ikawa, & Todokoro, 1991); *Abcb10* and *Mfrn1* (inner mitochondrial membrane proteins involved in iron import into the mitochondrial matrix for utilization in the synthesis of heme and iron-sulfur clusters) (Amigo et al., 2011; Shirihai, Gregory, Yu, Orkin, & Weiss, 2000); and globin chain genes (Gong, Stern, & Dean, 1991). It remains to be shown whether GATA1 also positively regulates *FECH* and *BLVRB*, as suggested by Scherzer and colleagues (Scherzer et al., 2008), but it is clear that GATA1 functions as a key regulator of mitochondrial iron import and utilization in erythroblasts (Figure 24a).

How *SNCA* relates to this process is unclear, but there are intriguing aspects of a-syn biology that may explain its inclusion in this group of GATA1-regulated genes. Alpha-synuclein interacts with membranes and is thought to function physiologically in the recycling of vesicles (Chua & Tang, 2011). Although most work examining the physiological function of a-syn has focused on synapses, where the protein is enriched, it is possible that a-syn has much broader roles in vesicle recycling and perhaps endosomal trafficking. In fact, it was shown recently in neural cell lines and in primary neurons that a-syn participates in clathrin-mediated endocytosis of the TfR (Ben Gedalya et al., 2009). Participation in endosomal vesicle trafficking could explain its inclusion in the group of iron-related erythroid genes regulated by GATA1. The primary means of importing iron into erythroblasts is in a bound state to the iron carrier protein transferrin, which interacts with plasma membrane transferrin receptor 1 (TfR1) and proceeds through a well-defined process of endocytic internalization (Horowitz & Greenamyre, 2010b). How endocytosed iron arrives at mitochondria is a contentious issue, but is believed, in most

cases, to proceed by a transient fusion of endocytotic vesicles with the mitochondrial outer membrane (Horowitz & Greenamyre, 2010b). The surprising abundance of a-syn in human blood (Scherzer et al., 2008) is consistent with it playing a role in a house-keeping process like endocytosis. It is therefore possible that GATA1 positively regulates *SNCA* in parallel with these mitochondrial iron import and heme synthesis genes in order to facilitate further delivery of iron to mitochondria via endocytosis of TfR1 in association with iron-bound transferrin (Figure 24a).

Like erythrocytes, dopaminergic neurons have a high requirement for iron since many cellular enzymes require iron-sulfur clusters or heme prosthetic groups for proper function and TH requires ferrous iron as a cofactor for its enzymatic activity (Haavik, Le Bourdelles, Martínez, Flatmark, & Mallet, 1991; Horowitz & Greenamyre, 2010b). It is possible that, similar to the role of GATA1 in erythroblasts, a similar transcriptional program driven by GATA2 is operational in SNc dopaminergic neurons in order to satisfy these cellular iron demands (Figure 24b). Although some of the GATA1-regulated genes mentioned above are erythroid specific (*e.g. ALAS2, Mfrn1*) or not expressed in SNc dopaminergic neurons (*e.g. TFR1*), ubiquitously expressed homologs or distinct genes may perform the same functions (e.g. *ALAS, Mfrn2*) in SNc dopaminergic neurons. We have previously demonstrated that transferrin receptor 2 (TfR2, *TFR2*), which binds iron-loaded transferrin like TfR1 and is likewise involved in cellular iron import, is expressed selectively in rat SNc dopaminergic neurons, is present in mitochondrial membranes as well as the plasma membrane, and thus may provide an efficient mechanism for mitochondrial iron import in these cells (Mastroberardino et al., 2009). TFR2 contains a GATA element in its promoter (Kawabata et al., 2001) and Bresnick's group has shown in hematopoietic cell lines that GATA2 positively and directly regulates *TFR2* (Emery Bresnick, personal communication to JT Greenamyre). It remains to be shown whether GATA2 regulates

TFR2 in SNc dopaminergic neurons. Again, like erythroblasts, SNc dopaminergic neurons express abundant SNCA, raising the possibility that a-syn participates in clathrin-mediated endocytosis of TfR2 and hence has a physiological role in facilitating iron import into mitochondria.

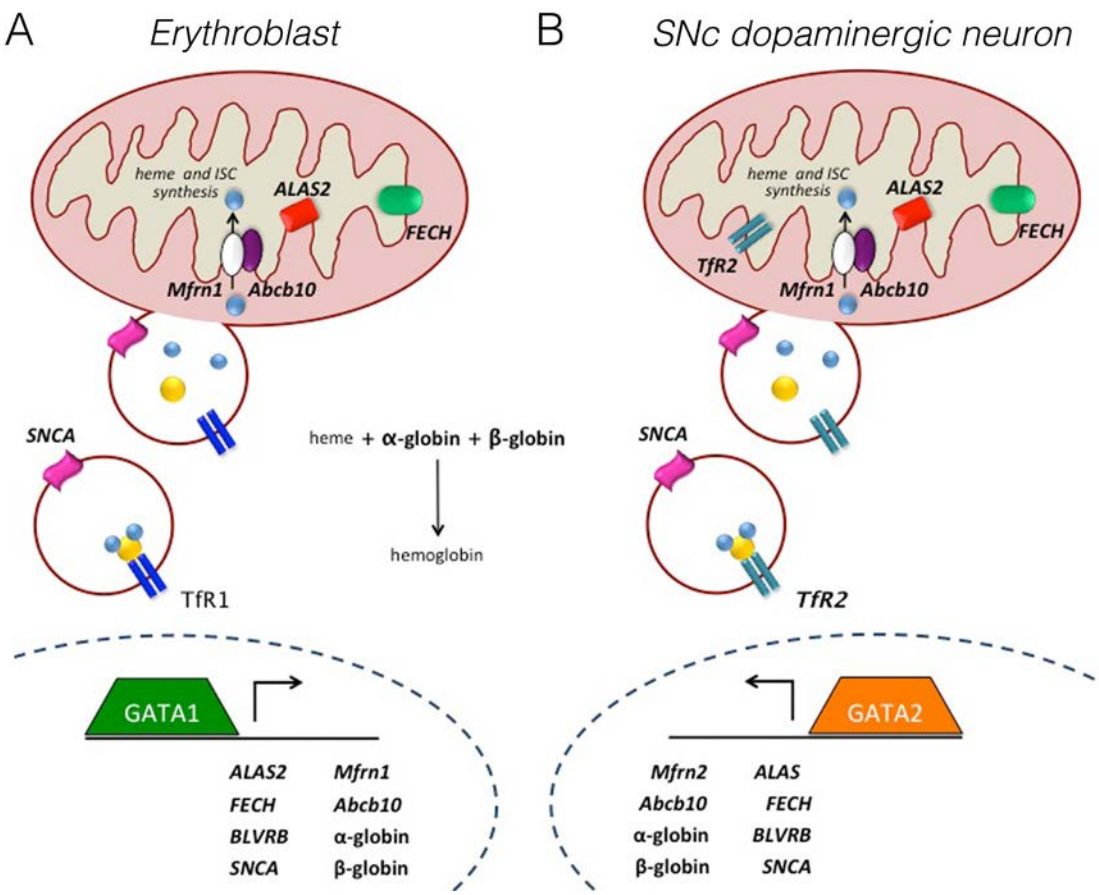

Figure 24. Proposed model for common role of GATA transcription factors in regulation mitochondrial iron import and utilization in erythroblasts and SNc dopaminergic neurons.

(A) Within early erythroblasts, GATA1 positively regulates genes involved in mitochondrial iron import (*Mfrn1, Abcb10*), heme synthesis (*ALAS2, FECH*), and hemoglobin subunits (α-globin, β-globin). Alpha-synuclein may play a role in trafficking of endocytotic vesicles containing iron to mitochondria. **(B)** Within SNc dopaminergic neurons, GATA2 may positively regulate similar gene sets to carry out mitochondrial iron import and utilization, including alternative target genes such as *Mfrn2* (as opposed to *Mfrn1*) , the non-erythroid isoform of *ALAS2* (*ALAS*), and the SNc dopaminergic neuron-specific iron import protein, *TfR2*. Alpha-synuclein may play a similar role in shuttling iron-containing vesicles to mitochondria. (*BLVRB*, which functions in catabolism of a heme degradation product may be positively regulated by respective GATA transcription factors in each cell type; not shown.)

More experiments are needed to explore a possible common role for GATA transcription factors in facilitating mitochondrial iron utilization in erythroblasts and SNc dopaminergic neurons. Specifically, it will be important to understand whether the several aforementioned iron-related genes are regulated by GATA2 in SNc dopaminergic neurons. Fortunately, midbrain sections from the animals infused with the viral vectors can be used to answer these questions with the same approach that we used to demonstrate that GATA2 regulates *SNCA in vivo* (Chapter 4). Not only can we examine expression levels of the particular iron-relevant genes, we can also determine whether iron defects are observable in SNc dopaminergic neurons following *GATA2* silencing by examining heme and iron levels *in situ* with histological stains. Such a finding would further support a functional role for GATA2 in SNc neuronal iron maintenance.

5.1.2 GATA2 silencing is not neuroprotective in the rotenone rat model of PD

In Chapter 4, we tested the hypothesis that silencing *GATA2* in SNc dopaminergic neurons protects these cells from rotenone-induced degeneration. The rationale for this hypothesis was that, since GATA2 had been shown to induce expression of *SNCA* and potentially genes involved in heme metabolism – two pathways that are dysregulated in PD – lowering GATA2 levels (and hence lowering GATA2 activity) might attenuate downstream toxicity of these target genes, as well as others that may be contributing to cytotoxicity. It is unknown whether GATA2 levels increase in PD or the rotenone rat model thereof, but *GATA2* silencing could theoretically be neuroprotective in either case – either by lowering basal expression of *GATA2* (and thus basal down-stream induction of target genes) or by normalizing aberrant *GATA2* expression to physiological levels.

