Developing Gene Therapy Methods to Inhibit a BAG Family Co-Chaperone Protein in Rat Substantia Nigra

by

Stanley Li

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Laboratory Medicine & Pathobiology
University of Toronto

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Abstract

Parkinson’s disease (PD) is a progressive neurodegenerative disorder affecting dopaminergic neurons in the substantia nigra (SN). Bcl2-associated athanogene 5 (BAG5) is a co-chaperone that promotes dopaminergic cell death in animal models of PD and dopaminergic degeneration. BAG5 also inhibits Hsp70 and parkin, which are protective in animal models of PD, and interacts with several other proteins implicated in PD pathogenesis. Thus, BAG5 represents a promising therapeutic target for PD. This thesis describes the development of a viral vector that can deliver shRNA against BAG5 to rat SN. We identified shRNAs that efficiently knocked down BAG5 in two cell lines and packaged them into lentivirus (LV) and adeno-associated virus (AAV) 1/2 vectors. AAV-shBAG5 demonstrated superior expression in SN compared to LV. In rat SN, AAV-shBAG5 produced significant BAG5 knockdown and protected neurons from axotomy injury. This work provides the foundation for future studies of BAG5 knockdown in rat models of PD.
Acknowledgments

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- My parents and family - For their unwavering support and encouragement of my studies in science.
Contributions

Hien Chau - helped perform AAV injection and MFBx surgeries; contributed to Figs. 2, 3, 5, and 11

Drs. Darius Ebrahimi-Fakhari & Mustafa Sahin - contributed to Fig. 5

Christopher Lozano - contributed to Fig. 7

Erik Friesen - contributed to Fig. 11

Mitch de Snoo - helped perform AAV injection surgeries

Drs. Ornella Pellerito & Sherri Thiele - helped perform lentivirus injection surgeries
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<tbody>
<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>AP</td>
<td>anteroposterior</td>
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<tr>
<td>ASO</td>
<td>antisense oligonucleotide</td>
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<tr>
<td>BAG5</td>
<td>Bcl2-associated athanogene 5</td>
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<tr>
<td>BGH</td>
<td>bovine growth hormone</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>CBA</td>
<td>chicken beta actin</td>
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<tr>
<td>CHIP</td>
<td>Carboxyl-terminus of Hsp70 interacting protein</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CRISPR</td>
<td>clustered regularly interspaced short palindromic repeats</td>
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<tr>
<td>DIV</td>
<td>days in vitro</td>
</tr>
<tr>
<td>DSB</td>
<td>double stranded break</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double-stranded RNA</td>
</tr>
<tr>
<td>DV</td>
<td>dorsoventral</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-Diaminobenzidine</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>GDNF</td>
<td>glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>gp/mL</td>
<td>genomic particles per milliliter</td>
</tr>
<tr>
<td>HDR</td>
<td>homology-directed repair</td>
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<tr>
<td>IRES</td>
<td>internal ribosomal entry site</td>
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<tr>
<td>LRRK2</td>
<td>Leucine-rich repeat kinase 2</td>
</tr>
<tr>
<td>LV</td>
<td>lentivirus</td>
</tr>
<tr>
<td>MFB</td>
<td>medial forebrain bundle</td>
</tr>
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<td>MFBx</td>
<td>medial forebrain bundle axotomy</td>
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<tr>
<td>miRNA</td>
<td>micro RNA</td>
</tr>
<tr>
<td>ML</td>
<td>mediolateral</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
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<td>NHEJ</td>
<td>non-homologous end joining</td>
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<td>phosphate buffered saline</td>
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<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethyleneimine</td>
</tr>
<tr>
<td>PGK</td>
<td>phosphoglycerate kinase</td>
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<td>PTEN-induced putative kinase 1</td>
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<td>precursor mRNA</td>
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<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
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<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>ROI</td>
<td>region of interest</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<tr>
<td>SN</td>
<td>substantia nigra</td>
</tr>
<tr>
<td>SNc</td>
<td>substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNr</td>
<td>substantia nigra pars reticulata</td>
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<tr>
<td>SSB</td>
<td>single stranded break</td>
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<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single-stranded RNA</td>
</tr>
<tr>
<td>TALE</td>
<td>transcription activator-like effector</td>
</tr>
<tr>
<td>TALEN</td>
<td>transcription activator-like effector nuclease</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
<tr>
<td>WPRE</td>
<td>woodchuck post-transcriptional regulatory element</td>
</tr>
<tr>
<td>ZFN</td>
<td>zinc finger nuclease</td>
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<tr>
<td>αSyn</td>
<td>alpha synuclein</td>
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Introduction

0.1 Parkinson’s disease overview

Parkinson’s disease (PD) is a common and progressive neurodegenerative disorder that affects 1% of people over age 60\(^1\). With the aging population and increasing life expectancy, PD is expected to become an even greater public health issue in the near future\(^2\).

PD is associated with the misfolding and aggregation of \(\alpha\)-synuclein (\(\alpha\)Syn) and the accompanying loss of dopaminergic neurons in the substantia nigra pars compacta (SNc). After a majority of these neurons are lost, motor symptoms including tremor, rigidity, bradykinesia, and postural instability begin to manifest. PD patients often also experience a variety of other non-motor symptoms, which include sleep disturbances, impulse control issues, and hallucinations\(^2,3\).

Most cases of PD are of unclear etiology (sporadic), but about 10% of cases are the result of mutations in specific genes. Autosomal dominant PD can be caused by mutations in SNCA (which encodes \(\alpha\)Syn), LRRK2, VPS35, EIF4G1, DNAJC13, CHCHD2, and the recently discovered TMEM230\(^2,4\). Parkin, PINK1, and DJ-1 are responsible for autosomal recessive PD. The identification of these genes has shed light on the molecular events and systems involved in PD pathogenesis, which include protein aggregation, the ubiquitin-proteasome system, the lysosome-autophagy pathway, mitophagy, and synaptic function\(^2\).

Although dopamine replacement drugs and deep-brain stimulation can effectively treat many of the motor symptoms, they do not change the progression of the disease\(^2,5\). Thus, the development of disease-modifying and neuroprotective therapeutics remains an important goal of PD research\(^2,5\).
0.2 Gene therapy for Parkinson’s disease

Gene therapy is a therapeutic technique in which nucleic acids are introduced into cells in vivo to treat or prevent disease. It can be used to replace a mutated gene in monogenic disorders, add gene(s) to help treat complex disorders, or modify endogenous gene expression by targeting RNA.

Gene therapy is a promising therapeutic strategy for PD. One gene therapy strategy that has been explored in animal models of PD is to drive increased expression of neuroprotective proteins. Many of these studies have reported favourable effects. Gene transfer of Hsp70 into SN was found to protect dopaminergic neurons in mice treated with MPTP, a toxin that selectively kills dopaminergic neurons. Rocha et al found that glucocerebrosidase overexpression in SN promoted clearance of αSyn aggregates and preserved dopaminergic neurons in both mice and rats overexpressing human αSyn. Promotion of the expression of growth factors such as vascular endothelial growth factor, glial cell-line derived neurotrophic growth factor (GDNF), and neurturin (a GDNF family member), has also demonstrated protection of dopaminergic neurons in SN in toxin-based animal models.

A different gene therapy strategy of reducing expression of harmful proteins using shRNA has been investigated as well. In rat models of PD, targets for silencing have included αSyn, ROCK2, and interleukin-1 receptor. One study using a human αSyn overexpression model reported that knockdown of human αSyn rescued forelimb deficit, but also led to enhanced dopaminergic cell death, possibly due to chronic shRNA (or GFP) expression. Another study found that knockdown of endogenous rat αSyn in the rotenone model of PD preserved dopaminergic neurons and rescued forelimb deficit, without causing toxicity. Saal et al experimented with toxin-based models of PD and reported that shRNA against ROCK2...
protected midbrain neurons, upregulated pro-survival markers, and improved motor behaviour\textsuperscript{14}. However, interleukin-1 receptor knockdown failed to demonstrate any therapeutic effect in the 6-hydroxydopamine model of PD\textsuperscript{15}.

Results from human clinical trials of gene therapy for PD are just beginning to emerge. These trials have focused on expressing neurotrophic factors or neurotransmitter synthesis enzymes, as the complex etiology of PD makes it difficult to pinpoint one defective gene to target\textsuperscript{16}. One study used AAV2 vector to bilaterally deliver glutamate decarboxylase I to the subthalamic nucleus. It was found to be a safe procedure and resulted in a significant improvement in clinical motor evaluations at 6 months after treatment\textsuperscript{17}. A second clinical trial used AAV2 to deliver neurturin to the putamen bilaterally. An initial group of 12 patients at one-year follow-up demonstrated safety and some motor improvement\textsuperscript{18}. A second group of patients showed no significant improvement at 12 months post-treatment, but mild improvement at 18 months\textsuperscript{19}. When the brains of four patients were analyzed post-mortem, very little neurturin expression was detected in SN, thus highlighting a challenge of delivering gene therapy to a structure that may be severely degenerated by the time treatment is initiated\textsuperscript{20}. In an attempt to improve expression in SN, additional patients were injected in both putamen and SN, but failed to demonstrate motor improvement after 2 years\textsuperscript{19}. A third study is currently recruiting patients with advanced PD. It will aim to deliver GDNF bilaterally to the putamen using AAV2 and follow up the patients for 5 years\textsuperscript{21}.

0.3 Medial forebrain bundle axotomy

The medial forebrain bundle (MFB) is an anatomical structure containing several different axon tracts, and in rat includes dopaminergic axons projecting from the SNC to the striatum\textsuperscript{22}. Medial forebrain bundle axotomy (MFBx) is a surgical procedure in which the axons
of the medial forebrain bundle are mechanically severed, resulting in retrograde degeneration of dopaminergic neurons in SNc\textsuperscript{23}. Weiser \textit{et al} reported that, at seven days following unilateral MFBx, the ipsilateral (transected side) SNC displayed 68\% tyrosine hydroxylase (TH) immunoreactivity compared to the contralateral control\textsuperscript{24}. A similar study by Brecknell \textit{et al} reported a similar figure of 72\%, again at 7 days post-MFBx\textsuperscript{25}.

In the past, MFBx has been used to model PD in rats because it results in cell death of the same cell population affected in PD\textsuperscript{26-28}. Recently however, αSyn-overexpression models have gained popularity, as they are believed to better represent some of the molecular features of PD (i.e. presence of human αSyn)\textsuperscript{29-33}. Nonetheless, MFBx remains a useful model of dopaminergic cell death.

0.4 BAG proteins

The Bcl2-associated athanogene (BAG) family is a group of co-chaperone proteins that function in cell growth and programmed cell death pathways\textsuperscript{34,35}. The name comes from the discovery of the first BAG protein, BAG1, which binds to B-cell lymphoma/leukemia 2 gene (Bcl2). Bcl2 itself promotes cell survival and has been found to increase neuronal survival, though its over-activation due to chromosomal translocations gives rise to cancer.

Six BAG proteins exist in humans: BAG1 through BAG6\textsuperscript{34,35}. Each BAG protein contains one evolutionarily conserved 78-amino acid BAG domain near the C-terminus\textsuperscript{36}, except BAG5, which has four\textsuperscript{34,35,36} or possibly five\textsuperscript{37-39} BAG domains spread along its length\textsuperscript{34,35}. BAG proteins are nucleotide exchange factors for the Hsp70/Hsc70 family of molecular chaperones, and BAG domains mediate direct interaction with the ATPase domain of Hsp70/Hsc70\textsuperscript{34,36}. Several other domains are also found within the different BAG proteins, allowing them to interact with specific proteins or to be targeted to specific intracellular locales\textsuperscript{34,35}. 

4
There are four isoforms of BAG1 in humans: BAG1L, BAG1M, BAG1, and BAG1S, in order from longest to shortest\textsuperscript{36}. Among the isoforms, BAG1L is the only one to have a nuclear localization signal at the N-terminus, so it is most likely found in the nucleus. BAG1 binding to Hsc/Hsp70 stimulates nucleotide exchange, thus promoting chaperone activity. Each isoform contains one ubiquitin-like domain that allows it to bring Hsc/Hsp70 chaperones to the proteasome, suggesting a role for BAG1 in protein degradation\textsuperscript{36}. BAG1 double knockout mice were found to have dramatic apoptosis of the fetal liver and nervous system\textsuperscript{40}. Recently, BAG1 overexpression in the red nucleus was found to protect neurons from dorsal hemisection injury\textsuperscript{41}. Thus, there is strong evidence for an anti-apoptotic role of BAG1.

