2015

Regulation of alpha-synuclein expression through beta-2-adrenoreceptor agonists: a novel approach towards treating Parkinson's disease

https://hdl.handle.net/2144/16203
Boston University
BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

REGULATION OF ALPHA-SYNUCLEIN EXPRESSION THROUGH BETA-2-ADRENORECEPTOR AGONISTS: A NOVEL APPROACH TOWARDS TREATING PARKINSON’S DISEASE

by

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B.S., University of Alabama, 2013

Submitted in partial fulfillment of the requirements for the degree of
Master of Science

2015
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I would like to dedicate this work to my late grandfather, Gregory Long, who suffered from Parkinson’s disease.
ACKNOWLEDGMENTS

I would like to thank every member of the Scherzer Lab for creating such a welcoming environment. I would especially like to thank Clemens Scherzer for his constant support and generosity and Shuchi Mittal for her patience and understanding. I learned an incredible amount working with Shuchi every day and would not have been able to successfully complete this without her.
ABSTRACT

The population of patients with Parkinson’s disease, already the second most common neurodegenerative disorder, is continuing to grow. Despite years of research, no cure or clear pathogenic pathway has been discovered. However, the \textit{SNCA} gene and its protein product, $\alpha$-synuclein, have emerged as an important focus in both inherited and sporadic Parkinson’s disease. Dosage effects created by duplication and triplication of the \textit{SNCA} locus can cause the death of dopaminergic neurons in the brain. Naturally occurring overexpression of $\alpha$-synuclein has been found to have the same devastating consequences. Most current drug development has focused on alleviating the overproduction of $\alpha$-synuclein, instead of stopping it. We have hypothesized that by repressing endogenous \textit{SNCA} gene expression at the transcription level we can prevent overexpression of $\alpha$-synuclein and its associated toxicity. The discovery that $\beta_2$-agonists, specifically clenbuterol hydrochloride, can reduce \textit{SNCA} mRNA abundance and protein expression has implicated the $\beta_2$-adrenergic receptor pathway as a potential regulatory target. We have further found that clenbuterol causes hypoacetylation of histone H3 that may downregulate \textit{SNCA} expression. Although, the precise mechanism by which $\beta_2$-agonists are regulating \textit{SNCA} expression needs to be further explained, our findings
present exciting data that could potentially lead to a novel treatment for not just Parkinson’s disease, but other synucleinopathies as well.
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LIST OF ABBREVIATIONS

AADC ................................................................. L-aromatic amino acid decarboxylase
AD ................................................................. autosomal dominant
ADRB2 ..................................................... β2-adrenergic receptor
ALS .................................................. Amyotrophic Lateral Sclerosis (Lou Gehrig’s disease)
AR ................................................................. autosomal recessive
AS ................................................................. α-synuclein
ATP ................................................................. adenosine triphosphate
ATP6AP2 .................................................. ATPase H+ transporting lysosomal accessory protein 2
β2AR ................................................................. protein phosphatase 2A
cAMP ................................................................. cyclic adenosine monophosphate
ChIP ................................................................. chromatin immunoprecipitation
COQ2 ........................................................... coenzyme Q2 4-hydroxybenzoate polyprenyltransferase
DBS ................................................................. deep brain stimulation
DLB ................................................................. Dementia with Lewy bodies
DMSO ................................................................. dimethyl sulfoxide
DNAJ6 ............................................................ dnaJ homolog subfamily C member 6
DNAJC13 .......................................................... dnaJ homolog subfamily C member 13
DPBS ................................................................. phosphate-buffered saline
EIF4GI ............................................................ eukaryotic translation initiation factor 4 gamma 1
ELISA ................................................................. enzyme-linked immunosorbent assay
GTP ................................................................. guanosine triphosphate
GWA .......................................................... genome-wide association studies
GWL .......................................................... genome-wide linkage
HDAC .......................................................... histone deacetylase
L .......................................................... lysine
LBs .................................................................. Lewy bodies
LNs .................................................................. Lewy neurites
LRRK2 .................................................. leucine-rich repeat kinase 2
M .......................................................... methionine
MSD ................................................................ Meso Scale Discovery
MPS ................................................................ massive parallel sequencing
PARK2 .......................................................... parkin RBR E3 ubiquitin protein ligase
PARK7 .......................................................... parkinson protein 7
PD .......................................................... Parkinson’s disease
PINK1 ................................................................ PTEN-induced putative kinase 1
PP2A ................................................................ protein phosphatase 2A
S .................................................................. serine
SNCA .................................................. synuclein, alpha (non A4 component of amyloid precursor)
SNP .......................................................... single-nucleotide polymorphism
SNYJ1 .......................................................... synaptojanin 1
TH .......................................................... tyrosine hydroxylase
VPS35 .......................................................... vacuolar protein sorting 35 homolog
Y .......................................................... tyrosine
INTRODUCTION