We found that silencing *GATA2* in SNc dopaminergic neurons did not protect them from rotenone toxicity, as we observed no difference in nigrostriatal integrity between the SNc that received the *GATA2* silencing vector and the SNc that received the negative control vector (Figure 23). Interestingly, there was no neuroprotection despite the strong consequent down-regulation of *SNCA*. When we compare this finding with the neuroprotection that was observed in a similar study in which we specifically silenced *SNCA* in rat SNc prior to rotenone treatment (Cannon et al., n.d.), we must conclude that either the degree of *SNCA* down-regulation that we achieved indirectly by silencing *GATA2* was insufficient to prevent rotenone toxicity or that GATA2 regulates other genes that may have deleterious consequences for the cell when down-regulated. In other words, the downstream beneficial effects of silencing *GATA2* (*e.g. SNCA* down-regulation) may be offset by the downstream detrimental effects of suppressing expression

of other genes (*e.g.* those involved in physiological iron uptake). Examining our results from another perspective, we can conclude that silencing *GATA2* does not render SNc dopaminergic neurons any more susceptible to rotenone toxicity than transduction with a control vector. This is noteworthy because it indicates that the gene sets that GATA2 regulates likely do not participate critically in cell survival. This interpretation is also consistent with our observation that silencing *GATA2* in the absence of rotenone does not result in nigrostriatal damage (Figure 18).

5.1.3 Future Directions

Several experiments would aid in clarifying what role GATA2 plays in SNc. It would be informative to perform ChIP-seq experiments on rat ventral midbrain homogenates to determine which GATA element-containing genes are bound by GATA2 *in vivo*. If further refinement is needed – *i.e.* specific isolation of SNc dopaminergic neurons – DNA could be isolated from these cells using laser-capture microdissection. In parallel with this approach, it would be necessary to examine changes in gene expression in these cells following silencing of *GATA2* in SNc. Combining the findings, as Bresnick's group has done for similar experiments on erythroid and endothelial cells (T. Fujiwara et al., 2009; Linnemann et al., 2011), would identify a set of genes that are occupied and regulated by GATA2. It would be interesting to understand whether some of the iron-related genes that were part of the transcriptional block containing *SNCA* based on Scherzer and colleagues' *in silico* data are identified (Scherzer et al., 2008). Such a finding would further strengthen the similarity between blood cells and neurons with respect to transcriptional regulation.

In addition to looking at downstream target genes of GATA2 in rat SNc, it would also be interesting to understand if environmental toxins such as rotenone or MPTP alter GATA transcription factor activity in SNc. Since activity of the GATA2 protein could theoretically increase or decrease without a change in protein levels – and since there is no clear post-translational modification that indicates active GATA2 (Bresnick et al., 2012) – we could use a viral gene delivery approach to transduce SNc dopaminergic neurons with a GATA factor activity reporter gene – *e.g.* a plasmid containing a GATA element in a promoter that drives expression of GFP when GATA factors are bound – or a negative control with a mutated GATA element. If GATA transcription factors are activated in SNc by a given treatment (*e.g.* rotenone), then SNc neurons would be GFP-positive.

The viral vectors that we created in this dissertation project could also be used to study GATA2 biology in other brain regions that express *GATA2*. It would be interesting to understand whether the positive regulation that GATA2 exerts on *SNCA* is specific to SNc neurons or occurs in other brain regions such as the cortex. These reagents could also be used to study GATA2 function outside of the central nervous system. GATA2 has been associated with an increased risk of developing early coronary artery disease and was recently shown in endothelial cells to regulate many genes related to inflammation, which is a central feature of atherosclerosis (Connelly et al., 2006; Linnemann et al., 2011). Because AAV2 has been shown to transduce vascular endothelial cells efficiently (Nicklin, 2001), it is possible to test the hypothesis that GATA2 contributes to atherosclerosis in a rat model of atherosclerosis by directing viral delivery to coronary arteries via intravenous infusion and then giving an atherogenic insult. We would expect less evidence of atherosclerosis in endothelial cells in which GATA2 has been silenced.

5.2 TAKING A STEP BACK: IMPORTANT UNANSWERED QUESTIONS IN PD RESEARCH

5.2.1 Why do SNc dopaminergic neurons degenerate in PD?

In his doctoral thesis published in 1919, Konstantin Tretiakoff reported that the SNc degenerates in PD, and while much has been learned about PD pathogenesis since then, it remains unclear why dopaminergic neurons in the SNc degenerate with relative selectivity. It must be emphasized that SNc dopaminergic neurons are not the only cells that degenerate in PD, since some degree of degeneration has been reported in the locus coeruleus, the raphe nucleus, dorsal motor nucleus of the vagus, nucleus basalis of Meynert, and some populations of catecholaminergic neurons in the brain stem (Lees et al., 2009). However, nigral degeneration is an essential neuropathological feature of the disease that accounts for the presenting motor symptoms, and, as such, has been the focal point for the majority of the PD research field.

Selective vulnerability suggests that these cells have some feature(s) that render(s) them more susceptible to toxic insults than other cells, and indeed many such features have been proposed. Interestingly, none of the genes that account for monogenic forms of PD are expressed specifically in dopaminergic neurons. This implies that additional factors must influence selective vulnerability. For the most part, the features that are thought to render SNc dopaminergic neurons vulnerable in PD center on oxidative stress and impaired proteostasis.

5.2.1.1 Oxidative stress

Dopamine, the neurotransmitter that nigrostriatal neurons use for intercellular communication, is prone to auto-oxidation and can thereby contribute to oxidative stress (Hastings, 2009). However, the ventral tegmental area (VTA), which is anatomically adjacent to the SNc, uses dopamine and does not degenerate in PD. Therefore, use of dopamine as a neurotransmitter is not a sufficient condition for selective vulnerability in PD. The fact that some non-dopaminergic neuronal populations also degenerate in PD means that use of dopamine as a neurotransmitter is not a necessary condition for selectively vulnerable cells in PD.

SNc dopaminergic neurons project to the caudate and putamen, with axons that are highly ramified with many terminals (Gauthier, Parent, Levesque, & Parent, 1999). Maintenance of these processes represents a great energetic demand, and not surprisingly, these cells are highly reliant on oxidative phosphorylation as a means of generating the requisite ATP (Dickson & Weller, 2011). Therefore, insults to mitochondria in general and oxidative phosphorylation in particular may have a larger impact on these cells than on others. The rotenone rat model of PD illustrates this point elegantly since rotenone is a highly lipophilic complex I inhibitor that enters all cells of the body when administered systemically yet induces selective degeneration of SNc dopaminergic neurons (Betarbet et al., 2000).

In contrast to VTA neurons, SNc dopaminergic neurons have intrinsic pacemaking activity which relies on a low-voltage L-type calcium channel, Ca(v)1.3 (Chan et al., 2007). The large calcium fluxes that these cells endure as a consequence of using these channels have been linked to oxidative stress via induction of calcium-responsive pro-oxidant enzymes (Guzman et al., 2010). Dopaminergic cells also seem to handle oxidative insults differently as compared to other cells. In a recent study, we examined how dopaminergic cells respond to challenge with

low concentrations of rotenone by examining changes in thiol oxidation over time using a novel redox histochemistry technique (Horowitz et al., 2011). We found a different oxidation profile in response to physiological oxidant exposure over time between midbrain dopaminergic neurons and cortical neurons. This pattern was consistent across dopaminergic neurons from multiple *in vitro* and *in vivo* systems, including rat primary midbrain culture, zebrafish larvae, and rat SNc (Horowitz et al., 2011). More studies are required in order to determine what accounts for these differences, but they may be due to differential utilization of various antioxidant systems, such as thiol buffering (*e.g.* glutathione) or induction of antioxidant enzymes (*e.g.* SOD1). Compounding these sources of oxidative stress is the fact that levels of antioxidants decrease with aging, limiting the ability of neurons to combat oxidative stress (Sohal & Orr, 2012).

5.2.1.2 Protein dyshomeostasis

A common feature of cells that selectively die in neurodegenerative diseases is accumulation and aggregation of protein (Chiti & Dobson, 2006; Saxena & Caroni, 2011). This would suggest that selectively vulnerable cells may have a diminished ability to properly re-fold or degrade mis-folded proteins as compared to non-degenerating cells. Protein dyshomeostasis often occurs in the context of oxidative stress, again suggesting that dysfunction in multiple crucial pathways can tip the balance within certain cells from stress adaptation to death. Aging neurons also have lower levels of protein chaperones, and a consequent reduced capacity for repairing and degrading damaged proteins (Ebrahimi-Fakhari, Wahlster, & McLean, 2011).

Lewy bodies (in neuronal somata) or Lewy neurites (in neuronal processes), are intracellular aggregates of various proteins, including misfolded and aggregation-prone proteins (*e.g.* a-syn, ubiquitinated proteins) as well as various proteins involved in protein homeostasis

(*e.g.* ubiquitin proteasome subunits, chaperones). Patients with *SNCA* locus multiplications develop a rare form of PD that is strikingly similar to sporadic PD when compared at the neuropathological levels – *i.e.* relatively selective loss of SNc dopaminergic neurons. Patients with triplications in the *SNCA* locus develop an earlier-onset, more severe form of PD than patients with locus duplication, suggesting that further exacerbation of protein homeostasis pathways results in a more severe cellular insult (Singleton et al., 2003). The subtle increase in a-syn levels may be enough to outstrip protein refolding and degradation machinery in SNc dopaminergic neurons and lead to aggregation in these cells, whereas other cells can handle the increased toxic load of misfolded protein.

These collective observations, as well as others, indicate that oxidative stress and impaired protein homeostasis are key factors in neuronal demise within the SNc. But just as very few cases of PD are due to one instigating pathological event (*e.g.* monogenic PD), SNc neuronal loss in PD is likely only rarely attributable to a single pathological insult sufficient to induce degeneration (*e.g.* MPTP exposure). A more likely reason for the relatively selective degeneration of SNc dopaminergic neurons that is observed in PD is multiple minor insults to pathways that render SNc dopaminergic neurons vulnerable – *i.e.* oxidative phosphorylation and protein homeostasis. These insults may be due to genetic variants or environmental exposures that, by themselves, impair these pathways only slightly, but collectively (and especially in the context of aging) are sufficient to push these cells beyond a critical threshold for survival.

5.3 HOW ARE WE GOING TO CURE PD?

There are currently no disease-modifying therapies for PD. Motor symptoms can be treated temporarily, but neurons invariably continue to degenerate and symptoms worsen over time, slowly eroding the patient's quality of life. Several challenges that beset therapeutic development for PD were mentioned earlier (1.1.6). Although the pharmacological outlook seems to be improving – with more potentially disease-modifying therapies in various stages of clinical trials – it is important that PD researchers collectively look beyond the pros and cons of candidate therapies and re-evaluate our goals and approach by focusing on a few key questions: 1) What are realistic goals for therapy in PD?, 2) What crucial technical or clinical advances are necessary to advance development and validation of disease-modifying therapeutics?, 3) What other changes will be important in sustaining strong translational PD research?