BAG2 is overexpressed in some cancers and may promote tumour formation by promoting the accumulation of other oncogenic mutations\textsuperscript{42}. However, BAG2 may play a different role in other contexts such as PD. In HEK293 cells, BAG2 inhibits C-terminus of Hsp70 interacting protein (CHIP), which normally ubiquitylates αSyn, PINK1, and other proteins for degradation\textsuperscript{43,44}. In this fashion, BAG2 stabilizes PINK1 and promotes mitophagy\textsuperscript{43}. Although it inhibits CHIP-mediated ubiquitylation, BAG2 was found to promote ubiquitin-independent Tau degradation\textsuperscript{45}.

BAG3 expression is induced by oxidative and proteasome stress. BAG3 knockout mice exhibit post-natal development issues, especially in muscle tissue, and die prematurely\textsuperscript{46}. In various cancer cell lines, BAG3 expression has been found to promote proliferation and enhance resistance to anti-cancer drugs\textsuperscript{47,48}. Recently, BAG3 was found to be important in autophagy pathways. As human fibroblast cells aged, they switched from predominantly BAG1 to BAG3 expression, corresponding to a switch from proteasome to selective macroautophagy based protein degradation\textsuperscript{49}. 
BAG4 is also called “silencer of death domain” and is well studied in cancer. It binds and inhibits “death domains” found in some tumour necrosis factor receptors, thus promoting cancer cell survival against radiation and anti-cancer drugs\textsuperscript{50,51}.

BAG6 may be unique amongst the BAG proteins in that its BAG domain may lack nucleotide exchange factor activity for Hsc70. Instead its BAG domain may help BAG6 form a complex that regulates membrane protein degradation\textsuperscript{52}. BAG6 inhibits Hsp70 protein refolding activity and appears to regulate Hsp70 induction after heat shock\textsuperscript{53}. It has been implicated in lung cancer and multiple sclerosis\textsuperscript{54,55}.

Unlike most other BAG proteins, several studies have suggested a pro-apoptotic function for BAG5. It has been shown to directly interact with several molecules associated with PD pathogenesis and it promotes dopaminergic cell death in two animal models of PD/dopaminergic injury\textsuperscript{56}. However, one recent study showed that BAG5 stabilized PINK1 by inhibiting its ubiquitylation in HEK293 cells, and also protected mitochondria from oxidative stress by upregulating PINK1\textsuperscript{57}. Nonetheless, another study also showed that BAG5 and BAG2 stabilized pathogenic ataxin proteins by inhibiting their ubiquitylation, providing further evidence of the role of BAG proteins in neurodegeneration\textsuperscript{42}. BAG5 may play a different role in cancer, where it was identified as a risk factor for non-Hodgkin’s lymphoma\textsuperscript{58}. In prostate cancer patients, BAG5 was found to be overexpressed and inhibit ER-stress induced apoptosis\textsuperscript{38}.

0.5 Rationale for targeting BAG5

BAG5 was originally discovered as a protein whose expression was upregulated in SNC at 24 hours following dopaminergic neuron injury, via medial forebrain bundle axotomy (MFBx), in rat\textsuperscript{56}. Adenovirus-mediated overexpression of BAG5 in rat SN significantly enhanced loss of TH immunoreactivity at 7 days post-MFBx\textsuperscript{56}, suggesting a positive feedback
mechanism. Similarly, BAG5 overexpression in the MPTP mouse model of PD resulted in greater amounts of TH loss at 2 weeks post-MPTP treatment, compared to controls\textsuperscript{56}. These animal experiments suggested that BAG5 could be a mediator of dopaminergic cell death.

BAG5 associates with several proteins that are mutated in familial forms of PD, including parkin\textsuperscript{56,59}, PINK1\textsuperscript{57}, and LRRK2\textsuperscript{60}. BAG5 directly interacts with parkin, sequesters it into protein aggregates, and inhibits its E3 ligase activity, which is necessary for the controlled removal of damaged mitochondria (mitophagy)\textsuperscript{56,59}. In addition, BAG5 forms a complex with the co-chaperone CHIP and αSyn, resulting in the inhibition of CHIP-mediated ubiquitylation of αSyn, thus leading to increased αSyn oligomerization\textsuperscript{61}. Furthermore, BAG5 has been identified as an interactor of LRRK2, though the functional consequences are unclear\textsuperscript{60,62} (also see Fig. 1).

By inhibiting Hsp70 chaperone function\textsuperscript{56} and inhibiting the E3 ligase activity of parkin\textsuperscript{56,59} and CHIP\textsuperscript{61}, BAG5 negatively affects the molecular chaperone and ubiquitin-proteasome systems, as well as the control of mitophagy, which are considered to be protective in the contexts of PD and neurodegeneration\textsuperscript{63-66}. Taken together with its role in mediating dopaminergic cell death \textit{in vivo}, BAG5 seems to be a common element in several pathways relevant to PD, and thus represents a promising therapeutic target.
Figure 1. BAG5 interactors and their involvement in pathways relevant to the pathogenesis of Parkinson’s disease. BAG5 directly interacts with Hsp70, parkin, PINK1, and LRRK2. It inhibits the chaperone activity of Hsp70 and also inhibits the E3 ligase activity of parkin\textsuperscript{56}. Through its interaction with Hsp70, BAG5 inhibits the E3 ligase activity of CHIP\textsuperscript{61}. BAG5 also stabilizes PINK1 by inhibiting its ubiquitylation\textsuperscript{57}. Finally, BAG5 is a director interactor of LRRK2, though the functional consequences of this interaction are unclear\textsuperscript{60}. Also depicted: PINK1 interacts with parkin to control mitophagy. CHIP ubiquitylates αSyn and reduces its oligomerization (though this effect is inhibited by BAG5, through Hsp70)\textsuperscript{61}. Molecular chaperones (such as Hsp70) refold misfolded proteins (such as αSyn) and prevent their aggregation.
0.6 RNA-targeting strategies for gene inhibition therapy

One approach to inhibiting gene expression in a biological system is to target mRNA transcripts before they can be translated into protein. With this approach, since the gene itself is not targeted, the risk of chromosomal mutation/damage is avoided, and inhibition is reversible. The main technologies for RNA-targeting gene inhibition are short hairpin and small interfering RNAs (via the endogenous RNA interference pathway) and antisense oligonucleotides.

0.6.1 RNA interference

RNA interference (RNAi) is a naturally occurring pathway that modulates gene expression through small RNA molecules, including the endogenous micro RNAs (miRNA)\textsuperscript{67}. The RNAi machinery can be exploited to reduce expression of a specific target gene by adding exogenous small RNAs, either in the form of short hairpin RNA (shRNA) or small interfering RNA (siRNA).

Short hairpin RNAs consist of a 19-29 base pair stem region that is linked by a single-stranded RNA loop of 4 or more nucleotides, plus a 2-nucleotide 3’ overhang\textsuperscript{68}. In the RNAi pathway, shRNAs are processed by two ribonucleases, Drosha and Dicer. Then, from the remaining 19-29 bp double-stranded RNA molecule (dsRNA) the sense (or passenger) strand is degraded and the antisense (or guide) strand is loaded into the RNA-induced silencing complex (RISC)\textsuperscript{68}. The guide strand directs RISC to bind and cleave mRNA transcripts that are fully complementary to the loaded guide strand. The cleaving is performed by the endonuclease Argonaute, a component of RISC\textsuperscript{67,68}. The similar siRNAs are artificial short dsRNA molecules designed to enter the RNAi pathway at the post-Dicer processing point and subsequently reduce expression of a target protein.
siRNAs are well suited for transient knockdown conditions in vitro because it is relatively cheap to synthesize and transfec siRNAs into cultured cells. For in vivo applications, siRNAs can struggle to avoid degradation and enter cells, and they must be constantly resupplied as they are (inefficiently) consumed⁶⁹. Viral vector-delivered shRNA can be a more sustainable strategy in the long term, as it provides long-lasting silencing of a target gene, and the viral vector allows for more efficient and controlled delivery.

0.6.2 Antisense oligonucleotides

Antisense oligonucleotides (ASO) are synthetic nucleic acids, 8-50 nucleotides in length, which are designed to bind to complementary RNA in order to alter gene expression⁷⁰. ASOs may operate through a variety of mechanisms, which may or may not lead to degradation of the target RNA. Unlike RNAi, ASOs can also target precursor mRNA (pre-mRNA) in the nucleus, before it has been exported and matured into mRNA. For instance, ASOs can bind near a splice site in pre-mRNA, causing exons to be skipped or included. They may also bind close to the 3’ end of the pre-mRNA and modulate polyadenylation⁷⁰,⁷¹. ASO binding on mature mRNA can lead to translation inhibition, and binding on either pre-mRNA or mature mRNA can recruit various nucleases that lead to transcript degradation⁷⁰,⁷¹.

One of the challenges facing in vivo use of ASOs is the natural instability of unmodified nucleic acids, due to the ubiquitous expression of nucleases. The chemical structure of ASOs may be modified in several different ways in order to improve nuclease stability⁷¹,⁷². Some modifications target the phosphate group in the backbone (swapping it for a sulfur-containing group) or the ribose/deoxyribose sugar (tagging it with additional functional groups or fusing it to a second ring that restricts conformation)⁷¹,⁷². A more extreme possibility is to produce nucleic acid mimics. These mimics can be either morpholinos (in which pentose sugars are replaced by
morpholine rings) or peptide nucleic acids (in which the sugar-phosphate backbone is replaced by a polypeptide backbone)\textsuperscript{70-72}. Another challenge is to deliver ASOs to the target cells in the body. This is usually attempted by attaching the ASOs to a ligand of, or antibody against, a target cell-surface receptor to allow the ASO to enter via receptor-mediated endocytosis\textsuperscript{70}.

0.7 DNA-targeting strategies for gene inhibition therapy

Rather than targeting RNA transcripts, genes themselves can be targeted in order to delete or silence the target gene, or to replace the target gene with an exogenously supplied DNA sequence. These alterations to the genome are mostly irreversible. The main technologies for gene editing are either recombinases (Cre/loxP system), or customizable nucleases (TALENs, zinc finger nucleases, CRISPR/Cas system) followed by endogenous DNA repair mechanisms.

0.7.1 Cre/loxP recombination

The Cre/loxP system, derived from machinery in P1 bacteriophage, uses Cre recombinase to create a targeted recombination event between two 34-bp loxP sites in the genome\textsuperscript{73}. Depending on the positions and directionality of the loxP sites, the recombination event may result in deletion or inversion of the interspacing DNA, or translocation (if the loxP sites are on different DNA molecules)\textsuperscript{74,75}. Several variations on the Cre/loxP setup make it quite flexible. Target genes can be silenced or overexpressed, either in the presence or absence of Cre expression. Genes can also be knocked in when Cre is present, via translocation or inversions. Additionally, Cre expression can be made inducible or tissue-specific by placing it under the control of various promoters, or it can be supplied via viral vectors\textsuperscript{74}. The major disadvantage of the Cre/loxP system is that it requires loxP sites to be inserted at the desired locations, resulting in relatively long turnaround times as cell lines or transgenic organisms (usually mice) are prepared\textsuperscript{74}. In addition, it is difficult to accurately achieve multiple Cre/loxP recombination
events in the same cell since there would be many possible combinations of pairings between multiple loxP sites.

The FLP-FRT recombination system, derived from machinery in yeast, can also produce targeted recombination events. It is conceptually similar to Cre/loxP\textsuperscript{76}.

0.7.2 TALEN

Transcription Activator-Like Effector (TALE) proteins are DNA-binding proteins found in several bacterial species. TALEs can be reprogrammed to bind any DNA sequence, as their binding specificity is conferred by just two amino acids in each DNA binding domain (DBD)\textsuperscript{67}. Customized TALEs can be fused to effector domains such as transcriptional repressors or endonucleases (creating TALE nucleases, or TALENs) to precisely direct the activity of the fused proteins. A particularly useful TALEN is the fusion of TALEs to FokI endonuclease, which functions only as a dimer and has separate binding and cleavage sites\textsuperscript{67,77}. The combination of two different TALEs that target adjacent sites on DNA can bring together two FokI to create a double-stranded break (DSB) at a highly specific target sequence\textsuperscript{67,77}.