Parkinson’s disease (PD) is estimated to effect six million people worldwide and more than one million in the United States alone, making it the second most frequent neurodegenerative disorder after Alzheimer’s disease (excluding macular degeneration) (Thomas & Beal, 2007; Lesage & Brice, 2009). Often diagnosed after the sixth decade of life, PD affects more than 1% of the world population aged 65 years and older (Lesage & Brice, 2009; Ozansoy & Başak, 2013). It is predicted that the number of individuals with PD will reach 9.3 million by 2030 due to aging populations, stressing the importance for continued research (Dorsey et al., 2007). PD is the most common form of Parkinsonism, a group of neurological syndromes clinically characterized by four essential motor symptoms – bradykinesia, muscle rigidity, resting tremor, and postural instability. The disease is clinically identified by the presence of these four motor impairments combined with positive responsiveness to levodopa therapy (Verstraeten, Theuns, & Van Broeckhoven, 2015). Gait difficulty is often an exhibited motor problem as well. In addition to these motor symptoms, some PD patients are affected by autonomic, cognitive, and psychiatric problems, which may include depression, sleep disturbances, constipation, hypertension, and even dementia in later stages (Thomas & Beal, 2007; Verstraeten, Theuns, & Van Broeckhoven, 2015).

James Parkinson first described Parkinson’s disease in 1817 in his work “An Essay of the Shaking Palsy.” The movement disorder is associated with the progressive loss of dopaminergic neurons in the nigrostratial pathway, the efferent connection between the
substantia nigra and corpus striatum (Thomas & Beal, 2007). As part of the basal ganglia motor loop, this major dopamine pathway in the brain is involved in the production of movement. Although there is widespread neurodegeneration throughout the entire Central Nervous System, the pathological hallmark of the disease is profound and selective loss of the dopaminergic neurons in the substantia nigra pars compacta in the midbrain with the presence of Lewy bodies (LBs) and Lewy neurites (LNs) in surviving neurons (Thomas & Beal, 2007; Lesage & Brice, 2009). LBs are eosinophilic, intracytoplasmic, and proteinaceous inclusions (Thomas & Beal, 2007). LNs are dystrophic nerve cell processes that contain protein aggregates. They are most widespread in the CA2 and CA3 of the hippocampus and in the substantia nigra. Both LBs and LNs can be identified in the brain using an antibody specific for the main structural component of LBs, \( \alpha \)-synuclein protein (Ozansoy & Başak, 2013; Verstraeten, Theuns, & Van Broeckhoven, 2015). LB pathology is not shared by all clinical PD patients and can found in other neurodegenerative diseases like Alzheimer’s disease and Dementia with Lewy bodies (DLB) (Verstraeten, Theuns, & Van Broeckhoven, 2015; (Beyer & Ariza, 2013).

Degeneration in other monoaminergic cell groups in the brainstem and microglial activation contributes to the development of PD as well (Lesage & Brice, 2009). Symptoms of Parkinson’s disease do not typically show until about 80-90% dopamine function is lost. Motor deficits typically manifest after approximately 80% of striatal dopaminergic neurons and 50% of nigral dopaminergic neurons are lost (Ozansoy & Başak, 2013). The late onset of symptoms suggests that there is likely a large population of undiagnosed patients with PD (Lesage & Brice, 2009). The neurodegeneration caused
by the disease can be described in a six-stage progression. In stages one and two the
damage is restricted to the medulla oblongata, the pontine tegmentum, and the olfactory
bulb. In stages three and four the nuclei in the midbrain, including the substantia nigra,
become affected. Finally in stages five and six the degeneration has spread into the
neocortex (Ozansoy & Başak, 2013).