5.3.1 What are realistic goals for therapy in PD?

One of the main challenges in PD therapy is the inability to intervene early in the course of disease. The demise of SNc dopaminergic neurons is insidious and motor signs do not appear until 50-70% of these neurons have died (Lesage & Brice, 2009). Whereas most neuroprotective studies in animal models follow a pre-treatment paradigm – in which animals receive the putative neuroprotective intervention prior to toxic insult – clinicians do not have the luxury of treating patients before their PD has progressed considerably. Therefore, the realistic goal of PD therapeutic development given the currently available tools is either to protect remaining neurons from degenerating (neuroprotection) or to replace neurons that have already died

(neurorestoration) (Yacoubian & Standaert, 2009). Of the main types of therapy in development for PD – drugs, gene therapy, and tissue transplantation – all may prove capable of staving off further neuron death, but only tissue transplantation may realistically achieve neurorestoration. Unfortunately, this approach suffered a set-back when it was demonstrated that fetal mesencephalic tissue grafts given to PD patients developed a-syn pathology within the fetal cells within 15 years of engraftment (Kordower & Brundin, 2009). These studies demonstrated that putting a healthy graft into an unhealthy environment may not lead to long-term neurorestoration, underscoring the need for a combined approach of neuroprotective and neurorestorative therapies.

Any therapeutic intervention requires some degree of physiologic function in the region or cells that it is targeting. However, many of these systems are compromised in PD. Furthermore, the course of PD progression can be variable, meaning that not all patients stand to benefit from these interventions. Some examples may clarify this point. The fetal mesecephalic tissue grafts succumbed to PD pathology because they were placed in an environment marked by inflammation and a-syn pathology (Kordower & Brundin, 2009). It is thought that the recent AAV2-neurturin trial failed to show neuroprotection likely due to impaired retrograde transport and consequent inability to deliver the therapeutic viral plasmid from nigrostriatal terminals to somata (Marks et al., 2010). These are not insurmountable challenges, but they are issues that must be kept in mind when designing therapeutics and enrolling patients in clinical trials.

5.3.2 What crucial technical or clinical advances need to be made?

Discovery of biomarkers – *i.e.* indicators of disease that can be measured in living individuals to assess risk for developing PD and/or disease progression – is the most important goal in PD research. Without a way to detect PD earlier (diagnostic biomarker) or track the progression of nigrostriatal degeneration accurately (response biomarker), evaluation of putative disease-modifying therapeutics is stymied. A major problem in PD clinical trials is determining whether clinical improvement is due to relief of symptoms only or due to *bona fide* disease modification. A good diagnostic biomarker would be one that sensitively and specifically identifies individuals in early stages of PD, far before the emergence of symptoms (Scherzer, 2009). Such a biomarker might be found in bodily fluids, in brain imaging, or possibly in the astute correlation of pre-motor symptoms. A good response biomarker would be one that accurately represents the state of disease over time and changes in response to improvement or decline (Scherzer, 2009). The discovery of biomarkers would have an enormous impact on PD clinical trials since they would allow for early intervention and meaningful evaluation of neuroprotection, both of which would improve the likelihood of successfully identifying disease-modifying therapies.

The constellation of symptoms and course of disease progression can vary markedly across PD patients. In theory, it is possible that a therapeutic that is neuroprotective for one group of PD patients may have no effect in another group. Yet these clinically disparate PD cases are often combined in clinical trials, which carries the risk that any signs of therapeutic improvement may be masked. In the absence of a biomarker that would help categorize PD patients for rational inclusion or exclusion from certain clinical trials, definition of clinical sub-

types of PD becomes paramount. Impressive efforts to do this are being undertaken, but more work is required (van Rooden et al., 2011).

Before any novel therapeutic can enter clinical trials for PD, it must first be evaluated for efficacy in animal models of PD. None of the currently used animal models of PD accurately recapitulates all features of human PD, and this is not surprising given the fundamental differences in biology and time-scale. However, animal modeling represents a third critical domain requiring improvement in order to advance translational PD research. The majority of PD cases are thought to arise from an interaction among genes, environment, and aging, however nearly all PD animal models are based on a single pathogenic insult – either a mutated gene or an administered toxin (Horowitz & Greenamyre, 2010a). Large genome-wide association studies have recently uncovered many common genetic variants associated with PD, creating the possibility of using such variants in combination with neurotoxins in animal models (Satake et al., 2009; Simón-Sánchez et al., 2009). An animal model that is more representative of PD etiology – *i.e.* arising from a combination of genetic and environmental insults – may prove more accurate in recapitulating disease and hence more useful in the evaluation of therapeutics in pre-clinical stages of development.

5.3.3 How do we sustain strong translational research?

In addition to innovation at the bench and bedside, the field of PD – and science more generally – needs innovation at the funding level for sustained support of translational research. Challenges in this domain include cuts to federal funding of research and a worrisome trend toward funding only “safe” projects. The two may be seen as going hand-in-hand: if federal research funding is

scant, then the projects with the highest likelihood of success are the ones that should receive support. However, this mode of operating can have a detrimental impact on innovation and translational progress.

As Dr. Anne Young rightly described in her lecture, “Neurodegenerative Diseases: The Path to Therapy,” at the 2011 Annual Meeting of the Society for Neuroscience, the trajectory of progress in neurodegenerative disease research has been marked by periodic bursts of innovation following key discoveries rather than steadfast work on the same problems (11-15-11, Washington DC, USA). If we keep this view in mind, then re-prioritizing funding to be more supportive of riskier projects with greater potential pay-off would lead to greater progress in translational PD research.

In summary, the most critical steps that must be taken to advance development of disease-modifying therapeutics for PD include: identification of reliable biomarkers for earlier diagnosis and accurate tracking of disease progression, evidence-based clinical sub-typing of PD patients for rational inclusion in clinical trials, and fostering of a funding environment that is more willing to support research that has the potential to lead to important breakthroughs.

APPENDIX A

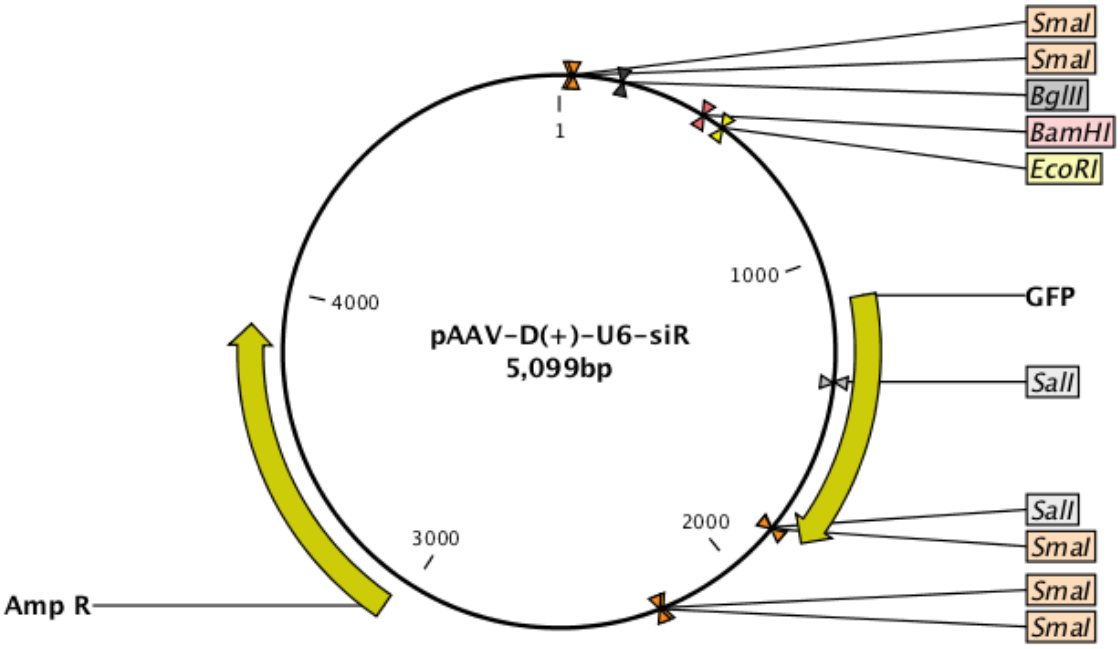

Figure 25. AAV2 expression plasmid used for construction of AAV2.shGATA2 and AAV2.shNeg

BIBLIOGRAPHY

Amigo, J. D., Yu, M., Troadec, M.-B., Gwynn, B., Cooney, J. D., Lambert, A. J., Chi, N. C., et al. (2011). Identification of distal cis-regulatory elements at mouse mitoferrin loci using zebrafish transgenesis. *Molecular and cellular biology*, *31*(7), 1344–1356. doi:10.1128/MCB.01010-10

Arthur, C. R., Morton, S. L., Dunham, L. D., Keeney, P. M., & Bennett, J. P. (2009). Parkinson's disease brain mitochondria have impaired respirasome assembly, age-related increases in distribution of oxidative damage to mtDNA and no differences in heteroplasmic mtDNA mutation abundance. *Molecular Neurodegeneration*, *4*(1), 37. doi:10.1186/1750-1326-4-37

Ascherio, A., Zhang, S. M., Hernán, M. A., Kawachi, I., Colditz, G. A., Speizer, F. E., & Willett, W. C. (2001). Prospective study of caffeine consumption and risk of Parkinson's disease in men and women. *Annals of neurology*, *50*(1), 56–63.

Banerjee, R., Starkov, A. A., Beal, M. F., & Thomas, B. (2009). Mitochondrial dysfunction in the limelight of Parkinson's disease pathogenesis. *Biochimica et biophysica acta*, *1792*(7), 651–663. doi:10.1016/j.bbadis.2008.11.007

Barroso, N., Campos, Y., Huertas, R., Esteban, J., Molina, J. A., Alonso, A., Guitierrez-Rivas, E., et al. (1993). Respiratory Chain Enzyme Activities in Lymphocytes from Untreated Patients with Parkinson disease. *Clinical chemistry*, 1–3.