DSBs are repaired with one of two endogenous and competing repair mechanisms: non-homologous end joining (NHEJ) or homology directed repair (HDR). NHEJ is an error-prone repair mechanism, resulting in frameshift (functional deletion) 2/3 of the time, and potential insertions/deletions the other 1/3 of the time. Thus it can result in genetic and phenotypic heterogeneity, and the outcome may be difficult to predict\textsuperscript{67}. On the other hand, HDR is a high fidelity repair system, but requires a template that shares homology with the area around the DSB. This template may be the other allele or an exogenously supplied homologous donor DNA sequence. In the latter case, a precise sequence can be inserted to bridge the DSB. While
TALENs are very specific with low off-target effects, it is expensive and time-consuming to design TALEs for each custom target\textsuperscript{78}.

**0.7.3 Zinc finger nucleases**

Zinc finger nucleases (ZFNs) are artificial restriction enzymes, formed by fusing eukaryotic transcription factors with a Cys2-His2 zinc-finger DNA binding domain and FokI\textsuperscript{67}. These DBDs bind DNA three nucleotides apart, and can be placed in tandem to confer better specificity for a target DNA sequence\textsuperscript{79}. Otherwise, ZFNs are conceptually similar to TALENs, though generally more difficult to program and less specific\textsuperscript{78}.

With either ZFNs or TALENs, one of the FokI domains can be mutated to inactivate its endonuclease activity. This creates a TALEN or ZFN “nickase” that generates a single-stranded break (SSB) at the target location\textsuperscript{67,77}. SSBs are repaired by HDR and not NHEJ, thus avoiding some of the challenges with NHEJ such as unintentional mutations and increased risk of apoptosis\textsuperscript{77}.

**0.7.4 CRISPR/Cas**

CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR associated protein) is a system found in the genome of bacteria and archaea that provides adaptive immunity against foreign nucleic acids\textsuperscript{80}. Unlike the protein domain-based binding of TALENs and ZFNs, the CRISPR/Cas system uses a guide RNA to target the nuclease to the target DNA site. It therefore has the major advantage of being easier to program to target custom DNA sequences\textsuperscript{78,81}.

The Cas9 endonuclease is most commonly used for CRISPR gene editing\textsuperscript{81}. It contains two nuclease domains and functions as a monomer, causing a DSB at the target location. Though
ZFNs and TALENs are more specific than normal Cas9\(^67\), Cas9 specificity can be improved in a few ways. For instance, one of its nuclease domains can be inactivated, producing a Cas9 nickase that may be paired with a second Cas9 nickase. Alternatively, both of Cas9’s nuclease domains can be inactivated to produce dCas9, which can then be fused to FokI domains. dCas9 can also be fused to transcriptional activators or repressors for additional control of gene expression\(^81,82\).

### 0.8 Viral vectors for gene delivery

In recent years, many viruses have been modified for *in vivo* gene therapy applications, including lentivirus and adeno-associated virus (AAV)\(^83\). Compared to physical or chemical delivery methods, viral vectors provide superior transduction efficiency, allow for better targeting, and naturally protect the gene product from nuclease degradation\(^83\). However, viral vectors have a genomic size limit, may induce an immune response, and can be costly.

#### 0.8.1 Lentivirus

Lentivirus (LV) is an enveloped ssRNA virus that has the unique ability among retroviruses to integrate into non-dividing cells such as neurons. It has strong tropism (i.e. tendency/ability to transduce) toward neural stem cells\(^83\). Lentivirus tropism can be modified by pseudotyping, a process in which lentivirus particles are generated with glycoproteins from other enveloped viruses. One of the most popular lentivirus pseudotypes uses vesicular stomatitis virus glycoprotein (VSV-G), resulting in very broad tropism, including neurons\(^84\). Lentivirus has the advantage of being able to deliver a relatively larger genomic payload of 8kb of RNA to infected cells\(^83\). These features of lentivirus, combined with its low immunogenicity and lack of side effects\(^83\), have made it a popular and effective gene therapy vector in CNS applications in animal experiments\(^85\).
0.8.2 Adeno-associated virus

AAV vectors have been another popular choice for gene therapy. They are non-enveloped ssDNA viruses that can transduce a wide range of dividing and non-dividing cells and produce long-lasting transgene expression\(^\text{16}\). AAVs can deliver up to 4.8kb of DNA\(^\text{86}\). Upon infection, AAV can either persist as a double-stranded episome or integrate into the host genome, most often at the AAVS1 site on chromosome 19\(^\text{16,83,86}\). AAVs are not associated with any disease, exhibit very low immunogenicity, and cannot replicate except in the presence of a helper virus (usually adenovirus), making them safe for human therapeutic applications\(^\text{16,83}\).

AAV tropism is determined by its serotype, or type(s) of capsid protein expressed. Different capsid proteins bind to specific mammalian cell surface receptors\(^\text{87}\). There are 11 natural AAV serotypes, but many hybrid serotypes (expressing capsid proteins from two different serotypes) and other variants have been generated, allowing for custom tropism\(^\text{16,87}\). Most AAV serotypes, but especially AAV1, 2, and 5, show strong neuronal tropism when injected into the brain\(^\text{16}\). AAV4 preferentially transduces ependymal cells, while AAV9 exhibits both neuronal and astrocytic tropism and has the unique ability to cross the blood-brain barrier\(^\text{16}\). To date, AAV2 has been the vector of choice in both human and animal studies of gene therapy in PD, mainly due to its historical safety record. For gene therapy applications, improved control of spatiotemporal expression can be achieved through the appropriate selection of AAV serotype, promoter, and injection strategy (i.e. systemic vs. targeted).
0.9 Research Objectives

The principal aim of this study is to develop and characterize an shRNA-expressing viral vector that can achieve efficient knockdown of BAG5 expression in the rat substantia nigra.

Chapter 1: Generation of viral vectors expressing BAG5-targeting shRNA.

The first chapter of this thesis describes the identification of several shRNAs that efficiently knock down rat BAG5 protein expression in two cell lines. Following this identification AAV and lentivirus vectors that express these shRNAs were generated for in vivo applications, as discussed in Chapter 2.

Chapter 2: Characterization of viral expression, target engagement, and biological consequences of BAG5 knockdown in vivo.

The second chapter of this thesis focuses on interrogating the effects of the viral vectors generated in Chapter 1 (especially the AAV) when injected into the rat substantia nigra. The effects of AAV-shRNA driven BAG5 knockdown in the context of SN dopaminergic neuron injury are also investigated.
Chapter 1: Generation of viral vectors expressing BAG5-targeting shRNA

1.1 Introduction

This chapter describes the process of designing and generating two viral vectors, adeno-associated virus and lentivirus, which express BAG5-targeting shRNA and GFP as an expression marker. It begins by detailing the initial screen of several candidate BAG5 shRNA target sequences in two cell lines and concludes with the generation and confirmation of AAV infectivity in primary neurons. Subsequent in vivo experiments are covered in Chapter 2.

1.2 Materials and Methods

1.2.1 BAG5 shRNA plasmid construction

All plasmids from Origene were constructed to express shRNA under control of the U6 promoter and GFP under the CMV (cytomegalovirus) promoter. The plasmids also contained the puromycin resistance gene under the SV40 promoter.

All Creative Biogene plasmids were constructed to express shRNA under control of the U6 promoter and GFP under the CMV promoter. The plasmids also contained the puromycin resistance gene under the PGK (phosphoglycerate kinase) promoter.

All Genecopoeia plasmids were constructed to express shRNA under control of the U6 promoter and both GFP and puromycin resistance under the SV40 promoter, using an intervening IRES (internal ribosomal entry site) sequence.

Targeting sequences for each of the shRNAs are found in Table 1.
1.2.2 Cell culture and transfection

Rat pheochromocytoma PC12 cells were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 10% FCS (fetal calf serum), penicillin (100 units/mL), streptomycin (100 µg/mL), and amphotericin B (250 ng/mL). Human embryonic kidney (HEK) 293T cells were maintained in DMEM (Dulbecco’s Modified Eagle Medium) supplemented with 10% FCS (fetal calf serum), penicillin (100 units/mL), streptomycin (100 µg/mL), and amphotericin B (250 ng/mL). All cells were incubated at 37°C in a humidified air atmosphere containing 5% carbon dioxide.

HEK and PC12 cells were plated on 6-well plates (1,000,000 cells/well for HEK; 2,000,000 cells/well for PC12) in antibiotic/antimycotic-free media. Cells were transfected with 1.5µg of plasmid DNA at 24 hours after plating (1.5µg each of shRNA and rat BAG5 plasmid for HEK cells), using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Media was replaced with fresh media at 24 hours post-transfection. Cells were harvested 48 hours post-transfection in RIPA (radioimmunoprecipitation assay) buffer containing protease inhibitor cocktail (Roche).

1.2.3 Immunoblotting and analysis

Cells were (re-) suspended and lysed in RIPA buffer containing protease inhibitor cocktail (Roche). Protein concentrations were determined using the DC Protein Assay (Bio-Rad). Equal amounts of protein were loaded and run through sodium dodecyl sulfate (SDS) polyacrylamide gels by electrophoresis, followed by wet transfer to polyvinylidene fluoride (PVDF) membranes. Membranes were probed with anti-BAG5 antibody from mouse (Santa Cruz, cat. #ab56738) diluted at 1:200 (for endogenous BAG5 in PC12 cells) or 1:1000 (for exogenous rat FLAG-BAG5 in HEK cells), and with anti-GAPDH antibody from rabbit (Cell
Signaling, cat. #2118) diluted at 1:1000. Membranes were then probed with secondary anti-mouse IgG or anti-rabbit IgG linked to horseradish peroxidase (Cell Signaling) diluted at 1:5000. Detection was performed with the enhanced chemiluminescence (ECL) method (Pierce) for GAPDH and with ECL+ (Pierce) for BAG5. Relative protein expression was determined by western blot densitometry (with background subtraction), with GAPDH serving as the loading control. Transfection efficiencies were assumed to be the same across all conditions, thus the bands were not normalized for transfection efficiency.

1.2.4 Adeno-associated virus generation

Adeno-associated virus 1/2 vectors were custom ordered from GeneDetect Ltd. (Auckland, New Zealand). Each vector expresses GFP under the control of the chicken beta actin (CBA) promoter hybridized with the CMV immediate early enhancer sequence. These vectors also include a woodchuck post-transcriptional regulatory element (WPRE) and a bovine growth hormone (BGH) polyadenylation sequence to improve protein expression. Each vector also expresses either Origene non-targeting Control, Origene C, or Genecopoeia G1 shRNA against BAG5, under the U6 promoter. Stock viral titers were determined by quantitative PCR to be $1.1 \times 10^{12}$ genomic particles/mL for all AAVs.

1.2.5 Lentivirus generation

Lentivirus vectors were custom ordered from the UHN Vector Core (Toronto, Canada). Each vector expresses either Origene non-targeting Control or Origene B shRNA under the U6 promoter, and also GFP under the CMV promoter. Stock viral titers were determined to be $9.0 \times 10^{7}$ IU/mL.
1.2.6 Rat primary hippocampal neuron culture and infection

Dissociated E18.5 rat hippocampal neurons were obtained and cultured as previously described by Nie & Sahin\textsuperscript{88}. In brief, rats were euthanized with CO\textsubscript{2} and embryos were recovered. Hippocampi and cortices were dissected and placed in chilled dissociation medium (Ca\textsuperscript{2+}-free HBSS with 100mM MgCl\textsubscript{2}, 10mM kynurenic acid (Sigma-Aldrich), and 100mM HEPES). Following enzymatic dissociation with papain (Worthington Biochemical Corporation) and L-cysteine (Sigma-Aldrich), trypsin inhibitor (Sigma-Aldrich) was added, and cell clumps were dissolved by trituration. Neurons were suspended in Neurobasal medium supplemented with B-27, L-glutamine, and penicillin/streptomycin/primocin, and plated at a density of 1x10\textsuperscript{6} per well in 6-well plates coated overnight with 20µg/ml poly-D-lysine (Sigma-Aldrich) and 3.5µg/mL laminin. At 2 days in vitro (DIV), neurons were infected with AAV vectors at an MOI of 2×10\textsuperscript{4} in a volume of 1 mL of conditioned medium. Half the medium was replaced with fresh medium on DIV5. On DIV8, cells were washed with cold PBS, collected in 500µL PBS and pelleted by centrifugation at 500g for 5 minutes at 4°C. Cell lysates were prepared for western blot as described previously (Section 1.2.3). Unless stated otherwise, all reagents were purchased from Thermo Fisher Scientific. This experiment (except western blot) was performed by Dr. Darius Ebrahimi-Fakhari in Dr. Mustafa Sahin’s lab at Boston Children’s Hospital, Harvard Medical School (Boston, MA, USA).