No cure for Parkinson’s disease has been discovered, nor any methods to slow
progression. Levodopa and dopamine agonists are the most common treatments used to
treat PD. Deep brain stimulation (DBS) is a more severe treatment that is occasionally
employed. All of the available treatments for PD only relieve the symptoms of the
disease; they do not repair the damage or delay the disease’s pathological progress
(Kakkar & Dahiya, 2015). PD is so difficult to treat because it is not fully understood.
While progress has been made, the molecular pathway causing the progression of the
disease and the death of the dopaminergic neurons remains unknown (Thomas & Beal,
2007).

The Genetics of Parkinson’s disease

Once believed to be solely a sporadically acquired disease, over the past twenty
years mutations of several genes were proved responsible for Mendelian forms of
Parkinson’s disease. While epidemiological studies show that less than 10% of cases are
strictly due to familial etiology, the role of genetics is no longer a controversial topic
(Thomas & Beal, 2007). PD is now believed to be a multifactorial disease resulting from
a combination of various environmental and genetics factors (Thomas & Beal, 2007;
Verstraeten, Theuns, & Van Broeckhoven, 2015). No specific environmental risk factor
has been proven to be directly responsible for the pathogenesis of the degenerative disorder (Ozansoy & Başak, 2013). There are no clear genetic links in occurrences considered sporadic, but the pathophysiology of sporadic PD is similar to that of genetically inherited PD. Parkinsonism with nigrostriatal dopaminergic degeneration characterizes both forms of the motor disorder, suggesting common pathogenic mechanisms (Thomas & Beal, 2007). The heritability of PD risk in over 500 nuclear families was analyzed in one study where it was discovered that in up to 60% of idiopathic PD patients, genetic factors could account for the phenotype (Verstraeten, Theuns, & Van Broeckhoven, 2015). So although 90% of patients have PD considered sporadic, understanding the molecular pathways in the genetic cases can lead to great strides in our understanding and treatment of this destructive disease.

When studies into the genetic causes of PD first began, research relied on classical molecular genetic approaches revolving around well-documented case families. Over the past decade exome sequencing has been combined with these classical approaches, leading to the identification of 15 causal genes for Parkinson’s disease and Parkinson-plus syndromes (disorders of multiple system degeneration). Genome-wide association (GWA) studies have determined over 25 genetic risk factors as well. While genetic research has been making strides, an estimated 30% of trait variance remains unexplained (Verstraeten, Theuns, & Van Broeckhoven, 2015).

The significances of the genes and loci recognized as influential in Parkinson’s disease are not always clear, but 5 genes have been established as well-defined causes of monogenic PD: synuclein, alpha (non A4 component of amyloid precursor) (SNCA),
parkin RBR E3 ubiquitin protein ligase (PARK2), parkinson protein 7 (PARK7), PTEN-induced putative kinase 1 (PINK1), and leucine-rich repeat kinase 2 (LRRK2). (Thomas & Beal, 2007; Lesage & Brice, 2009) These have been confirmed through genome-wide linkage (GWL) analyses or homozygosity mapping using highly polymorphic DNA markers followed by positional cloning (Verstraeten, Theuns, & Van Broeckhoven, 2015).

The SNCA gene has 3 identified missense mutations - A53T, A30P, E46K – that are correlated with autosomal dominant (AD) inheritance of PD (Thomas & Beal, 2007). Duplications and triplications of this gene have also been shown to cause this rare dominant form of PD in both familial and sporadic cases (Lesage & Brice, 2009). A fourth missense mutation in the SNCA gene, H50Q, was identified in 2013 (Proukakis et al., 2013). PARK2, PARK7, and PINK1 genes undergo loss-of-function mutations that are associated with autosomal recessive (AR) early onset Parkinsonism (Thomas & Beal, 2007; Lesage & Brice, 2009). Mutations in the LRRK2 gene cause AD Parkinson’s disease. Point mutations have been found in most of the identified domains of the protein and deletion or truncations are not present, suggesting a gain-of-function mechanism (Thomas & Beal, 2007).