Ben Gedalya, T., Loeb, V., Israeli, E., Altschuler, Y., Selkoe, D. J., & Sharon, R. (2009). α-Synuclein and Polyunsaturated Fatty Acids Promote Clathrin-Mediated Endocytosis and Synaptic Vesicle Recycling. *Traffic*, *10*(2), 218–234. doi:10.1111/j.1600-0854.2008.00853.x

Betarbet, R., Canet-Aviles, R. M., Sherer, T. B., Mastroberardino, P. G., McLendon, C., Kim, J.-H., Lund, S., et al. (2006). Intersecting pathways to neurodegeneration in Parkinson's disease: effects of the pesticide rotenone on DJ-1, alpha-synuclein, and the ubiquitin-proteasome system. *Neurobiology of disease*, *22*(2), 404–420. doi:10.1016/j.nbd.2005.12.003

Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, A. V., & Greenamyre, J. T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nature neuroscience*, *3*(12), 1301–1306. doi:10.1038/81834

Biskup, S., Moore, D. J., Celsi, F., Higashi, S., West, A. B., Andrabi, S. A., Kurkinen, K., et al. (2006). Localization of LRRK2 to membranous and vesicular structures in mammalian

brain. *Annals of neurology*, *60*(5), 557–569. doi:10.1002/ana.21019
Björklund, A., & Björklund, T. (2011). Gene therapy for Parkinson's disease shows promise. *Science translational medicine*, *3*(79), 79ed1. doi:10.1126/scitranslmed.3002430
Björklund, T., & Kirik, D. (2009). Scientific rationale for the development of gene therapy strategies for Parkinson's disease. *Biochimica et biophysica acta*, *1792*(7), 703–713. doi:10.1016/j.bbadis.2009.02.009
Block, M. L., Zecca, L., & Hong, J.-S. (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nature reviews. Neuroscience*, *8*(1), 57–69. doi:10.1038/nrn2038
Bonifati, V., Rizzu, P., van Baren, M. J., Schaap, O., Breedveld, G. J., Krieger, E., Dekker, M. C. J., et al. (2003). Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science (New York, N.Y.)*, *299*(5604), 256–259. doi:10.1126/science.1077209
Bowers, W. J., Breakefield, X. O., & Sena-Esteves, M. (2011). Genetic therapy for the nervous system. *Human molecular genetics*, *20*(R1), R28–41. doi:10.1093/hmg/ddr110
Böhm, M., Locke, W. J., Sutherland, R. L., Kench, J. G., & Henshall, S. M. (2009). A role for GATA-2 in transition to an aggressive phenotype in prostate cancer through modulation of key androgen-regulated genes. *Oncogene*, *28*(43), 3847–3856. doi:10.1038/onc.2009.243
Braak, H., Del Tredici, K., Rüb, U., de Vos, R. A. I., Jansen Steur, E. N. H., & Braak, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiology of aging*, *24*(2), 197–211.
Bresnick, E. H., Katsumura, K. R., Lee, H.-Y., Johnson, K. D., & Perkins, A. S. (2012). Master regulatory GATA transcription factors: mechanistic principles and emerging links to hematologic malignancies. *Nucleic acids research*. doi:10.1093/nar/gks281
Bresnick, E. H., Lee, H.-Y., Fujiwara, T., Johnson, K. D., & Keles, S. (2010). GATA switches as developmental drivers. *The Journal of biological chemistry*, *285*(41), 31087–31093. doi:10.1074/jbc.R110.159079
Breydo, L., Wu, J. W., & Uversky, V. N. (2012). **α**-Synuclein misfolding and Parkinson's disease. *Biochimica et biophysica acta*, *1822*(2), 261–285. doi:10.1016/j.bbadis.2011.10.002
Canet-Aviles, R. M., Wilson, M. A., Miller, D. W., Ahmad, R., McLendon, C., Bandyopadhyay, S., Baptista, M., et al. (2004). The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. *Proc Natl Acad Sci USA*, 1–6.
Cannon, J. R., & Greenamyre, J. T. (2011). The Role of Environmental Exposures in Neurodegeneration and Neurodegenerative Diseases. *Toxicological Sciences*, *124*(2), 225–250. doi:10.1093/toxsci/kfr239
Cannon, J. R., Bai, Q., Horowitz, M. P., Tapias, V., Shah, V., Sew, T., Ayadi, El, A., et al. (n.d.). Short hairpin RNA targeting endogenous **α**-synuclein prevents degeneration of dopaminergic neurons in the rotenone model of Parkinson’s disease. *Journal of clinical investigation (submitted)*.
Cannon, J. R., Sew, T., Montero, L., Burton, E. A., & Greenamyre, J. T. (2011). Pseudotype-dependent lentiviral transduction of astrocytes or neurons in the rat substantia nigra. *Experimental neurology*, *228*(1), 41–52. doi:10.1016/j.expneurol.2010.10.016
Cannon, J. R., Tapias, V., Na, H. M., Honick, A. S., Drolet, R. E., & Greenamyre, J. T. (2009). A highly reproducible rotenone model of Parkinson's disease. *Neurobiology of disease*, *34*(2), 279–290. doi:10.1016/j.nbd.2009.01.016
Cardo, L. F., Coto, E., de Mena, L., Ribacoba, R., Lorenzo-Betancor, O., Pastor, P., Samaranch, L., et al. (2011). A Search for SNCA 3“ UTR Variants Identified SNP rs356165 as a

Determinant of Disease Risk and Onset Age in Parkinson”s Disease. *Journal of molecular neuroscience : MN*. doi:10.1007/s12031-011-9669-1

Cederfjäll, E., Sahin, G., Kirik, D., & Björklund, T. (2012). Design of a Single AAV Vector for Coexpression of TH and GCH1 to Establish Continuous DOPA Synthesis in a Rat Model of Parkinson's Disease. *Molecular therapy : the journal of the American Society of Gene Therapy*. doi:10.1038/mt.2012.1

Chan, C. S., Guzman, J. N., Ilijic, E., Mercer, J. N., Rick, C., Tkatch, T., Meredith, G. E., et al. (2007). “Rejuvenation” protects neurons in mouse models of Parkinson’s disease. *Nature*, *447*(7148), 1081–1086. doi:10.1038/nature05865

Chaudhuri, K. R., & Schapira, A. H. V. (2009). Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. *Lancet neurology*, *8*(5), 464–474. doi:10.1016/S1474-4422(09)70068-7

Cheng, F., Vivacqua, G., & Yu, S. (2011). The role of alpha-synuclein in neurotransmission and synaptic plasticity. *Journal of chemical neuroanatomy*, *42*(4), 242–248. doi:10.1016/j.jchemneu.2010.12.001

Chesselet, M. (2009). Transcriptional regulation of α-synuclein: insights from blood? *Future Neurology*.

Chiba, T., Ikawa, Y., & Todokoro, K. (1991). GATA-1 transactivates erythropoietin receptor gene, and erythropoietin receptor-mediated signals enhance GATA-1 gene expression. *Nucleic acids research*, *19*(14), 3843–3848.

Chiba-Falek, O., & Nussbaum, R. L. (2001). Effect of allelic variation at the NACP-Rep1 repeat upstream of the alpha-synuclein gene (SNCA) on transcription in a cell culture luciferase reporter system. *Human molecular genetics*, 1–9.

Chiba-Falek, O., Lopez, G. J., & Nussbaum, R. L. (2006). Levels of alpha-synuclein mRNA in sporadic Parkinson disease patients. *Movement disorders : official journal of the Movement Disorder Society*, *21*(10), 1703–1708. doi:10.1002/mds.21007

Chiti, F., & Dobson, C. M. (2006). Protein Misfolding, Functional Amyloid, and Human Disease. *Annual review of biochemistry*, 1–37.

Chua, C. E. L., & Tang, B. L. (2011). Rabs, SNAREs and α-synuclein--membrane trafficking defects in synucleinopathies. *Brain research reviews*, *67*(1-2), 268–281. doi:10.1016/j.brainresrev.2011.03.002

Chung, J., Chen, C., & Paw, B. H. (2012). Heme metabolism and erythropoiesis. *Current opinion in hematology*, *19*(3), 156–162. doi:10.1097/MOH.0b013e328351c48b

Chung, S. J., Armasu, S. M., Biernacka, J. M., Lesnick, T. G., Rider, D. N., Lincoln, S., Ortolaza, A. I., et al. (2011). Common Variants in PARK Loci and Related Genes and Parkinson's Disease. *Movement disorders : official journal of the Movement Disorder Society*, 1–9. doi:10.1002/mds23376

Clark, I. E., Dodson, M. W., Jiang, C., Cao, J. H., Huh, J. R., Seol, J. H., Yoo, S. J., et al. (2006). Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature*, *441*(7097), 1162–1166. doi:10.1038/nature04779

Connelly, J. J., Wang, T., Cox, J. E., Haynes, C., Wang, L., Shah, S. H., Crosslin, D. R., et al. (2006). GATA2 is associated with familial early-onset coronary artery disease. *PLoS genetics*, *2*(8), e139. doi:10.1371/journal.pgen.0020139

Conway, K. A., Rochet, J. C., Bieganski, R. M., & Lansbury, P. T. (2001). Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. *Science (New York,*