1.3 Results

1.3.1 Screen of candidate BAG5 shRNA plasmids

Thirteen potential rat BAG5-targeting shRNA sequences and plasmids were generated from three vendors in order to increase our chances of identifying efficient shRNA clones. Based on the vendors, these shRNAs were termed Origene A through D, Creative Biogene 1 through 4,
and Genecopoeia G1, G4, G12, G13, and G14. Each candidate shRNA targeted a different site along the rat BAG5 mRNA transcript, within the open reading frame (ORF) (Fig. 2). None of the candidate shRNAs were located near a splice site; rat BAG5 has a single splice site located in the 5’ UTR (Ensembl genome browser89).

Candidate shRNAs were screened for ability to knock down BAG5 in two cell lines, and several promising shRNA sequences were identified. In rat pheochromocytoma PC12 cells (adrenal gland tumour; expresses endogenous rat BAG5), all four shRNA plasmids from Origene (clones A through D) generated substantial BAG5 knockdown 48h after transfection (Fig. 3A). Compared to control shRNAs, transfection with these targeting clones resulted in a proportion of BAG5 protein levels as follows (reported as mean ± standard deviation): Origene A (0.65 ± 0.18); Origene B (0.60 ± 0.23); Origene C (0.64 ± 0.27); Origene D (0.42 ± 0.07). All data are presented after removal of outliers using Grubbs’ test. Transfection efficiency for PC12 cells was observed to be roughly 30% (estimated proportion of GFP+ cells 48h post-transfection, based on visual inspection).

In human embryonic kidney (HEK) cells co-transfected with rat BAG5 and the candidate shRNA plasmids, substantial BAG5 knockdown was achieved 48 hours post-transfection by all four Origene clones, as well as Creative Biogene clone 2 and Genecopoeia clone G1 (Fig. 3B). Compared to controls, transfection with these targeting clones resulted in the following proportions of BAG5 protein levels: Origene A (0.69 ± 0.23); Origene B (0.71 ± 0.28); Origene C (0.51 ± 0.13); Origene D (0.35 ± 0.12); Creative Biogene 2 (0.59 ± 0.15); Genecopoeia G1 (0.46 ± 0.28). All data are presented after removal of outliers using Grubbs’ test. Transfection efficiency for HEK cells was observed to be roughly 70% (estimated proportion of GFP+ cells 48h post-transfection, based on visual inspection).
When the results from PC12 and HEK transfections are combined (Fig. 3C), again BAG5 knockdown was observed by all four Origene clones, Creative Biogene clone 2, and Genecopoeia clone G1. Compared to controls, transfection with these targeting clones resulted in the following proportions of BAG5 protein levels: Origene A (0.67 ± 0.19); Origene B (0.66 ± 0.25); Origene C (0.57 ± 0.20); Origene D (0.37 ± 0.10); Creative Biogene 2 (0.79 ± 0.29); Genecopoeia G1 (0.71 ± 0.48).

Origene D produces the most efficient knockdown in both PC12 and HEK cells. The other three Origene clones were also efficient at knocking down BAG5 in either cell line. Clone C in particular was quite effective at knocking down exogenous BAG5 in HEK cells, though not quite as well as clone D. None of the four targeting shRNAs from Creative Biogene were able to produce reliable endogenous BAG5 knockdown in PC12 cells. In HEK cells, Creative Biogene clone 2 showed some knockdown of exogenous BAG5, though the magnitude of knockdown was less than that produced by Origene C, Origene D, and Genecopoeia G1. The other Creative Biogene clones displayed either lack of efficiency or lack of reliability in HEK cells. None of the five Genecopoeia shRNAs were able to reliably knock down BAG5 in either PC12 or HEK cells, with the exception of G1 in the HEK condition.

Origene C, Origene D, and Genecopoeia G1 were selected as the three most promising candidate shRNAs. Although G1 showed similar overall efficiency (combined PC12 and HEK results) as Origene A and B, we chose to not select three shRNAs from a single vendor in order to avoid potential vendor-wide problems. Furthermore, despite the poor performance of G1 in PC12 cells, it displayed the second greatest average knockdown efficiency in HEK cells, just behind Origene D. This suggested that it may be quite effective at knocking down BAG5 under some conditions, and may have potential for our subsequent *in vivo* applications.
### TABLE 1.

**BAG5 shRNA targeting sequences and positions**

<table>
<thead>
<tr>
<th>shRNA</th>
<th>Targeting sequence (5’ to 3’)</th>
<th>Target position on rat BAG5 mRNA</th>
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<tr>
<td></td>
<td></td>
<td>5’</td>
</tr>
<tr>
<td><strong>Origene</strong></td>
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</tr>
<tr>
<td>Control</td>
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</tr>
<tr>
<td>A</td>
<td>ACTGATAAGAACTACATTCTGCTCTGGAGG</td>
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<tr>
<td>B</td>
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<tr>
<td>C</td>
<td>AGACTGGAGAGGATTCTGACGAAACACT</td>
<td>258</td>
</tr>
<tr>
<td>D</td>
<td>GTGCATCCTCTTCGCGCAAGATCAATTC</td>
<td>672</td>
</tr>
<tr>
<td><strong>Creative Biogene</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
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</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>3</td>
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<tr>
<td>4</td>
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</tr>
<tr>
<td><strong>Genecopoeia</strong></td>
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<tr>
<td>Control</td>
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<td>G14</td>
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</table>
Figure 2. Schematic showing target location of each of the 13 candidate BAG5-targeting shRNAs along the rat BAG5 mRNA transcript.
FIGURE 3.

A

BAG5 knockdown in PC12 (endogenous BAG5)

% Expression relative to scrambled

Origene A (n=3)  Origene B (n=3)  Origene C (n=3)  Creative Biogene 1 (n=3)  Creative Biogene 2 (n=4)  Creative Biogene 3 (n=4)  Creative Biogene 4 (n=4)  Genecopoeia G1 (n=1)  Genecopoeia G4 (n=1)  Genecopoeia G12 (n=1)  Genecopoeia G13 (n=1)  Genecopoeia G14 (n=1)

shRNA

B

BAG5 knockdown in HEK (exogenous FLAG-BAG5)

% Expression relative to scrambled

Origene A (n=4)  Origene B (n=4)  Origene C (n=4)  Creative Biogene 1 (n=2)  Creative Biogene 2 (n=2)  Creative Biogene 3 (n=2)  Creative Biogene 4 (n=2)  Genecopoeia G1 (n=2)  Genecopoeia G4 (n=2)  Genecopoeia G12 (n=2)  Genecopoeia G13 (n=2)  Genecopoeia G14 (n=2)

shRNA
Figure 3. Initial *in vitro* screen of knockdown efficiency of candidate shRNAs against rat BAG5, via western blot densitometry. (A) Knockdown of endogenous rat BAG5 in PC12 cells transfected with candidate shRNAs, 48h post-transfection. (B) Knockdown of exogenous rat BAG5 (co-transfected with the shRNAs) in HEK cells transfected with candidate shRNAs, 48h post-transfection. (C) Combined data from (A) and (B). All data are reported as mean ± standard deviation.
1.3.2 Confirmation of top three candidate shRNA plasmids

After identifying BAG5 shRNA clones C, D, and G1 as the three most promising candidate shRNAs, the knockdown experiment in PC12 and HEK (Fig. 3) was repeated, focusing on these top three shRNAs, in order to confirm their ability to knock down BAG5 in vitro.

In PC12 cells, densitometry analysis showed that, compared to the respective control shRNA, transfection with targeting clones resulted in the following proportions of BAG5 protein levels: Origene C (0.68); Origene D (0.64); Genecopoeia G1 (0.84) (Fig. 4A).

In HEK cells, transfection with targeting shRNA resulted in the following proportions of BAG5 protein levels, relative to the respective control shRNA: Origene C (0.73); Origene D (0.74); Genecopoeia G1 (0.22) (Fig. 4B).

The results here are similar to those observed in the initial shRNA screen, with clones C and D being able to knock down BAG5 in both PC12 and HEK cells, while G1 resulted in strong BAG5 knockdown in HEK cells, but not in PC12.
Confirmation of efficient BAG5 knockdown by top three shRNAs identified in the initial screen. (A) Knockdown of endogenous rat BAG5 in PC12 cells transfected with candidate shRNAs at 48h post-transfection. Left: western blot. Right: western blot densitometry (n=1). (B) Knockdown of exogenous rat BAG5 (co-transfected with the shRNAs) in HEK cells transfected with candidate shRNAs at 48h post-transfection. Left: western blot. Right: Western blot densitometry (n=1).
1.3.3 Generation of AAV1/2 and lentivirus vectors expressing BAG5 shRNA

Having confirmed the knockdown activity of clones C, D, and G1, these clones were packaged into adeno-associated virus (AAV) 1/2 vectors co-expressing GFP. A non-targeting control virus was also generated, using the shRNA sequence from Origene non-targeting Control. From here on, these vectors will be referred to as AAV-shBAG5 C, D, G1, and Control. We also had access to an AAV1/2 expressing only GFP and no shRNA, but otherwise identical to our AAV-shBAG5 vectors, and termed this AAV-GFP.

Preliminary in vivo testing of the different AAV-shBAG5s showed that C and G1 produced more reliable knockdown of BAG5 in injected rat substantia nigra compared to D (data not shown). Thus, all subsequent experiments focused on investigating only AAV-shBAG5 C and G1 compared to the non-targeting Control AAV.

Lentivirus (LV) vectors co-expressing shRNA and GFP were also generated, using shRNA sequences from Origene B and Origene non-targeting Control. From here on, the LV vectors will be called LV-shBAG5 and LV-Control. The LV vectors were generated well before the AAVs. At the time of LV generation, fewer candidate shRNAs were available for testing. In addition, fewer replicates of the in vitro knockdown experiments (as in Fig. 3) had been performed. Based on the data available at the time, we chose to package Origene clone B shRNA into the LV vector, rather than one of the shRNA sequences used in the AAVs.

1.3.4 AAV-shBAG5 knocks down BAG5 in rat primary hippocampal neurons

AAV-shBAG5 vectors were used to infect rat primary hippocampal neurons. GFP expression and BAG5 knockdown activity were assessed by western blot (Fig. 5A). All three AAV-shBAG5 vectors were all able to infect these neurons and drive expression of GFP. By 8
days in vitro, infection efficiency was observed to be approximately 75%, by visual inspection of the proportion of GFP+ neurons. Infection with AAV-shBAG5 Control resulted in considerably greater GFP expression compared to C (44% of Control) or G1 (68% of Control) (Fig. 5B). Both AAV-shBAG5 C and G1 were able to produce substantial knockdown of BAG5. Infection with AAV-shBAG5 C resulted in 28% BAG5 expression compared to Control AAV-infected neurons, while AAV-shBAG5 G1 resulted in 50% BAG5 expression compared to Control (Fig. 5C).
**Figure 5.**  

GFP expression and BAG5 knockdown by AAV-shBAG5 vectors in rat primary hippocampal neurons at 8 days post-infection. (A) Western blot for BAG5, GFP, and GAPDH from lysate of primary rat hippocampal neurons infected with AAV-shBAG5 C, AAV-shBAG5 G1, and AAV-shBAG5 Control at MOI of $2 \times 10^4$. (B) Densitometry analysis of GFP expression, relative to GAPDH. (C) Densitometry analysis of BAG5 expression, relative to GAPDH. (n=1).
1.4 Discussion

1.4.1 Choice of shRNA as gene inhibition strategy

There are several advantages to using shRNA as a gene inhibition strategy in vivo. Once it is delivered to cells via viral transduction, it results in sustained knockdown of its target mRNA. Thus, shRNA treatment requires only a single intervention, which saves on costs and reduces risk to the animal. Virus-delivered shRNA is also easier to target to a specific area of the brain. This accuracy can be further refined to specific cell types by carefully selecting viral serotype/tropism. By comparison, ASOs would require regular infusions and constant production of the therapeutic agent. Since these agents are usually delivered by intrathecal or intraventricular infusion, it is also more difficult to target them to specific locations, potentially resulting in unwanted effects in other regions of the CNS (not to mention the inefficiency). The similar siRNA can also be delivered in vivo, but has traditionally struggled to overcome issues of low stability and cellular uptake. Nonetheless, siRNAs and ASOs have shown promising results in animal models, and currently ASOs are being investigated in clinical trials for Huntington’s disease.