Less than 10% of inherited PD can be accredited to a mutation in one of these five genes. Other genetic mutations with unclear roles in PD etiology have been determined. Mutations in eukaryotic translation initiation factor 4 gamma 1 (EIF4GI) were identified as a potential cause of PD, but this is controversial due to a significant number of reported non-penetrant EIF4GI mutation carriers. Six more genes for PD and Parkinson-
plus syndromes were revealed using massive parallel sequencing (MPS) technologies combined with homozygosity mapping in consanguineous families or with GWL analyses in extended pedigrees: *vacuolar protein sorting 35 homolog (VPS35), dnaJ homolog subfamily C member 13 (DNAJC13), dnaJ homolog subfamily C member 6 (DNAJC6), ATPase H⁺ transporting lysosomal accessory protein 2 (ATP6AP2), synaptojanin 1 (SYNJ1), and coenzyme Q2 4-hydroxybenzoate polyprenyltransferase (COQ2).

The **SNCA** Gene

The **SNCA** gene encodes α-synuclein, a small soluble protein abundantly expressed in the brain (Ozansoy & Başak, 2013), specifically in the presynaptic terminal of the CNS (Yu, Uéda, & Chan, 2005). This protein product is also expressed in smaller amounts in the heart, muscles, and other tissues. Little is currently known about the trans-acting regulatory pathways and factors that modulate **SNCA** gene expression. The **GATA**-1 and **GATA**-2 transcription factors were discovered to directly regulate **SNCA** transcription via a conserved intron-1 GATA binding motif (Scherzer et al., 2008). NGF- and bFGF-mediated signal transduction may also regulate **SNCA** expression via the MAP/ERK and PI3 kinase pathways (Clough & Stefanis, 2007).

The **SNCA** gene is located on chromosome 4q22.1 and has six exons. Its protein product is 140 amino acids long and consists of three distinct regions: N terminal (residues 1-33), central (residues 34-100), and C-terminal (residues 101-140) (Ozansoy & Başak, 2013; Beyer & Ariza, 2013). Seven imperfect repeats of 11 amino acids containing the highly conserved KTKEGV motif are responsible for the formation of a N-terminal helix the
binds α-synuclein to membranes and a central helix that mediates protein-protein interactions. The hydrophobic central region encompasses the NAC domain (residues 61-95), considered the aggregation-prone component. The region between residues 71 and 82 is vital for α-synuclein fibrillization (Beyer & Ariza, 2013). The acidic C-terminal region contains an aggregation inhibition component induced by the low net charge of the protein (Ozansoy & Başak, 2013; Thomas & Beal, 2007). Formally believed to be natively unfolded, it is now evident that α-synuclein exists physiologically as a folded tetramer of 58kDa and does not show amyloid-like aggregation but is in fact aggregation-resistant, suggesting that the destabilization rather than the misfolding and aggregation of α-synuclein may play an important role in PD pathology (Beyer & Ariza, 2013; Ozansoy & Başak, 2013).

The neuronal protein is known to be vital to normal synaptic function, but has also been strongly linked to neurodegeneration with mutations resulting in PD and the aggregated protein being the foremost component of LBs found in sporadic PD (Vekrellis, Rideout, & Stefanis, 2004). The exact function of α-synuclein is somewhat obscure. It has been shown to be involved in dopamine metabolism, specifically in dopamine synthesis, storage, release, and uptake (Yu, Uéda, & Chan, 2005). Alpha-synuclein may be linked to apoptosis in the presence of reactive oxygen species and it may induce the fibrillation of tau protein leading to the filamentous and pathological inclusions found in PD. It has also been discovered that α-synuclein influences the trafficking of presynaptic vesicles, specifically the SNARE complex assembly. It is believed that it may function as a chaperone protein in a manner similar to the 14-3-3 protein (Ozansoy & Başak, 2013).
Alpha-synuclein has a possible influence on regulation of gene expression as well. Evidence supporting this includes localization of the molecule in the nuclei of normal brain neurons, nuclear translation in response to insults, and changes in gene expressions in cells with α-synuclein overexpression (Yu, Uéda, & Chan, 2005).