N.Y.), *294*(5545), 1346–1349. doi:10.1126/science.1063522
Cookson, M. R. (2011). A feedforward loop links Gaucher and Parkinson's diseases? *Cell*, *146*(1), 9–11. doi:10.1016/j.cell.2011.06.031
Costello, S., Cockburn, M., Bronstein, J., Zhang, X., & Ritz, B. (2009). Parkinson's Disease and Residential Exposure to Maneb and Paraquat From Agricultural Applications in the Central Valley of California. *American Journal of Epidemiology*, *169*(8), 919–926. doi:10.1093/aje/kwp006
Cronin, K. D., Ge, D., Manninger, P., Linnertz, C., Rossoshek, A., Orrison, B. M., Bernard, D. J., et al. (2009). Expansion of the Parkinson disease-associated SNCA-Rep1 allele upregulates human alpha-synuclein in transgenic mouse brain. *Human molecular genetics*, *18*(17), 3274–3285. doi:10.1093/hmg/ddp265
Cuervo, A. M., Stefanis, L., Fredenburg, R., Lansbury, P. T., & Sulzer, D. (2004). Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. *Science (New York, N.Y.)*, *305*(5688), 1292–1295. doi:10.1126/science.1101738
Dacshel, J., Lincoln, S., Gonzalez, J., Ross, O. A., Dickson, D. W., & Farrer, M. J. (2007). The Ups and Downs of alpha-Synuclein mRNA Expression. *Movement disorders : official journal of the Movement Disorder Society*, *22*(2), 293–293. doi:10.1002/mds.21190
Dawson, T. M., & Dawson, V. L. (2011). A lysosomal lair for a pathogenic protein pair. *Science translational medicine*, 1–4.
de Rijk, M. C., Launer, L. J., Berger, K., Breteler, M. M., Dartigues, J. F., Baldereschi, M., Fratiglioni, L., et al. (2000). Prevalence of Parkinson's disease in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group. *Neurology*, *54*(11 Suppl 5), S21–3.
de Rijk, M. C., Tzourio, C., Breteler, M. M., Dartigues, J. F., Amaducci, L., Lopez-Pousa, S., Manubens-Bertran, J. M., et al. (1997). Prevalence of parkinsonism and Parkinson“s disease in Europe: the EUROPARKINSON Collaborative Study. European Community Concerted Action on the Epidemiology of Parkinson”s disease. *Journal of neurology, neurosurgery, and psychiatry*, *62*(1), 10–15.
DePaolo, J., Goker-Alpan, O., Samaddar, T., Lopez, G., & Sidransky, E. (2009). The association between mutations in the lysosomal protein glucocerebrosidase and parkinsonism. *Movement disorders : official journal of the Movement Disorder Society*, *24*(11), 1571–1578. doi:10.1002/mds.22538
Desplats, P., Lee, H.-J., Bae, E.-J., Patrick, C., Rockenstein, E., Crews, L., Spencer, B., et al. (2009). Inclusion formation and neuronal cell death through neuron-to-neuron transmission of alpha-synuclein. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(31), 13010–13015. doi:10.1073/pnas.0903691106
Dickinson, R. E., Griffin, H., Bigley, V., Reynard, L. N., Hussain, R., Haniffa, M., Lakey, J. H., et al. (2011). Exome sequencing identifies GATA-2 mutation as the cause of dendritic cell, monocyte, B and NK lymphoid deficiency. *Blood*, *118*(10), 2656–2658. doi:10.1182/blood-2011-06-360313
Dickson, D. W., Braak, H., Duda, J. E., Duyckaerts, C., Gasser, T., Halliday, G. M., Hardy, J., et al. (2009). Neuropathological assessment of Parkinson's disease: refining the diagnostic criteria. *Lancet neurology*, *8*(12), 1150–1157. doi:10.1016/S1474-4422(09)70238-8
Dickson, D., & Weller, R. O. (Eds.). (2011). *Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders* (2nd ed. p. 496). Wiley-Blackwell.

Dore, L. C., Chlon, T. M., Brown, C. D., White, K. P., & Crispino, J. D. (2012). Chromatin occupancy analysis reveals genome-wide GATA factor switching during hematopoiesis. *Blood*, *119*(16), 3724–3733. doi:10.1182/blood-2011-09-380634

Dorfman, D. M., Wilson, D. B., Bruns, G. A. P., & Orkin, S. H. (1992). Human Transcription Factor GATA-2. *The Journal of biological chemistry*, Evidence for regulation of preproendothelin-1 gene expression in endothelial cells, 1–7.

Doxakis, E. (2010). Post-transcriptional regulation of alpha-synuclein expression by mir-7 and mir-153. *The Journal of biological chemistry*, *285*(17), 12726–12734. doi:10.1074/jbc.M109.086827

Dubow, J. S. (2007). Autonomic dysfunction in Parkinson's disease. *Disease-a-month : DM*, *53*(5), 265–274. doi:10.1016/j.disamonth.2007.02.004

Dykxhoorn, D. M., Novina, C. D., & Sharp, P. A. (2003). Killing the messenger: short rnas that silence gene expression. *Nature Reviews Molecular Cell Biology*, *4*(6), 457–467. doi:10.1038/nrm1129

Ebrahimi-Fakhari, D., Wahlster, L., & McLean, P. J. (2011). Molecular Chaperones in Parkinson's disease. *Journal of Parkinsons Disease*, Present and Future, 1–30.

Elbaz, A., & Tranchant, C. (2007). Epidemiologic studies of environmental exposures in Parkinson's disease. *Journal of the neurological sciences*, *262*(1-2), 37–44. doi:10.1016/j.jns.2007.06.024

Elbaz, A., Bower, J. H., Maraganore, D. M., McDonnell, S. K., Peterson, B. J., Shlskog, E. J., Schaid, D. J., et al. (2002). Risk tables for parkinsonism and Parkinson's disease. *Journal of clinical epidemiology*, 1–7.

Farrer, M., Maraganore, D. M., Lockhart, P., Singleton, A., Lesnick, T. G., de Andrade, M., West, A., et al. (2001). alpha-Synuclein gene haplotypes are associated with Parkinson's disease. *Human molecular genetics*, *10*(17), 1847–1851.

Ferreira, R., Ohneda, K., Yamamoto, M., & Philipsen, S. (2005). GATA1 function, a paradigm for transcription factors in hematopoiesis. *Molecular and cellular biology*, *25*(4), 1215–1227. doi:10.1128/MCB.25.4.1215-1227.2005

Fuchs, J., Tichopad, A., Golub, Y., Munz, M., Schweitzer, K. J., Wolf, B., Berg, D., et al. (2007). Genetic variability in the SNCA gene influences -synuclein levels in the blood and brain. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *22*(5), 1327–1334. doi:10.1096/fj.07-9348com

Fujiwara, T., O'Geen, H., Keles, S., Blahnik, K., Linnemann, A. K., Kang, Y.-A., Choi, K., et al. (2009). Discovering hematopoietic mechanisms through genome-wide analysis of GATA factor chromatin occupancy. *Molecular cell*, *36*(4), 667–681. doi:10.1016/j.molcel.2009.11.001

Gash, D. M., Rutland, K., Hudson, N. L., Sullivan, P. G., Bing, G., Cass, W. A., Pandya, J. D., et al. (2008). Trichloroethylene: Parkinsonism and complex 1 mitochondrial neurotoxicity. *Annals of neurology*, *63*(2), 184–192. doi:10.1002/ana.21288

Gauthier, J., Parent, M., Levesque, M., & Parent, A. (1999). The axonal arborization of single nigrostriatal neurons in rats. *Brain Research*, 1–5.

Giacca, M. (2010). *Gene Therapy* (1st ed. p. 328). Springer.

Glass, C. K., Saijo, K., Winner, B., Marchetto, M. C., & Gage, F. H. (2010). Mechanisms Underlying Inflammation in Neurodegeneration. *Cell*, *140*(6), 918–934. doi:10.1016/j.cell.2010.02.016

Goetz, C. G., Wuu, J., McDermott, M. P., Adler, C. H., Fahn, S., Freed, C. R., Hauser, R. A., et al. (2008). Placebo response in Parkinson's disease: Comparisons among 11 trials covering medical and surgical interventions. *Movement disorders : official journal of the Movement Disorder Society*, *23*(5), 690–699. doi:10.1002/mds.21894

Goldman, S. M., Kamel, F., Ross, G. W., Jewell, S. A., Bhudhikanok, G. S., Umbach, D., Marras, C., et al. (2012). Head injury, alpha-synuclein Rep1, and Parkinson's disease. *Annals of neurology*, *71*(1), 40–48. doi:10.1002/ana.22499

Gong, Q. H., Stern, J., & Dean, A. (1991). Transcriptional role of a conserved GATA-1 site in the human epsilon-globin gene promoter. *Blood*, 1–10. doi:10.1128/MCB.11.5.2558

Gordon, D. F., Lewis, S. R., Haugen, B. R., James, R. A., McDermott, M. T., Wood, W. M., & Ridgway, E. C. (1997). Pit-1 and GATA-2 Interact and Functionally Cooperate to Activate the Thyrotropin Beta-Subunit Promoter. *The Journal of biological chemistry*, 1–9.

Greenamyre, J. T., & Hastings, T. G. (2004). Biomedicine. Parkinson's--divergent causes, convergent mechanisms. *Science (New York, N.Y.)*, *304*(5674), 1120–1122. doi:10.1126/science.1098966

Greenamyre, J. T., Cannon, J. R., Drolet, R., & Mastroberardino, P. G. (2010). Lessons from the rotenone model of Parkinson's disease. *Trends in pharmacological sciences*, *31*(4), 141–2; author reply 142–3. doi:10.1016/j.tips.2009.12.006

Gründemann, J., Schlaudraff, F., Haeckel, O., & Liss, B. (2008). Elevated alpha-synuclein mRNA levels in individual UV-laser-microdissected dopaminergic substantia nigra neurons in idiopathic Parkinson's disease. *Nucleic acids research*, *36*(7), e38. doi:10.1093/nar/gkn084

Guilarte, T. R. (2011). Manganese and Parkinson's Disease: A Critical Review and New Findings. *Cien Saude Colet*, 1–18.

Guzman, J. N., Sanchez-Padilla, J., Wokosin, D., Kondapalli, J., Ilijic, E., Schumacker, P. T., & Surmeier, D. J. (2010). Oxidant stress evoked by pacemaking in dopaminergic neurons is attenuated by DJ-1. *Nature*, *468*(7324), 696–700. doi:10.1038/nature09536

Haas, R. H., Nasirian, F., Nakano, K., Ward, D., Pay, M., Hill, R., & Shults, C. W. (1995). Low platelet mitochondrial complex I and complex II/III activity in early untreated Parkinson's disease. *Annals of neurology*, *37*(6), 714–722. doi:10.1002/ana.410370604

Haavik, J., Le Bourdelles, B., Martínez, A., Flatmark, T., & Mallet, J. (1991). Recombinant human tyrosine hydroxylase isozymes. *European Journal of biochemistry*, Reconstitution with iron and inhbitory effects of other metal ions, 1–8.

Hadaczek, P., Eberling, J. L., Pivirotto, P., Bringas, J., Forsayeth, J., & Bankiewicz, K. S. (2009a). Eight Years of Clinical Improvement in MPTP-Lesioned Primates After Gene Therapy With AAV2-hAADC. *Molecular therapy : the journal of the American Society of Gene Therapy*, *18*(8), 1458–1461. doi:10.1038/mt.2010.106

Hadaczek, P., Forsayeth, J., Mirek, H., Munson, K., Bringas, J., Pivirotto, P., McBride, J. L., Davidson, B. L., et al. (2009b). Transduction of Nonhuman Primate Brain with Adeno-Associated Virus Serotype 1: Vector Trafficking and Immune Response. *Human gene therapy*, 1–16.