Another strategy to inhibit BAG5 could be to target the protein rather than the mRNA. A BAG5 mutant, BAG5-DARA, has already been characterized. The DARA mutant is unable to interact with Hsp70, and prevents normal BAG5 from interacting with Hsp70. The BAG5-Hsp70 interaction is critical for some of BAG5’s functions, including its interaction with CHIP, which prevents CHIP from ubiquitylating αSyn. However, the DARA mutant does not affect BAG5’s interaction with parkin or its inhibition of parkin’s E3 ligase activity. In addition, BAG5 may have other, potentially deleterious functions that are unaffected by DARA. Thus, inhibiting the expression of BAG5 may result in a different phenotype that may have more comprehensive effects than DARA-based inhibition.
At the time of this project’s initiation, reports of in vivo CRISPR strategies were just beginning to emerge, so we chose a more traditional technology in shRNA. Going forward, CRISPR will be an interesting alternative strategy to pursue, though gene knockdown and knockout may be differ in phenotype, and CRISPR has its own off-target effects and disadvantages to consider. For instance, it may be less efficient than RNAi given that it must successfully cleave and functionally knock out both alleles of the target gene. Additionally, unrepaired double-stranded breaks can cause cell death, and off-target effects could result from cleaving at untargeted loci\(^1\).

### 1.4.1.4.2 BAG5 antibody validation

For application in western blot, our lab had previously tested several BAG5 antibodies from different vendors. Of these antibodies, the antibody used in Chapter 1 (Santa Cruz, cat. #ab56738) provided the cleanest detection of endogenous BAG5 in PC12 cell lysate. It detected a single band at the predicted molecular weight of 51 kDa in HEK cell lysate expressing FLAG-BAG5, and in the S2 (cytosolic) fraction of rat brain lysate (data not shown).

### 1.4.21.4.3 Transfection efficiency and interpretation of apparent knockdown

In PC12 cells, we achieved approximately 30% transfection efficiency using 1.5µg of plasmid and Lipofectamine 2000, after trying several different cationic lipid-based transfection reagents that were no more efficient. Reports of PC12 transfection efficiency in the literature are quite variable. Lee et al reported 14% efficiency with 1 µg of DNA and Lipofectamine 2000, and 15% with polyethyleneimine (PEI)\(^2\). Cogli et al achieved a 30% transfection efficiency using a different cationic lipid product (Metafectene Pro)\(^3\). Covello et al achieved a transfection efficiency of 46% using 1 µg of plasmid Lipofectamine 2000\(^4\). However, they were able to increase transfection efficiency to 90% when they used an electroporation method instead\(^4\).
In HEK cells, we were able to achieve a relatively high transfection efficiency of approximately 70% using 1.5µg of plasmid and Lipofectamine 2000. This value is similar to literature reports, which range from 60% efficiency with Lipofectamine 2000 and 1-2 µg of DNA\textsuperscript{95}, to 75% with PEI (with suspension cells)\textsuperscript{96}.

Since we analyzed cell lysate from the entire population of cells and not just the transfected cells, the maximum expected knockdown in our population of cells should be equal to the transfection efficiency. The level of knockdown observed in our shRNA screen (Fig. 3) generally followed this rule, with the most efficient shRNA clones achieving roughly 30% knockdown in PC12 cells (Fig. 3A), and 70% knockdown in HEK cells (Fig. 3B). Thus, the actual knockdown produced by the most efficient shRNAs would be close to 100%. However, Origene clone D produced a mean knockdown of 58% (i.e. 0.42 times the BAG5 band density compared to Control), which is substantially greater than the expected maximum knockdown of 30%. The reason for this is unclear. It is possible that transfection efficiency was underestimated, or that clone D produced an off-target effect increasing GAPDH expression, causing the amount of BAG5 in lysate to be underestimated. On the other extreme, we also observed that several less efficient shRNAs from Genecopoeia produced an apparent mean upregulation of BAG5 compared to non-targeting Control, in both HEK and PC12 cells (Fig. 3). This result may be attributable to the low sample size with high variance, poorly designed Control sequence, or off-target effects.

It is also important to consider protein half-life in knockdown experiments because shRNA only reduces mRNA levels without affecting existing protein. Previous studies of the proteome in various mammalian cell types have found that protein half-lives can be highly variable and depends on cell type, but are mostly on the order of hours to tens of hours\textsuperscript{97-99}. The
half-life of BAG5 has not been studied, but if actual BAG5 knockdown were indeed close to 100% at 48h post-transfection, our results would suggest that BAG5 half-life would be on the order of several hours, such that the vast majority would be degraded within 48 hours. Protein half-life can be more accurately determined by pulse-chase assay or by cycloheximide blocking.  

It is unclear why some of the candidate shRNAs appeared to not produce appreciable knockdown, especially the clones from Creative Biogene (in PC12) and the non-G1 clones from Genecopoeia (in PC12 and HEK). These shRNAs may simply not have been effective at binding the target sequence or associating with RISC. It is unlikely that there is an issue with transfection or with the plasmids’ stability or interaction with the lipofection reagent, since cells transfected with these plasmids demonstrated similar GFP expression levels and transfection efficiencies compared to shRNA clones that produced efficient knockdown. One possible explanation of poor knockdown efficiency is the dilution of plasmids as cells divide, since the plasmids are not designed to replicate in mammalian cells. The plasmids from Creative Biogene and Genecopoeia may produce efficient knockdown only at high plasmid copy numbers, perhaps because the shRNA targeting sequences bind less effectively to BAG5 mRNA. Different doubling times between PC12 and HEK cells (and thus plasmid dilution rates), and also different transfection efficiencies, may also explain the efficient knockdown produced by Creative Biogene plasmids in HEK cells, but not in PC12 cells. In addition, the Genecopoeia plasmids use a different GFP promoter than the plasmids from the other vendors. Therefore, although the GFP expression and transfection efficiency from Genecopoeia plasmids may seem comparable to that produced by other plasmids, the actual amount of plasmid delivered to each cell and the expression of shRNA may not be.
1.4.3.1.4.4 Limitations of western blot and densitometry analysis

Knockdown in vitro was quantified by western blot densitometry, which relies on the assumption that all bands of interest fall within the linear range (i.e. band intensity is directly proportional to amount of protein). We tried to adjust X-ray film exposure times to remain in the linear range, but did not use a protein standard to confirm. BAG5 bands were normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase) bands as a loading control. While this is standard practice, it assumes that all cells express equal amounts of GAPDH, and that this expression is not altered by the experimental treatment of shRNA transfection. While our shRNAs did not target GAPDH, there remains the risk of off-target effects. Recently, several groups have suggested that analyzing total protein (using a stain such as Ponceau S or Coomassie Brilliant Blue) instead of a specific high-abundance protein such as GAPDH or actin would be a more accurate method of normalizing.

1.4.4.1.4.5 Alternative techniques to quantify knockdown

There is some ambiguity in assessing knockdown in a heterogeneous population of transfected and non-transfected cells. New variations on transfection methods can achieve higher rates of transfection, but not 100% efficiency. Instead, it is possible to isolate transfected cells (GFP+) from a population using fluorescence activated cell sorting (FACS), followed by analysis (or subculture) of this highly purified subpopulation. Flow cytometry methods could also to used to obtain a more accurate measurement of transfection efficiency. Another method of quantifying shRNA-mediated knockdown is RT-qPCR, though reported mRNA levels are not always reflective of the amount of protein available. Unlike western blot, RT-qPCR is feasible even when good antibodies for the target protein are unavailable.
Chapter 2: Characterization of BAG5 shRNA viral vectors in vivo

2.1 Introduction

In the previous chapter, we identified shRNA sequences that can efficiently knock down BAG5 in vitro, and subsequently generated AAV and LV vectors that co-express these shRNAs along with a GFP expression marker. The next aim is to establish in vivo expression and BAG5 knockdown capabilities of these BAG5 shRNA viral vectors in rat SN. In this chapter, we aim to identify a suitable viral titer for infection, characterize the spatiotemporal expression of these vectors, and establish successful BAG5 knockdown in the rat SN. We also observe significant differences in expression of AAV compared to LV. Finally, we conduct a preliminary analysis of some of the effects of BAG5 knockdown using the medial forebrain bundle axotomy (MFBx) rat model of dopaminergic neuron injury.

Dopaminergic neurons, which express and positively stain for tyrosine hydroxylase (TH), are present in the SNc and in the ventral tegmental area (VTA), a medial structure in the rat ventral midbrain. It can be difficult to distinguish the boundary between the SNc and VTA in certain coronal sections (“levels”) of the rat midbrain where these structures are continuous with each other from left to right of the ventral region in coronal sections. However, another anatomical structure called the medial terminal nucleus (MTN), which does not contain dopaminergic neurons, exists between SNc and VTA at some levels of the midbrain. On TH-stained coronal sections, the MTN appears as a small TH-negative area that extends dorsolaterally from the ventral surface, separating SNc from VTA. Thus, at the level of the MTN, the SNc is easily distinguishable from the VTA using TH staining, allowing subsequent analysis to accurately examine SNc and not mistakenly include VTA in the analysis (or exclude
medial SNC). For this reason, all microscopy images and analysis included in this chapter have been taken/conducted at the level of the medial terminal nucleus (MTN).

Anatomically, SNC extends from -4.56mm to -6.60mm relative to bregma (Paxinos & Watson, 1997). The VTA is particularly challenging to distinguish from SNC in more anterior sections, though VTA exists from -4.68mm to -6.24mm, nearly the entire anteroposterior extent of the SNC. MTN is a useful divider of SNC and VTA from -4.92mm to -5.28mm. Since the anteroposterior coordinate of injection is -5.2mm, the MTN-containing sections included in our analysis tend to be located very close but slightly anterior to the ideal injection site.

2.2 Materials and Methods

2.2.1 Animals

All animal experiments were conducted using female adult Sprague-Dawley rats (Charles River), weighing 275-300g at the time of virus injection. Rats were co-housed in pairs on a regulated 12-hour light/dark cycle at standard temperature (21–22 °C) and allowed food and water ad libitum.

2.2.2 Stereotactic injection of AAV or LV into rat SN

All procedures were performed in accordance with the rules and regulations of the Animal Resources Centre in the University Health Network. Animals were deeply anaesthetized with Anafen (Merial) at 5mg/kg delivered subcutaneously. Animals were mounted on a stereotactic frame and a craniotomy was made relative to bregma according to the brain atlas of Paxinos and Watson (1997). Rats received a single 2µL, unilateral injection of AAV or lentivirus targeted to the SN by stereotactic injection. AAVs were injected at either 1/2 of stock titer (resulting in a titer of $5.5 \times 10^{11}$ gp/mL) or at 1/3 of stock (resulting in a titer of $3.7 \times 10^{11}$ gp/mL). AAV-GFP (used in Fig. 7) was injected at a titer of $5.1 \times 10^{12}$ gp/mL. Lentivirus was injected at a
titer of $9.0 \times 10^4$ IU/µL (or $9.0 \times 10^7$ IU/mL). Viruses were delivered via microinjector (Harvard Apparatus) at a rate of 0.5 µL/min, using the following coordinates relative to bregma: AP, -5.2mm; ML, -2.0mm; DV, -7.5mm (Paxinos & Watson, 1997). After surgery, animals were housed individually on a regulated 12-hour light/dark cycle and allowed food and water *ad libitum*.

2.2.3 Medial forebrain bundle axotomy

Three weeks following AAV injection, rats received unilateral axotomy of the medial forebrain bundle (MFBx), ipsilateral to the side of injection. A retractable wire knife was inserted directly into the brain at the following coordinates relative to bregma: AP, -3.8mm; ML, -2.4mm; DV, -8.0mm (Paxinos & Watson, 1997). The blade was extended, then raised 2.5mm, then lowered 3.0mm. The blade was retracted and removed. After surgery, animals were housed individually on a regulated 12-hour light/dark cycle and allowed food and water *ad libitum*. Rats were sacrificed 7 days post-MFBx (see Section 2.2.4).