Alpha-synuclein interacts with the rate-limiting enzyme in dopamine synthesis, tyrosine hydroxylase (TH). Binding of α-synuclein to TH prevents phosphorylation, increasing the protein phosphatase 2A (PP2A) activity and thus, inhibits TH activation. In this manner overexpression of α-synuclein reduces TH activity, while low levels of α-synuclein leads to an increase in TH activity through higher levels of TH phosphorylation. Alpha-synuclein also interacts with L-aromatic amino acid decarboxylase (AADC), an enzyme that catalyzes the conversion of L-DOPA to dopamine. The protein inhibits phosphorylation of AADC, so the enzyme’s activity is reduced when α-synuclein is overexpressed. It is possible that even though α-synuclein is widely expressed throughout the entire brain, that dopaminergic neurons are most effected because these cells are most vulnerable due to the proteins involvement in dopamine metabolism. It is likely that age is a crucial factor in the maintenance of dopamine metabolism as well, lending an explanation to the age group most afflicted with PD (Ozansoy & Başak, 2013).

\textit{SNCA} was the first gene linked to PD, specifically the PARK1 locus. It was discovered through the Contursi kindred, a linkage analysis of a large multigenerational family exhibiting PD in an autosomal dominant inheritance pattern. PD attributed to \textit{SNCA} is early onset and rapidly progressing. Two different loci have been described on the gene, PARK1 and PARK 4. Three missense mutations, duplications, and triplications
have proven to be significant. The A53T mutation was identified in the Contursi kindred, the A30P mutation in a German family, and the E46K mutation in a Spanish family. Multiplications of the normal α-synuclein gene are considered rare but are noteworthy and prove that the wild-type α-synuclein is pathogenic if overexpressed, not just the mutated protein (Ozansoy & Başak, 2013).

The clinical progression of SNCA-linked PD varies based on the mutation present. The A53T and E46K point mutations cause similar progression marked by early onset and severe Parkinsonism with frequent dementia. The A30P mutation typically causes a late-onset form of PD with mild dementia that comes much later. SNCA duplications are responsible for late onset PD that shows dopa-responsiveness. SNCA triplications lead to a more severe form of PD with dementia that is earlier onset and rapidly progresses. It is possible that the differences in development of PD are caused by dosage differences of SNCA (Ozansoy & Başak, 2013). In patients with familial PD due to a duplication or triplication of the SNCA locus, copies of functionally normal SNCA mRNA and subsequent protein level in brain in blood are increased by 50-100% (Miller et al., 2004).

Research has shown that posttranslational modifications and alternative splicing of α-synuclein may trigger neurodegeneration that leads to Parkinson’s disease. The aggregation-prone properties of α-synuclein are augmented by posttranslational modifications including phosphorylation, ubiquination, nitration, and truncation. This is supported by the ample amounts of phosphorylated, monoubiquinated, and nitrated α-synuclein protein found in LBs (Beyer & Ariza, 2013).
The most common and likely most important posttranslational modification of α-synuclein is protein phosphorylation. Serine (S) residues on α-synuclein are constitutively phosphorylated with S129 being most significant site and S87 being a secondarily noteworthy site. It was found that greater than 90% of insoluble α-synuclein in DLB brains was phosphorylated at S129, while only 4% of normal α-synuclein was phosphorylated, implying the extreme phosphorylation is a meaningful pathogenic event. Tyrosine (Y) phosphorylation has also been noted at Y125, Y133, and Y135 (Beyer & Ariza, 2013).

Alpha-synuclein purified from LBs is partly ubiquinated, and probably occurs at three lysine (K) residues *in vivo* - K6, K10, and K12. *SIAH-1*, *SIAH-2*, and *CHIP* are the E3 ligases determined to able to ubiquinated α-synuclein and have all been found accompanying the aggregated protein in LBs (Beyer & Ariza, 2013).

Four tyrosine residues on α-synuclein – Y39, Y125, Y133, and Y136 – are nitrated under enhanced oxidative stress. Four methionine (M) residues – M1, M5, M116, and M127 – are modified by oxidation as well. It has been confirmed that nitrated α-synuclein can be neurotoxic and accelerate the death of dopaminergic neurons (Beyer & Ariza, 2013).