Hahn, C. N., Chong, C.-E., Carmichael, C. L., Wilkins, E. J., Brautigan, P. J., Li, X.-C., Babic, M., et al. (2011). Heritable GATA2 mutations associated with familial myelodysplastic syndrome and acute myeloid leukemia. *Nature genetics*, *43*(10), 1012–1017. doi:10.1038/ng.913

Hastings, T. G. (2009). The role of dopamine oxidation in mitochondrial dysfunction: implications for Parkinson's disease. *Journal of bioenergetics and biomembranes*, *41*(6), 469–472. doi:10.1007/s10863-009-9257-z

Hatano, Y., Li, Y., Sato, K., Asakawa, S., Yamamura, Y., Tomiyama, H., Yoshino, H., et al. (2004). NovelPINK1 mutations in early-onset parkinsonism. *Annals of neurology*, *56*(3), 424–427. doi:10.1002/ana.20251

Healy, D. G., Falchi, M., O'Sullivan, S. S., Bonifati, V., Durr, A., Bressman, S., Brice, A., et al. (2008). Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: a case-control study. *The Lancet Neurology*, *7*(7), 583–590. doi:10.1016/S1474-4422(08)70117-0

Hebert, S. S., & De Strooper, B. (2007). MOLECULAR BIOLOGY: miRNAs in Neurodegeneration. *Science (New York, N.Y.)*, *317*(5842), 1179–1180. doi:10.1126/science.1148530

Herzog, C. D., Brown, L., Gammon, D., Kruegel, B., Lin, R., Wilson, A., Bolton, A., et al. (2009). EXPRESSION, BIOACTIVITY, AND SAFETY 1 YEAR AFTER ADENO-ASSOCIATED VIRAL VECTOR TYPE 2–MEDIATED DELIVERY OF NEURTURIN TO THE MONKEY NIGROSTRIATAL SYSTEM SUPPORT CERE-120 FOR PARKINSON'S DISEASE. *Neurosurgery*, *64*(4), 602–613. doi:10.1227/01.NEU.0000340682.06068.01

Horowitz, M. P., & Greenamyre, J. T. (2010a). Gene-environment interactions in Parkinson's disease: the importance of animal modeling. *Clinical pharmacology and therapeutics*, *88*(4), 467–474. doi:10.1038/clpt.2010.138

Horowitz, M. P., & Greenamyre, J. T. (2010b). Mitochondrial iron metabolism and its role in neurodegeneration. *Journal of Alzheimer's disease : JAD*, *20 Suppl 2*, S551–68. doi:10.3233/JAD-2010-100354

Horowitz, M. P., Milanese, C., Di Maio, R., Hu, X., Montero, L. M., Sanders, L. H., Tapias, V., et al. (2011). Single-cell redox imaging demonstrates a distinctive response of dopaminergic neurons to oxidative insults. *Antioxidants & redox signaling*, *15*(4), 855–871. doi:10.1089/ars.2010.3629

Hosoya, T., Maillard, I., & Engel, J. D. (2010). From the cradle to the grave: activities of GATA-3 throughout T-cell development and differentiation. *Immunological reviews*, *238*(1), 110–125. doi:10.1111/j.1600-065X.2010.00954.x

Jackson-Lewis, V., Blesa, J., & Przedborski, S. (2012). Animal models of Parkinson's disease. *Parkinsonism & related disorders*, *18 Suppl 1*, S183–5. doi:10.1016/S1353-8020(11)70057-8

Jellinger, K. A. (2009). A critical evaluation of current staging of alpha-synuclein pathology in Lewy body disorders. *Biochimica et biophysica acta*, *1792*(7), 730–740. doi:10.1016/j.bbadis.2008.07.006

Junn, E., Lee, K.-W., Jeong, B. S., Chan, T. W., Im, J.-Y., & Mouradian, M. M. (2009). Repression of alpha-synuclein expression and toxicity by microRNA-7. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(31), 13052–13057. doi:10.1073/pnas.0906277106

Kala, K., Haugas, M., Lilleväli, K., Guimera, J., Wurst, W., Salminen, M., & Partanen, J. (2009). Gata2 is a tissue-specific post-mitotic selector gene for midbrain GABAergic neurons. *Development (Cambridge, England)*, *136*(2), 253–262. doi:10.1242/dev.029900

Kamel, F., Tanner, C., Umbach, D., Hoppin, J., Alavanja, M., Blair, A., Comyns, K., et al. (2006). Pesticide Exposure and Self-reported Parkinson's Disease in the Agricultural Health Study. *American Journal of Epidemiology*, *165*(4), 364–374. doi:10.1093/aje/kwk024

Kang, Y.-A., Sanalkumar, R., O'Geen, H., Linnemann, A. K., Chang, C.-J., Bouhassira, E. E., Farnham, P. J., et al. (2012). Autophagy driven by a master regulator of hematopoiesis. *Molecular and cellular biology*, *32*(1), 226–239. doi:10.1128/MCB.06166-11

Kawabata, H., Germain, R. S., Ikezoe, T., Tong, X., Green, E. M., Gombart, A. F., & Koeffler, H. P. (2001). Regulation of expression of murine transferrin receptor 2. *Blood*, *98*(6), 1949–1954.

Kim, J., Inoue, K., Ishii, J., Vanti, W. B., Voronov, S. V., Murchison, E., Hannon, G., et al. (2007). A MicroRNA feedback circuit in midbrain dopamine neurons. *Science (New York, N.Y.)*, *317*(5842), 1220–1224. doi:10.1126/science.1140481

Kingsbury, A. E., Daniel, S. E., Sangha, H., Eisen, S., Lees, A. J., & Foster, O. J. F. (2004). Alteration in alpha-synuclein mRNA expression in Parkinson's disease. *Movement disorders : official journal of the Movement Disorder Society*, *19*(2), 162–170. doi:10.1002/mds.10683

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., et al. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*, 1–4.

Kordower, J. H., & Brundin, P. (2009). Lewy body pathology in long-term fetal nigral transplants: is parkinson's disease transmitted from one neural system to another? *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *34*(1), 254–254. doi:10.1038/npp.2008.161

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

Kordower, J. H., Chu, Y., Hauser, R. A., Olanow, C. W., & Freeman, T. B. (2008b). Transplanted dopaminergic neurons develop PD pathologic changes: A second case report. *Movement disorders : official journal of the Movement Disorder Society*, *23*(16), 2303–2306. doi:10.1002/mds.22369

Kordower, J. H., Herzog, C. D., Dass, B., Bakay, R. A. E., Stansell, J., III, Gasmi, M., & Bartus, R. T. (2006). Delivery of neurturin by AAV2 (CERE-120)-mediated gene transfer provides structural and functional neuroprotection and neurorestoration in MPTP-treated monkeys. *Annals of neurology*, *60*(6), 706–715. doi:10.1002/ana.21032

Kramer, M. F., Gunaratne, P., & Ferreira, G. C. (2000). Transcriptional regulation of the murine erythroid-specific 5-aminolevulinate synthase gene. *Gene*, 1–14.

Kumar, A., & Cookson, M. R. (2011). Role of LRRK2 kinase dysfunction in Parkinson disease. *Expert Reviews in Molecular Medicine*, *13*. doi:10.1017/S146239941100192X

Lang, A. E., & Lozano, A. (1998a). Parkinson's disease: First of two parts. *NEJM*, 1–10.

Lang, A. E., & Lozano, A. (1998b). Parkinson's disease: Second of two parts. *NEJM*, 1–14.

Langston, J. W., Ballard, P., Tetrud, J. W., & Irwin, I. (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science (New York, N.Y.)*, *219*(4587), 979–980.

Lee, B. D., Shin, J.-H., VanKampen, J., Petrucelli, L., West, A. B., Ko, H. S., Lee, Y.-I., et al. (2010). Inhibitors of leucine-rich repeat kinase-2 protect against models of Parkinson's disease. *Nature medicine*, *16*(9), 994–996. doi:10.1038/nm.2199

Lees, A. J., Hardy, J., & Revesz, T. (2009). Parkinson's disease. *Lancet*, *373*(9680), 2055–2066. doi:10.1016/S0140-6736(09)60492-X

Lesage, S., & Brice, A. (2009). Parkinson's disease: from monogenic forms to genetic susceptibility factors. *Human molecular genetics*, *18*(R1), R48–59. doi:10.1093/hmg/ddp012

Lewis, T. B., & Standaert, D. G. (2011). Parkinson's disease, primates, and gene therapy: vive la différence? *Movement disorders : official journal of the Movement Disorder Society*, *26*(1), 2–3. doi:10.1002/mds.23544

LeWitt, P. A., Rezai, A. R., Leehey, M. A., Ojemann, S. G., Flaherty, A. W., Eskandar, E. N., Kostyk, S. K., et al. (2011). AAV2-GAD gene therapy for advanced Parkinson's disease: a double-blind, sham-surgery controlled, randomised trial. *Lancet neurology*, *10*(4), 309–319. doi:10.1016/S1474-4422(11)70039-4

Li, J.-Y., Englund, E., Holton, J. L., Soulet, D., Hagell, P., Lees, A. J., Lashley, T., et al. (2008). Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. *Nature medicine*, *14*(5), 501–503. doi:10.1038/nm1746

Linnemann, A. K., O'Geen, H., Keles, S., Farnham, P. J., & Bresnick, E. H. (2011). Genetic framework for GATA factor function in vascular biology. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(33), 13641–13646. doi:10.1073/pnas.1108440108

Liu, M., Choi, D.-Y., Hunter, R. L., Pandya, J. D., Cass, W. A., Sullivan, P. G., Kim, H.-C., et al. (2010). Trichloroethylene induces dopaminergic neurodegeneration in Fisher 344 rats. *Journal of Neurochemistry*, *112*(3), 773–783. doi:10.1111/j.1471-4159.2009.06497.x

Liu, Z., Hamamichi, S., Dae Lee, B., Yang, D., Ray, A., Caldwell, G. A., Caldwell, K. A., et al. (2011). Inhibitors of LRRK2 kinase attenuate neurodegeneration and Parkinson-like phenotypes in Caenorhabditis elegans and Drosophila Parkinson's disease models. *Human molecular genetics*, *20*(20), 3933–3942. doi:10.1093/hmg/ddr312

Lundvig, D., Lindersson, E., & Jensen, P. H. (2005). Pathogenic effects of alpha-synuclein aggregation. *Brain research. Molecular brain research*, *134*(1), 3–17. doi:10.1016/j.molbrainres.2004.09.001

Lurie, L. J., Boyer, M. E., Grass, J. A., & Bresnick, E. H. (2008). Differential GATA factor stabilities: implications for chromatin occupancy by structurally similar transcription factors. *Biochemistry*, *47*(3), 859–869. doi:10.1021/bi701692p

Ma, G. T., Roth, M. E., Groskopf, J. C., Tsai, F. Y., Orkin, S. H., Grosveld, F., Engel, J. D., et al. (1997). GATA-2 and GATA-3 regulate trophoblast-specific gene expression in vivo. *Development (Cambridge, England)*, 1–8.