2.2.4 Processing of rat brains

Rats were anaesthetized, and then sacrificed by exsanguination and transcardial perfusion with cold saline, followed by cold 4% paraformaldehyde (PFA). Brains were then removed and stored at 4°C, first in 4% PFA for 24 hours, then put through a 15% to 30% sucrose-PBS gradient. In order to generate sections, brains were frozen on a stage and then six series of 40µm coronal sections were generated using a sliding microtome. Each series of sections was stored in cryoprotectant (30% glycerol, 30% ethoxyethanol, 40% PBS) at -20°C.

2.2.5 Immunofluorescence staining of rat brain sections for TH and BAG5

Cryoprotectant was washed off with several rinses of 0.2% PBS-Tween 20 (PBS-T), then sections were blocked in 10% NGS + 2% BSA in 0.2% PBS-T for one hour at room temperature.
Sections were then incubated at room temperature overnight in primary antibody: rabbit polyclonal anti-BAG5, 1:1000 (Imgenex cat. #IMG-5678, identical to Novus Biologicals cat. #NB100-56091); mouse anti-TH, 1:1000 (Millipore cat. #MAB318). After several washings in PBS-T, sections were incubated in goat anti-mouse IgG biotin-SP at 1:1000 (Jackson ImmunoResearch cat. #115-065-146) for 1 hour at room temperature, in order to amplify TH staining using the biotin-streptavidin system. After another several washings in PBS-T, sections were incubated for 1 hour at room temperature in either secondary fluorescent antibody (for BAG5 staining) goat Alexa Fluor anti-rabbit 594, 1:500 (Invitrogen cat. #A-11037); and/or in either Alexa Fluor streptavidin 350, 1:500 (Molecular Probes cat. #S11249) or Alexa Fluor streptavidin 594, 1:500 (Molecular Probes cat. #S32356). Sections were again washed in PBS-T, then mounted on glass slides, allowed to dry, then covered by a coverslip with fluorescent mounting medium (Dako cat. #S302380-2).

2.2.6 Immunostaining of rat brain sections for TH and NeuN

Cryoprotectant was washed off with several rinses of 0.1% PBS-T. Endogenous peroxidase was quenched by washing sections in 3% H$_2$O$_2$ (Sigma cat. #H-1009) for 3 minutes, followed by another several rinses in PBS-T. Sections were blocked in 10% NGS + 2% BSA in 0.2% PBS-T for one hour at room temperature. Sections were then incubated at room temperature overnight in primary mouse anti-NeuN antibody, 1:1000 (Chemicon cat. #MAB377). After several washings in PBS-T, sections were incubated in secondary antibody for 2 hours at room temperature: biotinylated anti-mouse IgG from goat, 1:400 (Vector cat. #BA-9200). After washing in PBS-T, sections were incubated for 1 hour at room temperature in ABC Elite mix according to the manufacturer’s instructions (Vector cat. #PK-6100). Sections were rinsed in 0.1M Dulbecco’s PBS (Sigma), and then stained with DAB (3,3’-Diaminobenzidine) according to the manufacturer’s instructions (Vector cat. #SK-4105). This was followed by
another several rinses in PBS-T and overnight, room temperature incubation in the second
primary antibody, rabbit anti-TH, 1:2000 (Chemicon cat. #AB152). Sections were rinsed and
incubated in secondary antibody as previously described, using alkaline phosphatase-conjugated
anti-rabbit IgG from goat, 1:400 (Jackson ImmunoResearch cat. #111-055-144). This was
followed by another series of PBS-T rinses, then by incubation in Vector Blue substrate
according to the manufacturer’s instructions (Vector cat. #SK-5300). Sections were again
washed in PBS-T, then mounted on glass slides, allowed to dry overnight, then covered by a
coverslip with Vectamount (Vector cat. #H-5000).

2.2.7 Semi-quantification of BAG5 expression in SN using ImageJ

Paired images of rat SN stained for BAG5 were acquired at 100x magnification using a
deconvolution microscope (Zeiss Axioplan 2). Paired images were captured using identical
exposure times, set to the automatically determined optimal exposure time for the uninjected SN
using AxioVision 4.8 software (Zeiss). These images were opened in grayscale using ImageJ
software (Schneider et al., 2012). A freeform region of interest (ROI) was used to enclose the
area of the SNc on the image taken contralateral to virus injection, and this ROI was mirrored
onto the paired image. Using the “Find Maxima” tool with noise tolerance value arbitrarily set to
8, the number of points of local pixel intensity maxima within each ROI was identified, as a
proxy for overall intensity (see discussion and Fig. 6).

2.2.8 Statistical analyses

Statistical analysis was conducted using GraphPad Prism 6.0 software (GraphPad
Software, La Jolla, California, USA, www.graphpad.com). Data containing three or more groups
were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test.
Statistical significance was arbitrarily set to p < 0.05.
Figure 6. Demonstration of semi-quantitative method of assessing BAG5 immunofluorescence in SN, using “find maxima” function in ImageJ.
2.3 Results

2.3.1 AAV1/2 achieves greater expression in rat SN than LV

As an initial step in our in vivo experiments, LV and AAV1/2 vectors were tested for expression in rat brain. We used AAV-GFP rather than AAV-shBAG5 vectors here because the latter were not yet available. Four weeks following unilateral injection of virus into the rat SN, coronal midbrain sections were examined for GFP expression (Fig. 7). Substantial GFP signal was observed in and around the SN in rats injected with AAV-GFP vector, but LV-Control and LV-shBAG5 injected brains showed very poor GFP signal. These sections were also stained by immunofluorescence as an attempt to amplify any GFP signal that may be too weak to see by eye. However, even after signal amplification, GFP signal in the LV sections was poor. There was occasionally some GFP signal near the injection track, but not in SN, and signal was generally highly inconsistent. The reason for the LV vectors’ poor expression is unclear, as they efficiently transduced cultured cells (data not shown). Based on these results, we decided to use only the AAV vectors in subsequent experiments.
Figure 7. GFP expression in SN-containing sections of LV- or AAV-injected rat brains, at four weeks post-injection. Top row: GFP signal without amplification, in green channel. Second row: immunofluorescence-amplified GFP signal, in red channel. Bottom row: merge of green and red channels.

Figure also contributed by C. L
2.3.2 AAV-shBAG5 vectors express throughout SN at level of MTN

All three AAVs provided excellent mediolateral coverage of the SNC, as visualized by GFP expression throughout the SNC, in MTN-containing sections (Fig. 8). All three AAVs also provided fairly comprehensive SN coverage along the anteroposterior axis (images not shown), though GFP expression tended to appear slightly weaker in the most posterior sections. With the BAG5-targeting AAVs, GFP expression was well localized to SNC, though some green signal was observed in the surrounding tissues, especially those immediately dorsal to the SN. By contrast, AAV-shBAG5 Control resulted in a large spread of strong GFP expression dorsal to the SN, often covering nearly half the injected hemisphere.
Figure 8. SNc-containing sections from rat brains injected with AAV-shBAG5 Control, C, or G1 at 1/2 stock dilution (resulting in $5.5 \times 10^{11}$ gp/mL), at three weeks post-injection. Immunofluorescence staining of tyrosine hydroxylase (TH) in red channel, merged with endogenous GFP in green channel, reveal the coverage of AAV expression in and around SNc at the level of the medial terminal nucleus (MTN), the area of non-TH immunoreactivity indicated by the white arrow.
2.3.3 AAV-shBAG5 vectors at 1/2 dilution knock down BAG5 in vivo

Having confirmed excellent AAV coverage at the level of the MTN at three weeks following a 1/2 of stock AAV dilution (resulting in $5.5 \times 10^{11}$ gp/mL), we next sought to establish knockdown of BAG5 in these same animals by immunostaining for BAG5. Most MTN-containing sections from rats injected with either of the targeting AAV-shBAG5 vectors displayed clear BAG5 knockdown in the injected SN, by visual inspection and comparison against the uninjected SN as an internal control (Fig. 9A-F). Furthermore, the overall degree of BAG5 knockdown was judged to be roughly 50% with both AAV-shBAG5 C and G1, again by inspection.

In order to confirm BAG5 knockdown and to eliminate potential observer bias, we developed a semi-quantitative image analysis method that could be used to compare BAG5 staining in the injected SN to the uninjected SN (see section 2.2.7). Based on this method, which uses an algorithm to identify the number of local pixel intensity maxima within a user-defined region of interest, it was confirmed that both AAV-shBAG5 C and G1 resulted in a significantly greater BAG5 knockdown in the injected SN than that produced by AAV-shBAG5 Control (Fig. 9G). Specifically, the ratio of total maxima in injected SN to total maxima in uninjected SN was found to be the following (reported as mean ± standard deviation): Control (0.62 ± 0.25); C (0.34 ± 0.19); G1 (0.33 ± 0.21). Paired sections judged to have exceptionally poor BAG5 staining were excluded from the analysis. All data are presented after removal of outliers using Grubbs’ test.
FIGURE 9.
FIGURE 9 (CONT’D)

E

Injected hemisphere  Un-injected hemisphere

BAG5

TH

GFP

Merge

100x magnification. Scale bar is 200µm.

F

Injected hemisphere  Un-injected hemisphere

BAG5

TH

GFP

Merge

400x magnification. Scale bar is 50µm.

G

Relative BAG5 staining to contralateral side

RS/LS maxima values

Control  G  G1

*
Figure 9. AAV-shBAG5 mediated BAG5 knockdown at three weeks post-injection of AAVs at 1/2 dilution (5.5×10^{11} gp/mL). Representative immunofluorescence staining of BAG5 and TH in SNc of rats injected with AAV-shBAG5 Control, at low magnification (A) and high magnification (B); AAV-shBAG5 C, at low magnification (C) and high magnification (D); or AAV-shBAG5 G1, at low magnification (E) and high magnification (F). Maxima-based semi-quantification of BAG5 staining in SNc of MTN-containing sections, expressed as the ratio between right side (RS; injected) and left side (LS; uninjected) total maxima (n=6 rats injected with each AAV; three MTN-containing sections analyzed for each rat). Statistical analysis performed by ANOVA with Tukey’s post-hoc test, * indicates p < 0.05.
2.3.4 AAV-shBAG5 vectors at 1/3 dilution knock down BAG5 in vivo

Since our AAV-shBAG5 vectors also express GFP, which has previously been shown to be toxic to neurons when overexpressed in rat SN\textsuperscript{33}, we next assessed the expression and knockdown capabilities of our AAV-shBAG5 vectors when injected at a lower dilution (1/3 of stock, resulting in 3.7×10\textsuperscript{11} gp/mL) but otherwise using the same experimental parameters as before. At three weeks following AAV injection at this lower dilution, immunostaining for BAG5 demonstrated substantial BAG5 knockdown in the injected SN, by visual inspection and comparison against the uninjected SN (Fig. 10A-F). Similar to observations with the 1/2 dilution, the degree of knockdown achieved by C and G1 was judged to be roughly 50\% for both AAVs, by inspection. As before, the maxima-based semi-quantitative method was applied to confirm knockdown, and again showed that significantly greater BAG5 knockdown was achieved by the two targeting AAVs compared to AAV-shBAG5 Control (Fig. 10G). Specifically, the ratio of total maxima in injected SN to total maxima in uninjected SN was found to be the following (reported as mean ± standard deviation): Control (1.03 ± 0.36); C (0.59 ± 0.19); G1 (0.57 ± 0.27). Paired sections judged to have exceptionally poor BAG5 staining were excluded from the analysis. All data are presented after removal of outliers using Grubbs’ test.

By inspection, the overall magnitude of BAG5 knockdown produced by the 1/3 dilution appeared to be slightly less than that produced by the 1/2 dilution. However, the difference between the magnitudes of knockdown achieved by the two dilutions is difficult to quantify, and the maxima-based method does not provide a suitable measure of this.
FIGURE 10 (CONT’D)

E

F

G

Relative BAG5 staining to contralateral side

RS/LS maxima values

Control  G  G1

*  *

Scale bar is 200μm.