Truncated α-synuclein makes up about 15% of α-synuclein species in LBs. Cell and animal modes have repeatedly shown that truncation of the C-terminal of α-synuclein enhances the protein’s fibril assembly and stimulates full length species to aggregate. Proteasomal and lysosomal pathways have been associated with this truncation of α-

Alternative splicing of α-synuclein (AS) results in four related forms of the α-synuclein protein. Of the six exons that make up the protein, exon 1, 2a, 2b, and 2c are changeably included as 5’ UTR in different transcripts while exons 3-6 are transcribed. Exons 4 and 6 are alternatively spliced resulting in at least four transcripts for each of the four 5’UTR differing isoforms of α-synuclein. The main α-synuclein variant is the normal AS140 in which all exons are encoded. When exon 6 is left out of the transcript, the C-terminal amino acids 103-130 are deleted giving rise to AS112. Overexpression of AS112 in a human dopaminergic cell line led to inhibited proteasomal function and induced cell death. Splicing out of exon 4 results in AS126 created by a 14 amino acid deletion alters both the N-terminal helices and the central helix. This isoform has a lower net charge than the normal AS140 protein that reduces aggregation and lends protection. The function of this isoform in the brain is currently unknown. When both exon 4 and exon 6 are spliced out α-synuclein, the AS98 isoform with a very shortened N-terminal and an interrupted C-terminal helical domain is created. Like AS112, AS98 is aggregation-prone. Overexpression of this shortest variant of α-synuclein seems to enhance the susceptibility of neurons to oxidative insults by increasing steady-state levels of oxidative stress (Beyer & Ariza, 2013).

GWA studies have recognized four other genetic variants of the \textit{SNCA} gene, in addition to the four confirmed mutations, that may present susceptibility to the development of PD. These variants can cause distorted control of transcription levels of
the SNCA gene, regulation of alternative splicing, or mRNA stability in post-transcriptional mechanisms. A polymorphic dinucleotide repeat site, REP1, located 10kb upstream of the transcriptional start site of SNCA affects the expression level of SNCA (Ozansoy & Başak, 2013). Three single-nucleotide polymorphisms (SNPs) can modify AS112 and AS140 mRNA expression rates in the frontal cortex. At the 3’end is rs356219, the 3’UTR is rs365165, and in intron 5 is rs2736990 (Beyer & Ariza, 2013).

**β2-Adrenergic Receptors**

The evident role of α-synuclein to the pathogenesis of PD has made it the focus of most current Parkinson’s research. The overexpression of α-synuclein in particular has been shown to lead to neurodegeneration through both aggregation and dosage effects (Ozansoy & Başak, 2013). Using an innovate gene expression high-throughput screening method for identifying small molecules that repress SNCA gene expression in neuroblastoma cells, my lab has identified six drugs that repress the expression SNCA at the transcriptional level. β-adrenergic agonists, specifically β2-agonists, emerged as a class of drugs that could possibly regulate α-synuclein mRNA levels.

Adrenergic receptors are a class of cellular receptors shown to regulate the response of the sympathetic nervous system through their interactions with catecholamines such as epinephrine and norepinephrine. There are two discrete subtypes of adrenergic receptors, α and β, distinguished by the proteins with which they interact (Stiles, Caron, & Lefkowitz, 1984). β-receptors are further subdivided into β1-, β2-, and β3-receptors. They are typical G-protein coupled receptors (Johnson, 2001).
A gene on the long arm of chromosome 5 encodes the human β-adrenergic receptor. It is member of the 7-transmembrane family of receptors and is composed of 413 amino acids. β2-adrenoceptors (β2AR) exist in both active and inactive states. Their activation is mediated by increases in intracellular cyclic adenosine monophosphate (cAMP) levels. β2ARs are associated with the α-subunit of the Gs protein and a molecule of guanosine triphosphate (GTP) in the active state. When guanosine diphosphate replaces GTP it catalyzes the conversion of adenosine triphosphate (ATP) to cAMP and significantly lowers the affinity of the α-subunit for the receptor, causing it to dissociate and return to its inactive, low-energy state. It is likely that β2-agonists do not cause a conformation change in the receptor, but that they bind to receptors in their activated state, temporarily stabilizing them. β-antagonists move the receptor equilibrium away toward the inactive form by binding with high affinity to the inactive, low-energy form of the receptor (Johnson, 2001).