Maraganore, D. M., de Andrade, M., Elbaz, A., Farrer, M. J., Ioannidis, J. P., Krüger, R., Rocca, W. A., et al. (2006). Collaborative analysis of alpha-synuclein gene promoter variability and Parkinson disease. *JAMA : the journal of the American Medical Association*, *296*(6), 661–670. doi:10.1001/jama.296.6.661

Marder, K. (2010). Cognitive impairment and dementia in Parkinson's disease. *Movement disorders : official journal of the Movement Disorder Society*, *25 Suppl 1*, S110–6. doi:10.1002/mds.22787

Marks, W. J., Bartus, R. T., Siffert, J., Davis, C. S., Lozano, A., Boulis, N., Vitek, J., et al. (2010). Gene delivery of AAV2-neurturin for Parkinson's disease: a double-blind, randomised, controlled trial. *Lancet neurology*, *9*(12), 1164–1172. doi:10.1016/S1474-4422(10)70254-4

Mastroberardino, P. G., Hoffman, E. K., Horowitz, M. P., Betarbet, R., Taylor, G., Cheng, D., Na, H. M., et al. (2009). A novel transferrin/TfR2-mediated mitochondrial iron transport system is disrupted in Parkinson's disease. *Neurobiology of disease*, *34*(3), 417–431. doi:10.1016/j.nbd.2009.02.009

Mazzulli, J. R., Xu, Y.-H., Sun, Y., Knight, A. L., McLean, P. J., Caldwell, G. A., Sidransky, E., et al. (2011). Gaucher disease glucocerebrosidase and **α**-synuclein form a bidirectional pathogenic loop in synucleinopathies. *Cell*, *146*(1), 37–52. doi:10.1016/j.cell.2011.06.001

McLean, J. R., Hallett, P. J., Cooper, O., Stanley, M., & Isacson, O. (2011). Transcript expression levels of full-length alpha-synuclein and its three alternatively spliced variants in Parkinson's disease brain regions and in a transgenic mouse model of alpha-synuclein overexpression. *Molecular and cellular neurosciences*, *49*(2), 230–239. doi:10.1016/j.mcn.2011.11.006

Meissner, W. G., Frasier, M., Gasser, T., Goetz, C. G., Lozano, A., Piccini, P., Obeso, J. A., et al. (2011). Priorities in Parkinson's disease research. *Nature reviews. Drug discovery*, *10*(5), 377–393. doi:10.1038/nrd3430

Miller, D. W., Hague, S. M., Clarimon, J., Baptista, M., Gwinn-Hardy, K., Cookson, M. R., & Singleton, A. B. (2004). Alpha-synuclein in blood and brain from familial Parkinson disease with SNCA locus triplication. *Neurology*, *62*(10), 1835–1838.

Minegishi, N., Suzuki, N., Kawatani, Y., Shimizu, R., & Yamamoto, M. (2005). Rapid turnover of GATA-2 via ubiquitin-proteasome protein degradation pathway. *Genes to cells : devoted to molecular & cellular mechanisms*, *10*(7), 693–704. doi:10.1111/j.1365-2443.2005.00864.x

Mittermeyer, G., Christine, C. W., Rosenbluth, K. H., Baker, S. L., Starr, P., Larson, P., Kaplan, P. L., et al. (2012). Long-Term Evaluation of a Phase 1 Study of AADC Gene Therapy for Parkinson's Disease. *Human gene therapy*, *23*(4), 377–381. doi:10.1089/hum.2011.220

Nardelli, J., Thiesson, D., Fujiwara, Y., Tsai, F. Y., & Orkin, S. H. (1999). Expression and genetic interaction of transcription factors GATA-2 and GATA-3 during development of the mouse central nervous system. *Developmental biology*, *210*(2), 305–321. doi:10.1006/dbio.1999.9278

Narendra, D., Tanaka, A., Suen, D. F., & Youle, R. J. (2008). Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *The Journal of cell biology*, *183*(5), 795–803. doi:10.1083/jcb.200809125

Nicklin, S. (2001). Efficient and Selective AAV2-Mediated Gene Transfer Directed to Human Vascular Endothelial Cells. *Molecular Therapy*, *4*(3), 174–181. doi:10.1006/mthe.2001.0424

Nozawa, D., Suzuki, N., Kobayashi-Osaki, M., Pan, X., Engel, J. D., & Yamamoto, M. (2009). GATA2-dependent and region-specific regulation of Gata2 transcription in the mouse midbrain. *Genes to cells : devoted to molecular & cellular mechanisms*, *14*(5), 569–582. doi:10.1111/j.1365-2443.2009.01289.x

Nutt, J. G., & Wooten, G. F. (2005). Clinical practice. Diagnosis and initial management of Parkinson's disease. *The New England journal of medicine*, *353*(10), 1021–1027. doi:10.1056/NEJMcp043908

Ostergaard, P., Simpson, M. A., Connell, F. C., Steward, C. G., Brice, G., Woollard, W. J., Dafou, D., et al. (2011). Mutations in GATA2 cause primary lymphedema associated with a predisposition to acute myeloid leukemia (Emberger syndrome). *Nature genetics*, *43*(10), 929–931. doi:10.1038/ng.923

Paisán-Ruíz, C., Jain, S., Evans, E. W., Gilks, W. P., Simón, J., van der Brug, M., de Munain, A. L., et al. (2004). Cloning of the Gene Containing Mutations that Cause PARK8-Linked Parkinson's Disease. *Neuron*, *44*(4), 595–600. doi:10.1016/j.neuron.2004.10.023

Pals, P., Lincoln, S., Manning, J., Heckman, M., Skipper, L., Hulihan, M., Van den Broeck, M., et al. (2004). Alpha-Synuclein promoter confers susceptibility to Parkinson's disease. *Annals of neurology*, *56*(4), 591–595. doi:10.1002/ana.20268

Park, J., Lee, S. B., Lee, S., Kim, Y., Song, S., Kim, S., Bae, E., et al. (2006). Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. *Nature*, *441*(7097), 1157–1161. doi:10.1038/nature04788

Pascual, A., Hidalgo-Figueroa, M., Piruat, J. I., Pintado, C. O., Gómez-Díaz, R., & López-Barneo, J. (2008). Absolute requirement of GDNF for adult catecholaminergic neuron survival. *Nature neuroscience*, *11*(7), 755–761. doi:10.1038/nn.2136

Pevny, L., Simon, M. C., Robertson, E., Klein, W. H., Tsai, S.-F., D'Agati, V., Orkin, S. H., et al. (1991). Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature*, 1–4.

Pi, W., Zhu, X., Wu, M., Wang, Y., Fulzele, S., Eroglu, A., Ling, J., et al. (2010). Long-range function of an intergenic retrotransposon. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(29), 12992–12997. doi:10.1073/pnas.1004139107

Pizzolato, G., & Mandat, T. (2012). Deep brain stimulation for movement disorders. *Frontiers in Integrative Neuroscience*, 1–5. doi:10.3389/fnint.2012.00002/abstract

Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., et al. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science (New York, N.Y.)*, *276*(5321), 2045–2047.

Priyadarshi, A., Khuder, S. A., Schaub, E. A., & Shrivastava, S. (2000). A meta-analysis of Parkinson's disease and exposure to pesticides. *Neurotoxicology*, *21*(4), 435–440.

Quik, M., O'Leary, K., & Tanner, C. M. (2008). Nicotine and Parkinson's disease: Implications for therapy. *Movement disorders : official journal of the Movement Disorder Society*, *23*(12), 1641–1652. doi:10.1002/mds.21900

Reid, D. M., Perry, V. H., Andersson, P. B., & Gordon, S. (1993). Mitosis and apoptosis of microglia in vivo induced by an anti-CR3 antibody which crosses the blood-brain barrier. *Neuroscience*, *56*(3), 529–533.

Richter, F., Meurers, B. H., Zhu, C., Medvedeva, V. P., & Chesselet, M.-F. (2009). Neurons express hemoglobin alpha- and beta-chains in rat and human brains. *The Journal of comparative neurology*, *515*(5), 538–547. doi:10.1002/cne.22062

Ritz, B., & Yu, F. (2000). Parkinson's disease mortality and pesticide exposure in California 1984-1994. *International journal of epidemiology*, 1–7.