Scale bar is 50μm.
Figure 10. AAV-shBAG5 mediated BAG5 knockdown at three weeks post-injection of AAVs at 1/3 dilution (3.7×10^{11} gp/mL). Representative immunofluorescence staining of BAG5 and TH in SNC of rats injected with AAV-shBAG5 Control, at low magnification (A) and high magnification (B); AAV-shBAG5 C, at low magnification (C) and high magnification (D); or AAV-shBAG5 G1, at low magnification (E) and high magnification (F). Maxima-based semi-quantification of BAG5 staining in SNC of MTN-containing sections, expressed as the ratio between right side (RS; injected) and left side (LS; uninjected) total maxima (n=6 rats injected with each AAV; three MTN-containing sections analyzed for each rat). Statistical analysis performed by ANOVA with Tukey’s post-hoc test, * indicates p < 0.05.
2.3.5 AAV-shBAG5 vectors protect against MFBx injury

Following confirmation that our AAV-shBAG5 vectors are able to knock down BAG5 in rat SN at the tested dilutions, we next conducted a preliminary investigation into the neuroprotective potential of BAG5 knockdown in the MFBx model of dopaminergic injury.\(^{23}\)

Injection of either targeting AAV three weeks prior to MFBx resulted in significant protection from loss of TH in the ipsilateral SN at 7 days post-MFBx (Fig. 11A). The percentages of TH+ profiles in the injected SN compared to the uninjected SN (reported as mean ± SEM) are as follows: Control (41.9 ± 11.7); C (70.9 ± 11.0); G1 (70.2 ± 10.1).

In addition, AAV-shBAG5 C significantly rescued the loss of mature neurons (NeuN+ cells) in the SN, while AAV-shBAG5 G1 trended towards rescue but did not reach statistical significance (Fig. 11B). The percentages of NeuN+ profiles in the injected SN compared to the uninjected SN (reported as mean ± SEM) are as follows: Control (30.8 ± 5.0); C (46.1 ± 3.6); G1 (42.5 ± 8.0). All data are presented after removal of outliers using Grubbs’ test.
Figure 11. Effect of AAV-shBAG5 injected at 1/2 dilution (5.5×10^{11} \text{ gp/mL}) on TH and NeuN expression in SN of rats receiving medial forebrain bundle axotomy at 7 days post-axotomy (four weeks post-injection). Ratio of TH+ (A) and NeuN+ (B) profiles on injected vs. contralateral (i.e. uninjected) side at the level of the MTN (n=6 for Control, n=8 each for C and G1; three MTN-containing sections analyzed per animal). All data are reported as mean ± SEM. Statistical analysis performed by ANOVA with Tukey’s post-hoc test, * indicates p < 0.05.
2.4 Discussion

2.4.1 Rationale for selecting AAV1/2 serotype and injection titers

Although AAV2 has typically been the vector of choice for clinical gene therapy trials for CNS disorders\textsuperscript{16}, we chose to use AAV1/2 vector in our work for several reasons. Firstly, it combines the strong neuronal tropism of AAV2 with the widespread penetrance in brain tissue of AAV1\textsuperscript{33,110} to ensure full coverage of the SN. In addition, expertise about this specific vector was readily available from our collaborators, who developed a rat model of PD using AAV1/2 expressing the A53T mutant of human αSyn\textsuperscript{33,111}. Based on their previous reports, we were able to choose an appropriate injection titer to minimize GFP-mediated toxicity by comparing titers to the previously characterized AAV1/2-GFP in rat SN\textsuperscript{111}. Our first choice of titer (Fig. 9) of 1/2 stock (5.5×10\textsuperscript{11} gp/mL) was chosen to closely match the 5.1×10\textsuperscript{11} gp/mL titer of AAV1/2-GFP used by Koprich \textit{et al.}\textsuperscript{111}, which showed no trend toward toxicity at either 3 or 6 weeks post-injection, as determined by TH expression and forepaw asymmetry\textsuperscript{111}. Finally, using an AAV1/2 vector for shRNA delivery would enable us, as a future experiment, to test our vectors in the AAV1/2-hA53T-SNCA model while ensuring consistency in spatiotemporal expression (see Section 3.3.1).

2.4.2 Comparison of AAV to LV

It is unclear why the LV vectors failed to express any detectable amounts of GFP in the rat SN or around the injection site in the midbrain, even after signal amplification. Our injection titer of 9.0×10\textsuperscript{4} IU/µL (or 9.0×10\textsuperscript{7} IU/mL) falls within the range of titers used by other groups to achieve lentivirus-delivered GFP expression in rat SN. For instance, Harms \textit{et al.} successfully expressed GFP with a 2µL injection of LV at a titer of 3.3×10\textsuperscript{7} IU/mL, at both 2 and 5 weeks post-injection\textsuperscript{112}. Similarly, Yin \textit{et al.} found success with a 1µL injection of 1.0×10\textsuperscript{8} IU/mL of LV, and detected GFP expression in SN as early as 72 hours post-injection\textsuperscript{113}. A third group was
successful using a higher titer of $9.0 \times 10^8$ IU/mL of LV, at 8 weeks post-injection\textsuperscript{114}. Besides the injection titer, we also chose a well-established mammalian constitutive promoter (CMV) for protein expression, used standard stereotactic coordinates for SN injection, and allowed a reasonable time frame (4 weeks post-injection) for expression. Indeed, the LVs were injected using the same techniques and parameters as AAV-GFP (Fig. 7), which did show robust GFP expression at 4 weeks post-injection.

Given the lack of GFP expression following LV injection, it seemed likely that the shRNAs from the LV would also be poorly expressed. We therefore did not investigate BAG5 knockdown in the LV-injected brains. Based on these results, we conclude that at least in our hands, AAV1/2 is superior to LV as a viral gene delivery vector in the rat SN, and we thus proceeded with AAV for the remainder of the experiments.

### 2.4.3 Spatial expression of AAV and analysis at level of MTN

The excellent coverage of the SN in sections containing MTN (Fig. 8) reinforces our decision to analyze SN at that level. We can be confident that our analysis of the SN includes AAV-infected neurons along the full extent of the SN while it also excludes TH+ neurons of the VTA based on the anatomy defined by the MTN. By narrowing the focus of analysis toward an area relatively dense in nigral dopaminergic neurons infected with AAV, differences between the injected and uninjected sides become easier to detect, since the suppressing effect of irrelevant background is minimized.

The widespread mediolateral SN coverage pattern in MTN-containing sections was observed at a relatively early time point of three and four weeks post-injection (as in Figs. 8-10, and Figs. 7 & 11, respectively). Although this is the only time window of AAV expression reported here, others have reported sustained protein expression with AAV1/2 at six\textsuperscript{33} and
twenty-four\textsuperscript{110,115} weeks after injection in rat brain. AAV expression in humans has been shown to last even longer: in one patient enrolled in a hemophilia gene therapy trial, AAV2 continued expressing after 10 years in non-dividing cells (skeletal muscle)\textsuperscript{116}.

Based on the spatiotemporal expression of GFP, we expect that AAV-shBAG5 vectors will also be able to deliver widespread and sustained expression of shRNA well beyond the demonstrated three-week window. The ability to produce sustained BAG5 knockdown over several months would allow these vectors to be delivered in combination with one of several rat models of PD in which pathology and behavioural deficits may take several weeks to fully develop\textsuperscript{33,111,117} (also see section 3.3). It also better supports experimental designs where AAV-shBAG5 is injected prior to the induction of the PD model, rather than a simultaneous or delayed injection.

There are several limitations to narrowing the analysis of the SN to MTN-containing sections. Firstly, this approach ignores SN in non-MTN containing sections, which together represent a large part of the SN structure. We did not formally evaluate or confirm BAG5 knockdown on sections without MTN. If BAG5 is only knocked down in MTN-containing sections and not elsewhere, the functional consequences of BAG5 knockdown may be difficult to detect. Furthermore, it is possible that BAG5 knockdown in different regions of the SN can have different effects on the animal, and certain regions may be more critical for normal physiological functioning than others. Going forward, it may be important to analyze the other regions of the SN to see how a single injection at our target coordinates affects BAG5 knockdown and pathology. If knockdown is insufficient in some areas of the SN, multiple injections and/or increased injection titer may be needed. Based on our observations however, most brains injected
with AAV-shBAG5 have displayed fairly comprehensive anteroposterior SN coverage, which should alleviate some of the concerns outlined above.

2.4.4 ImageJ fluorescence semi-quantification method

We have been unable to find methods in the literature to rapidly quantify or semi-quantify protein knockdown in the SN. In fact, many studies do not quantify in vivo knockdown at all, possibly due to the technical challenges involved.

We sought to develop a relatively rapid and unbiased approach to compare levels of BAG5 protein in the injected SN versus the contralateral SN, free from observer bias and more quantitative than a simple visual assessment of staining intensity. The “find maxima” function in ImageJ\textsuperscript{109} identifies local intensity maxima within a user-specified region of interest. This simple and efficient method fairly consistently produces results that match relative staining intensity as judged by eye (albeit subjectively). It is more reliable and less subject to human bias compared to sampling-based strategies, and is better able to capture small differences compared to mean intensity based methods, in which non-specific/background staining tends to wash out intensity differences between the two SN. This issue is especially problematic in the case of BAG5, which does not stain very strongly by immunofluorescence. In addition, unlike mean intensity based methods, the maxima method is mostly unaffected by technical imperfections such as tissue cracking or overlapping.

One caveat of the maxima method is that it becomes unreliable when the staining is extremely weak (i.e. almost indistinguishable from background), as this results in a low number of total maxima, which could more easily skew the ratio of maxima between the injected and uninjected SN. For this reason we have excluded such sections from the analysis. In addition, due to the relatively weak detection of BAG5 by immunostaining, SN images must be captured
at 100x magnification to ensure clarity. However, it is not possible to capture the entire SN in a single image at this magnification with the equipment available to us (only around 80% of the SN can be captured at this magnification), thus excluding analysis of the most medial and most lateral portions of the structure. The maxima method somewhat relies on the assumption that the areas of high light intensity in a given area are spread out. For instance, if all the areas of intensity are very tightly clustered together, this method will report a low number of total maxima, even if the mean intensity in the area is very high.

2.4.5 Analysis of BAG5 knockdown in rat SN

At both the 1/2 and 1/3 titer, the BAG5 targeting AAVs had very similar injected/uninjected ratios, suggesting they have a similar knockdown efficiency. At both tested titers, the targeting AAVs resulted in significantly greater knockdown than the non-targeting Control, according to the maxima analysis method. Though we did not quantify magnitude of knockdown, our roughly estimated knockdown of 50% for both targeting AAVs is similar to previous reports of in vivo intranigral delivery of shRNA against αSyn (35% knockdown with AAV2)\textsuperscript{13} and interleukin-1 receptor (40% knockdown with lentivirus)\textsuperscript{15}. Unlike our non-targeting Control AAV, control vectors in these two studies resulted in no appreciable knockdown of the target protein, as determined by single-cell densitometry analysis.

The maxima count does not necessarily scale linearly with the total brightness in a given image or region of interest, because it depends on the pattern of intensity distribution throughout the ROI. Thus, the maxima ratio between injected and uninjected SN derived from this method should not be interpreted as the magnitude of knockdown. Instead, it provides an unbiased method to support protein knockdown already observed by eye.
In theory the maxima counts in uninjected SN and SN injected with AAV-shBAG5 Control should be the same, since the Control shRNA should not produce BAG5 knockdown. As a result, the maxima ratio between injected and uninjected SN for Control AAV brains should theoretically be 1. This ratio was indeed very close to 1 when Control AAV was injected at 1/3 dilution (Fig. 10). At 1/2 dilution however, the mean injected to uninjected ratio across SN pairs of 0.62 suggests that BAG5 signal tended to be weaker in the injected SN vs. the uninjected SN (Fig. 9). One potential explanation is that the Control shRNA had some off-target effect leading to down-regulation of BAG. Partial complementation between an shRNA targeting sequence and a stretch of mRNA can result in translation repression or mRNA degradation\textsuperscript{67,118}. This type of “specific off-target effect” can depend on the dose of shRNA\textsuperscript{119}, which fits our observations of apparent knockdown in the higher dilution only. As well, cells that are overloaded with siRNA/shRNA may exhibit “non-specific off-target effects” as a result of endogenous miRNAs, which normally help control gene expression, being displaced from RISC\textsuperscript{120}. Again, this would fit our observation that only the higher titer of Control AAV results in apparent BAG5 knockdown.