β2ARs are widely expressed in the brain, including the substantia nigra, the main region of the brain affected by PD (Rainbow, Parsons, & Wolfe, 1984). Both β1-receptors and β2-receptors have been found in the brain and the spinal cords of humans. Autoradiographic distribution revealed that the cerebellum and the hippocampus had the densest distribution of receptors. Moderate densities were found in the thalamic nuclei, midbrain, basal ganglia, and cerebral cortex. The substantia nigra showed 38.4 ± 4.00 fmol/mg iodopindolol protein binding to β-receptors during the autoradiographic study (Reznikoff, Manaker, Rhodes, Winokur, & Rainbow, 1986). In mice, quantitative radiography selectively labeling each β-receptor subtype revealed high levels of β2 ARs
were found in the cerebellum, thalamic nuclei, olfactory tubercle, cerebral cortex, medial preoptic nucleus, nuclei of the medulla, and substantia nigra (Rainbow, Parsons, & Wolfe, 1984). A similar distribution of β-receptors was demonstrated in rat and monkey models as well (Nicholas, Pieribone, & Hökfelt, 1993; Slesinger et al., 1988). A study on post-mortem human brains discovered that the distribution of both β-receptor subtypes was in agreement with the findings in mouse, rat, and monkey brains (De Paermentier, Cheetham, Crompton, & Horton, 1989).

The number of people affected by Parkinson’s disease continues to grow every year as people continue to live longer and the population continues to age. With no known cure and limited understanding of the pathogenesis of this prevalent neurodegenerative disease. It is important that research continues to search for answers and ways to treat the progressive movement disorder. Genetic studies have demonstrated that PD is not only a sporadic event and have created new avenues for scientists to explore. While familial PD only makes up a small percentage of cases, it has been shown that similar pathways and pathogenic events likely cause both familial and sporadic PD. *SNCA* has emerged as one of the genetic links that has a significant role in sporadic PD pathogenesis. Its protein product, α-synuclein has been established as a central topic in current PD research, as it makes up LBs and can cause neurodegeneration when overexpressed. Most drug development efforts have focused on dealing with the consequences of α-synuclein overproduction by clearing or breaking down aggregation, preventing transformation into toxic species, or lessening the toxicity symptoms. We
instead have decided to tackle the problem of α-synuclein overexpression at its source.

We have hypothesized that the most direct and precise solution is to find a compound that reduces SNCA expression in order to prevent excessive transcription and eventual overexpression and aggregation. A specialized drug screening revealed that six drugs have can repress SNCA expression, with the exciting discovery that β₂AR agonists can do so significantly.
METHODS

Cells and Reagents
Human neuroblastoma SK-N-MC cells were acquired from American Type Culture Collection. All drugs were from Sigma. High Capacity cDNA Reverse Transcription and Taqman universal qPCR reagents were from Applied Biosystems.

Cell Treatments and qPCR
Approximately 1.5x10^4 SK-N-MC cells per well were plated in 6-well culture plates and allowed to grow for 96 hours. Cells were independently treated with three β2AR agonists - metaproterenol (10uM), clenbuterol (10uM), and salbutamol (5um); a β2AR antagonist - propranolol (100uM); a positive control - valproic acid (200um); and a negative control – dimethyl sulfoxide (DMSO). Cells were also treated with each β2AR agonist + propranolol. After incubating for 48 hours cells were washing once in cold phosphate-buffered saline and then lysed at room temperature with lysis buffer (Applied Biosystems) using the Cell-to-CT procedures according to the manufacturer’s instructions (Applied Biosystems). After 5 min, lysis was terminated by incubation with stop solution (Applied Biosystems). 11μL of each lysate was transferred to 384-well plates for RT in a total volume of 25μl. 4μL of RT reaction was then transferred to new 384-well plates for qPCR.

Using the Cell-to-CT kit (Applied Biosystems) we performed expression analysis directly from SK-N-MC neuroblastoma cells cultured in 384-well plates through in situ cDNA synthesis. In this miniaturized assay, high copy numbers of SNCA and of endogenous
controls were detected as indicated by raw cycle threshold values of 25.11 ± 0.02 (mean CT value ± SEM) and 20.2 ± 0.02 for SNCA and UBC, respectively. Technical variation (using identical cDNA loaded in multiple wells) and biological variation (cDNA synthesized in situ in each of 384 wells of cultured SK-N-MC catecholaminergic cells treated with DMSO) were low, with coefficients of variation of 1% and 5% respectively. Cells were plated and grown again in the same manner as before. Cells were independently and collectively treated with clenbuterol (10μM), propranolol (100μM), and the positive and negative controls for four days. Medium was changed and the drugs were re-administered after 48 hours. Cells were lysed, collected, and analyzed in the same way.