Ross, O. A., Braithwaite, A. T., Skipper, L. M., Kachergus, J., Hulihan, M. M., Middleton, F. A., Nishioka, K., et al. (2008). Genomic investigation of **α**-synuclein multiplication and parkinsonism. *Annals of neurology*, *63*(6), 743–750. doi:10.1002/ana.21380

Rott, R., Szargel, R., Haskin, J., Bandopadhyay, R., Lees, A. J., Shani, V., & Engelender, S. (2011). **α**-Synuclein fate is determined by USP9X-regulated monoubiquitination. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(46), 18666–18671. doi:10.1073/pnas.1105725108

Sanders, L. H., Cooper, O., Mastroberardino, P. G., Hu, X., Dickinson, B. C., Chang, C., Schule, B., et al. (n.d.). Mitochondrial DNA damage: an early biomarker in environmental (rotenone)

and genetic (LRRK2) Parkinson's disease models. *Annals of neurology (submitted)*.
Satake, W., Nakabayashi, Y., Mizuta, I., Hirota, Y., Ito, C., Kubo, M., Kawaguchi, T., et al. (2009). Genome-wide association study identifies common variants at four loci as genetic risk factors for Parkinson's disease. *Nature genetics*, *41*(12), 1303–1307. doi:10.1038/ng.485
Savitt, J. M., Dawson, V. L., & Dawson, T. M. (2006). Diagnosis and treatment of Parkinson disease: molecules to medicine. *The Journal of clinical investigation*, *116*(7), 1744–1754. doi:10.1172/JCI29178
Saxena, S., & Caroni, P. (2011). Selective Neuronal Vulnerability in Neurodegenerative Diseases: from Stressor Thresholds to Degeneration. *Neuron*, *71*(1), 35–48. doi:10.1016/j.neuron.2011.06.031
Scherzer, C. R. (2009). Chipping away at diagnostics for neurodegenerative diseases. *Neurobiology of disease*, *35*(2), 148–156. doi:10.1016/j.nbd.2009.02.016
Scherzer, C. R., Grass, J. A., Liao, Z., Pepivani, I., Zheng, B., Eklund, A. C., Ney, P. A., et al. (2008). GATA transcription factors directly regulate the Parkinson's disease-linked gene alpha-synuclein. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(31), 10907–10912. doi:10.1073/pnas.0802437105
Shimahara, A., Yamakawa, N., Nishikata, I., & Morishita, K. (2010). Acetylation of lysine 564 adjacent to the C-terminal binding protein-binding motif in EVI1 is crucial for transcriptional activation of GATA2. *The Journal of biological chemistry*, *285*(22), 16967–16977. doi:10.1074/jbc.M110.102046
Shirihai, O. S., Gregory, T., Yu, C., Orkin, S. H., & Weiss, M. J. (2000). ABC-me: a novel mitochondrial transporter induced by GATA-1 during erythroid differentiation. *The EMBO journal*, *19*(11), 2492–2502. doi:10.1093/emboj/19.11.2492
Sian-Hülsmann, J., Mandel, S., Youdim, M. B. H., & Riederer, P. (2011). The relevance of iron in the pathogenesis of Parkinson's disease. *Journal of Neurochemistry*, *118*(6), 939–957. doi:10.1111/j.1471-4159.2010.07132.x
Simón-Sánchez, J., Schulte, C., Bras, J. M., Sharma, M., Gibbs, J. R., Berg, D., Paisan-Ruiz, C., et al. (2009). Genome-wide association study reveals genetic risk underlying Parkinson's disease. *Nature genetics*, *41*(12), 1308–1312. doi:10.1038/ng.487
Singleton, A. B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., et al. (2003). alpha-Synuclein locus triplication causes Parkinson's disease. *Science (New York, N.Y.)*, *302*(5646), 841. doi:10.1126/science.1090278
Snow, J. W., Trowbridge, J. J., Johnson, K. D., Fujiwara, T., Emambokus, N. E., Grass, J. A., Orkin, S. H., et al. (2011). Context-dependent function of “GATA switch” sites in vivo. *Blood*. doi:10.1182/blood-2010-10-313031
Sohal, R. S., & Orr, W. C. (2012). The redox stress hypothesis of aging. *Free radical biology & medicine*, *52*(3), 539–555. doi:10.1016/j.freeradbiomed.2011.10.445
Spillantini, M. G., Schmidt, M. L., Lee, V. M., Trojanowski, J. Q., Jakes, R., & Goedert, M. (1997). Alpha-synuclein in Lewy bodies. *Nature*, *388*(6645), 839–840. doi:10.1038/42166
Surinya, K. H., Cox, T. C., & May, B. K. (1997). Transcriptional regulation of the human erythroid 5-aminolevulinate synthase gene. Identification of promoter elements and role of regulatory proteins. *The Journal of biological chemistry*, *272*(42), 26585–26594.
Tanner, C. M., Kamel, F., Ross, G. W., Hoppin, J. A., Goldman, S. M., Korell, M., Marras, C., et al. (2011). Rotenone, Paraquat, and Parkinson’s Disease. *Environmental health perspectives*, *119*(6), 866–872. doi:10.1289/ehp.1002839

Thomas, B. (2009). Parkinson's disease: from molecular pathways in disease to therapeutic approaches. *Antioxidants & redox signaling*, *11*(9), 2077–2082. doi:10.1089/ars.2009.2697

Thomas, B., & Beal, M. F. (2007). Parkinson's disease. *Human molecular genetics*, *16 Spec No. 2*, R183–94. doi:10.1093/hmg/ddm159

Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W., et al. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature*, *371*(6494), 221–226. doi:10.1038/371221a0

Tsang, A. H. K., & Chung, K. K. K. (2009). Oxidative and nitrosative stress in Parkinson's disease. *Biochimica et biophysica acta*, *1792*(7), 643–650. doi:10.1016/j.bbadis.2008.12.006

Tsarovina, K., Pattyn, A., Stubbusch, J., Müller, F., van der Wees, J., Schneider, C., Brunet, J.-F., et al. (2004). Essential role of Gata transcription factors in sympathetic neuron development. *Development (Cambridge, England)*, *131*(19), 4775–4786. doi:10.1242/dev.01370

Valente, E. M., Abou-Sleiman, P. M., Caputo, V., Muqit, M. M. K., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., et al. (2004a). Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science (New York, N.Y.)*, *304*(5674), 1158–1160. doi:10.1126/science.1096284

Valente, E. M., Salvi, S., Ialongo, T., Marongiu, R., Elia, A. E., Caputo, V., Romito, L., Albanese, A., et al. (2004b). PINK1 mutations are associated with sporadic early-onset parkinsonism. *Annals of neurology*, *56*(3), 336–341. doi:10.1002/ana.20256

Van Laar, V. S., Arnold, B., Cassady, S. J., Chu, C. T., Burton, E. A., & Berman, S. B. (2011). Bioenergetics of neurons inhibit the translocation response of Parkin following rapid mitochondrial depolarization. *Human molecular genetics*, *20*(5), 927–940. doi:10.1093/hmg/ddq531

van Rooden, S. M., Colas, F., Martínez-Martín, P., Visser, M., Verbaan, D., Marinus, J., Chaudhuri, R. K., et al. (2011). Clinical subtypes of Parkinson's disease. *Movement disorders : official journal of the Movement Disorder Society*, *26*(1), 51–58. doi:10.1002/mds.23346

Vekrellis, K., Xilouri, M., Emmanouilidou, E., Rideout, H. J., & Stefanis, L. (2011). Pathological roles of α-synuclein in neurological disorders. *Lancet neurology*, *10*(11), 1015–1025. doi:10.1016/S1474-4422(11)70213-7

Viger, R. S., Guittot, S. M., Anttonen, M., Wilson, D. B., & Heikinheimo, M. (2008). Role of the GATA Family of Transcription Factors in Endocrine Development, Function, and Disease. *Molecular endocrinology (Baltimore, Md.)*, *22*(4), 781–798. doi:10.1210/me.2007-0513

Volpicelli-Daley, L. A., Luk, K. C., Patel, T. P., Tanik, S. A., Riddle, D. M., Stieber, A., Meaney, D. F., et al. (2011). Exogenous α-synuclein fibrils induce Lewy body pathology leading to synaptic dysfunction and neuron death. *Neuron*, *72*(1), 57–71. doi:10.1016/j.neuron.2011.08.033

Wallach, I., Zhang, J., Hartmann, A., van Landeghem, F. K. H., Ivanova, A., Klar, M., & Dame, C. (2009). Erythropoietin-receptor gene regulation in neuronal cells. *Pediatric research*, *65*(6), 619–624. doi:10.1203/PDR.0b013e31819ea3b8

West, A. B., Moore, D. J., Biskup, S., Bugayenko, A., Smith, W. W., Ross, C. A., Dawson, V. L., et al. (2005). Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. *Proc Natl Acad Sci USA*, 1–6.

Westerlund, M., Hoffer, B., & Olson, L. (2010). Parkinson's disease: Exit toxins, enter genetics.

Progress in neurobiology, *90*(2), 146–156. doi:10.1016/j.pneurobio.2009.11.001
Willett, R. T., & Greene, L. A. (2011). Gata2 Is Required for Migration and Differentiation of Retinorecipient Neurons in the Superior Colliculus. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *31*(12), 4444–4455. doi:10.1523/JNEUROSCI.4616-10.2011
Wozniak, R. J., Keles, S., Lugus, J. J., Young, K. H., Boyer, M. E., Tran, T. M., Choi, K., et al. (2008). Molecular Hallmarks of Endogenous Chromatin Complexes Containing Master Regulators of Hematopoiesis. *Molecular and cellular biology*, *28*(21), 6681–6694. doi:10.1128/MCB.01061-08
Xilouri, M., Vogiatzi, T., Vekrellis, K., Park, D., & Stefanis, L. (2009). Abberant **α**-Synuclein Confers Toxicity to Neurons in Part through Inhibition of Chaperone-Mediated Autophagy. (H. E. Gendelman, Ed.)*PloS one*, *4*(5), e5515. doi:10.1371/journal.pone.0005515.g009
Yacoubian, T. A., & Standaert, D. G. (2009). Targets for neuroprotection in Parkinson's disease. *Biochimica et biophysica acta*, *1792*(7), 676–687. doi:10.1016/j.bbadis.2008.09.009
Youle, R. J., & Narendra, D. P. (2011). Mechanisms of mitophagy. *Nature Reviews Molecular Cell Biology*, *12*(1), 9–14. doi:10.1038/nrm3028
Zhao, G.-Y., Li, Z.-Y., Zou, H.-L., Hu, Z.-L., Song, N.-N., Zheng, M.-H., Su, C.-J., et al. (2008). Expression of the transcription factor GATA3 in the postnatal mouse central nervous system. *Neuroscience research*, *61*(4), 420–428. doi:10.1016/j.neures.2008.04.014
Zhou, Y., Lim, K.-C., Onodera, K., Takahashi, S., Ohta, J., Minegishi, N., Tsai, F. Y., et al. (1998). Rescue of the embryonic lethal hematopoietic defect reveals a critical role for GATA-2 in urogenital development. *The EMBO journal*, 1–12.
Zimprich, A., Biskup, S., Leitner, P., Lichtner, P., Farrer, M., Lincoln, S., Kachergus, J., et al. (2004). Mutations in LRRK2 Cause Autosomal-Dominant Parkinsonism with Pleomorphic Pathology. *Neuron*, *44*(4), 601–607. doi:10.1016/j.neuron.2004.11.005