2.4.6 Alternative methods to measure BAG5 knockdown in vivo

It is also possible to quantitatively assess BAG5 knockdown in vivo, though these methods tend to be more complicated and time-consuming. One such method is single-cell densitometry analysis\textsuperscript{13,15}, a technique in which many individual cells in each SN would be imaged followed by densitometry analysis of each cell. Single-cell densitometry has been used to quantify levels of αSyn\textsuperscript{13} and interleukin-1 receptor 1\textsuperscript{15} in rat SN following delivery of shRNA against those molecules. However, it relies on having a strong antibody signal and may still be subject to selection bias when the individual cells are selected. Since BAG5 displays a fairly weak signal by immunostaining, single-cell densitometry may not be the most appropriate
method for our purposes. Another option for quantifying BAG5 knockdown in the SN is to dissect out the SN and western blot the tissue lysate\textsuperscript{121,122}. This option surveys the entire SN without having to select a sample of cells, and may function without a strong antibody. It may be a viable way to further validate BAG5 knockdown in rat SN. As a third option is to use laser capture micro-dissection to excise individual cells from the SN, followed by protein (or RNA) analysis\textsuperscript{123,124}. One benefit of this technique is the ability to analyze only infected neurons, avoiding other cell types and uninfected cells, which could dilute the measured knockdown effect.

As previously suggested, an alternate approach is to examine BAG5 mRNA rather than protein. Analyzing the levels of BAG5 mRNA more directly measures the effects of shRNA, and would be a valuable complement to protein analysis. One of the most popular techniques to quantify a specific mRNA molecule from a tissue sample is qPCR. Another option is \textit{in situ} hybridization (followed by quantification), which can show the localization of the mRNA of interest, unlike traditional PCR methods that require RNA isolation. However, this technique is also limited by the nature of overlapping cells of mixed populations in tissue sections.

\textbf{2.4.7 Potential toxicity from GFP overexpression}

As shown by both western blot of infected primary hippocampal cells (Fig. 5) and by visual inspection of sections from AAV-shBAG5 injected SN (Figs. 8-10), the Control AAV tends to produce greater GFP expression than either of the two targeting AAVs, which appear to produce comparable levels of GFP expression compared to each other. The reason for the difference in GFP expression is unclear, given that all three AAVs are completely identical in sequence, aside from the shRNA targeting sequence. One possibility is that the Control AAV had a greater functional titer (i.e. more AAV able to infect cells) than the other AAVs, despite all
three AAVs having the same physical titer (in terms of genomic particles per mL). Another explanation could be that both shRNA targeting mRNAs exhibit off-target effects that reduce GFP expression.

High levels of GFP expression have been reported to be toxic to dopaminergic neurons in the rat SN, in a dose-dependent fashion\textsuperscript{33,111,125}. Koprich \textit{et al} reported that delivery of AAV1/2-GFP vector to the rat SN produced a 24% loss of TH+ neurons at three weeks post-injection using a titer of $5.1 \times 10^{12}$ gp/mL, but no loss was observed when AAV titer was diluted down to $1.7 \times 10^{12}$ gp/mL\textsuperscript{33,111}. We chose to use relatively low injection titers in an attempt to avoid GFP-associated toxicity. Our higher titer ($5.5 \times 10^{11}$ gp/mL) was more than three times less concentrated than the “safe” titer of $1.7 \times 10^{12}$ gp/mL reported by Koprich \textit{et al}\textsuperscript{111}. Despite this precautionary measure, it is possible that the reduction of BAG5 in SN injected with our higher titer of Control AAV is (at least partially) a result of GFP-mediated toxicity (Fig. 9). If this is indeed the case, the efficiency of BAG5 knockdown may be even greater than originally observed. The Control AAV (presumably causing increased cell death) would reduce the amount of BAG5 available for detection, but the targeting AAVs (presumably resulting in less cell death) still resulted in apparent knockdown compared to Control.

The intended purpose of the AAV-shBAG5 Control vector was to control for both shRNA and GFP expression. However, assuming the functional titers of all three AAVs are identical, the GFP expression difference makes it such that the Control vector is only (presumably) appropriately controlling for shRNA load, but not GFP load. It is important for the AAVs to co-express a marker in order to easily confirm and visualize infection and expression, as shRNA expression itself is difficult to detect. Ideally a less toxic expression marker could be used, but GFP already seems to be the marker of choice, with relatively low toxicity.
Unfortunately, dopaminergic neurons tend to be particularly susceptible to insult\textsuperscript{125}, so other protein markers may present the same problem. AAV titer can always be reduced further, but this risks insufficient shRNA expression to achieve a detectable effect.

2.4.8 Effects of AAV-shBAG5 on TH expression

The effect of AAV-shBAG5 vectors on TH immunoreactivity was found to be highly variable across brain sections and animals, with either injection titer. We did not formally quantify TH expression in the immunofluorescence-stained sections (Figs. 9 & 10) partly for this reason. While many sections showed robust and equal levels of TH immunoreactivity in both SN (with any of the three AAVs), several sections also showed very poor TH staining. The TH signal in these sections appeared ghostlike, with the fluorescence signal in apparently TH+ cells being significantly weaker than truly TH+ cells in the same section or same animal.

This issue may be related to tissue damage resulting from the injection of several microliters of liquid into the brain parenchyma. Indeed, the “ghosting” phenomenon was observed mainly in sections near the injection site and in the middle of the mediolateral extent of the SN structure, where the injections were targeted. The mediolateral and anteroposterior extremes of the SN structure tended to be relatively spared.

We stained TH with two fluorophores, Alexa 594 (red) and Alexa 350 (blue) to try and rule out problems with a specific fluorophore or fluorescence channel, but the results were identical in both channels. The pattern of TH immunoreactivity following AAV-shBAG5 injection will require further investigation with a larger sample size.
2.4.9 Effects of pre-injected AAV-shBAG5 following MFBx

Seven days following MFBx, AAV-shBAG5 Control-injected rats demonstrated a 42% preservation of TH immunoreactivity (Fig. 11). This figure is very similar to the report by Crocker et al of 44% TH preservation using adeno-virus overexpressing β-galactosidase (as a control in their study)\(^2^3\). However, others have reported that in non-injected SNc at 7 days post-MFBx, TH immunoreactivity only falls to around 70% of contralateral\(^2^4,^2^5\), implying that virus injection and/or protein overexpression does result in some toxicity in rat SN.

Despite this apparent toxicity, both targeting AAVs significantly mitigated TH loss following MFBx, suggesting a protective effect of BAG5 knockdown (Fig. 11). In support of this, clone C significantly preserved loss of mature neurons (NeuN+) in the injected SN, while clone G1 trended toward reducing NeuN loss. This difference between the two targeting AAVs could be due to a lack of statistical power, in which case future studies may consider expanding the sample size. It is also possible that the two targeting AAVs have slightly different off-target effects that impact neuronal survival/NeuN expression, since the shRNA targeting sequences are different.

These results again confirm successful BAG5 knockdown and demonstrate that the levels of BAG5 knockdown produced by our AAVs, though not formally quantified, were sufficient to achieve measurable and relevant biological effects. Additionally, they show that the protective effects of the targeting AAVs in the MFBx model outweigh potential GFP-mediated toxicity at the 1/2 titer.
Chapter 3: General Discussion and Conclusion

3.1 Summary

The aim of this thesis was to develop and characterize a viral vector that could produce shRNA-mediated BAG5 knockdown in rat substantia nigra. We set out to accomplish this in two chapters. In chapter 1, we identified efficient shRNAs against BAG5 from a pool of candidates and packaged them, along with a non-targeting Control, in lentivirus and AAV vectors. We confirmed that the AAVs were able to infect primary neurons and knock down BAG5.

In chapter 2, we tested the LV and AAV vectors in rats, but discovered that the LVs did not express in the rat brain, despite LV being an established vector for gene delivery in the rat brain. By contrast, the AAV vectors achieved strong expression throughout the extent of the substantia nigra. Upon analysis of sections close to the injection site, both clones of targeting AAV-shBAG5 were able to knock down BAG5 at two different dilutions. Finally, pre-injection of either targeting AAV-shBAG5 vectors resulted in significant protection from axotomy-induced dopaminergic degeneration.

To our knowledge, this is work is among the first to study shRNA delivery to the rat substantia nigra using the AAV1/2 serotype. The only published study to date using specifically AAV1/2 to deliver shRNA in vivo targeted the red nucleus in rats. Our work contributes to a rapidly emerging body of research demonstrating the effectiveness of AAV1/2 as an shRNA delivery vector in animal experiments. As gene therapy delivery research moves forward, it will be important to understand the performance of different AAV serotypes in vivo as one of several dimensions of spatiotemporal control.
3.2 Limitations

Firstly, it is worth noting that we studied knockdown but not knockout, and these two conditions may have different phenotypes\textsuperscript{67}. We did not formally quantify BAG5 knockdown, and did not formally demonstrate knockdown throughout the entire SN, since we only analyzed MTN-containing sections. Thus, it is possible that our current injection target and titer does produce sufficiently great or widespread knockdown to exert the full phenotypic effects of BAG5 inhibition. Performing multiple injections or increasing titer may resolve some of these potential issues, but these modifications come with their own risks, such as increased toxicity from GFP load, or physical damage to the tissue from injecting larger volumes of liquid.

Despite having demonstrated some protective effects of BAG5 knockdown with histological analyses, we did not show that this effect translates to functional improvement with behavioural tests. Again, it is thus unclear how impactful our attained levels of BAG5 knockdown will be in terms of improving behavioural deficits when applied in animal models of PD.

As with any animal model, the MFBx model is limited in how well it can represent human disease states. Other rat models that better recapitulate some of the molecular features of PD, such as the presence of human αSyn, have recently gained favour over MFBx as models of PD in rats. However, MFBx is still a useful model of dopaminergic degeneration, and we have shown that BAG5 knockdown does have biologically relevant consequences in the context of this particular injury. Accordingly, it has potential to protect dopaminergic neurons in other contexts of insult, including toxin and protein overexpression-based models of Parkinson’s disease, though this remains an open question for future investigation.
3.3 Future Directions

3.3.1 Human A53T-SNCA overexpression model of PD

Having established successful BAG5 knockdown using our AAV-shBAG5 vectors, and demonstrated protective effects in the MFBx model of dopaminergic injury, the next step would be to test this therapeutic strategy in a rat model of PD. The human A53T mutant αSyn overexpression model of PD (delivered by AAV1/2) is well characterized in terms of motor phenotype and timeframe of dopaminergic neuron loss, both of which are viral titer-dependent\(^{33,111}\). We attempted to test the effects of BAG5 knockdown in this model by co-injecting our AAV-shBAG5 vectors with AAV-A53T-SNCA. However, at 6 weeks post-injection, the hA53T-SNCA overexpression model did not behave as previously characterized in terms of behaviour and pathology\(^{33,111}\), so we were unable to interpret the influence of BAG5 knockdown in this system (data not shown). Thus some technical challenges remain to be addressed before testing our AAV-shBAG5 vectors in this model.

3.3.2 VenusYFP-SNCA bimolecular protein complementation system

The αSyn bimolecular protein complementation system can be used as another rat model of PD\(^{117}\). In this model, an AAV encoding the N-terminus half of VenusYFP fused to full-length wild-type human αSyn is co-injected with an AAV encoding αSyn fused to the C-terminus half of VenusYFP. When the protein products of both AAVs are expressed in the same cell, the oligomerization of αSyn joins the halves of Venus and produces a fluorescent signal, allowing for the visualization of αSyn oligomers \textit{in vivo}. This complementation system has previously been implemented in rats using an AAV8 vector for delivery\(^{117}\). We are currently working on characterizing the behavioural and histopathological outcomes of this model of PD when delivered using AAV1/2 vector. After this, as a future experiment, we could pair this Venus-αSyn model with our AAV-shBAG5 vectors (expressing RFP rather than GFP to avoid
confusion with Venus signal) to evaluate the effects of BAG5 knockdown on motor behaviour (by rescue of forepaw asymmetry), dopaminergic cell death, and synuclein pathology.

3.4 Conclusion

We have created and characterized an AAV1/2 vector capable of selectively and quite comprehensively transducing neurons in the rat substantia nigra, producing substantial knockdown of BAG5 in neurons as early as three weeks post-injection. This work establishes the foundation for future investigations into the role of BAG5 in the SN, especially in relation to neurodegeneration and Parkinson’s disease.
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