**Protein Estimation**

Meso Scale Discovery (MSD) assay technology was utilized for endogenous α-synuclein protein estimation. This technology provides a rapid and convenient method for measuring one or more protein targets within a single small volume of sample. The MSD standard plate was coated with 30μL (100ng) of capture antibody (2F12) diluted in TBS-T. The plate was then incubated at 4°C overnight and blocked with 150μL per well (5% MSD blocker A in TBS-T), sealed, and shaken for 1 hour at room temperature (22–24°C). The plate was then washed three times with TBS-T and 30μL of sample diluted (1:15) in TBS-T was added along with 1% MSD blocker A and 0.5% NP40 and subsequently incubated overnight at 4°C. The plate was washed three times with TBS-T and 30μL of detection antibody (SOY1: 100ng sulfotag antibody) was added to each
well. The plate was incubated on the shaker for 1 hour at room temperature, and then washed with TBS-T. Finally 150 µL of MSD reader buffer (1X) was added and the plate was read.

In a different experiment SK-N-MC cells were treated with clenbuterol, propranolol, and valproic acid. The detection antibody used was Ac-H3 in order to observe the effects of these compounds on the acetylation of histone H3.

Quantitative ChIP Assay

Chromatin immunoprecipitations were completed on SK-N-MC cells the protocol for SimpleChIP® Enzymatic Chromatin IP Kit with magnetic beads from Cell Signaling Technology. Three cell treatments were used – clenbuterol (10uM), valproic acid (100uM), and DMSO. Four immunoprecipitations were completed from each cell treatment – H3K27ac antibody (3uM), H3 antibody (10uM), Rabbit IgG antibody (1uM), and no antibody. H3 acted as the positive control and Rabbit IgG acted as the negative control. Each immunoprecipitation required 4x10⁶ cells. The purified DNA from eluted chromatin was quantified by RT-qPCR using the KAPA SYBR® FAST Universal qPCR Kit from KAPA Biosystems. Ten sets of primers, created by Shuchi Mittal, that probed five areas of the SNCA gene believed to be important acetylation sites were used in the quantitative RT-qPCR.
**Experimental Animals**

Eight-week-old C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME) were obtained for the study. Mice were exposed to a 12-hour light and 12-hour dark cycle and maintained at a constant temperature of 22°C. The mice were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Eight-week-old Sprague Dawley male rats (Jackson Laboratory, Bar Harbor, ME) were obtained for the study as well. They were exposed to the same conditions and cared for in the same manner.

**Experimental Protocols**

Clenbuterol and riluzole were purchased from Sigma (St. Louis, MO). Clenbuterol dissolved in saline was administered to mice intraperitoneally. Saline was administered as a vehicle treatment. Riluzole dissolved in saline was administered to rats intraperitoneally. Saline was administered as a vehicle treatment. Different doses of each drug, ranging from 1mg/kg – 10mg/kg were administered for different experiments. Whole brains were removed at 24, 72, or 96 hours post injection for various experiments. When mice were euthanized with CO₂ at the designated times, brains were isolated immediately and frozen in dry ice. Muscle tissue and blood was also removed and frozen on dry ice. The substantia nigra regions were dissected from the frozen brain tissue. Tissues and blood were stored at -80°C until mRNA extraction and protein extraction were performed.
RNA Extraction

RNA extraction was performed on SK-N-MC neuroblastoma cells, brain and muscle tissue from C57BL/6 mice and brain tissue from Sprague Dawley rats following a modified version of the protocol for TRIzol® Reagent from Life Technologies.

*Tissue Samples:* TRIzol® Reagent was added to individual samples of frozen tissues to saturate and break up the tissue. For larger tissue samples 1.0mL of reagent was used, and for smaller tissue samples 0.5mL of reagent was used. Plastic hand homogenizers were used to grind the tissue into a homogenous liquid. The homogenized samples sat at room temperature for approximately 5 minutes in order to permit complete dissociation of the nucleoprotein complex. Chloroform was added to each sample to induce phase separation, 0.2mL for larger tissues samples and 0.1mL for smaller tissue samples. Tubes were closed and shaken vigorously by hand to mix. Samples were then incubated at room temperature for 3 minutes, and then centrifuged at 10,000 rpm at 4°C for 15 minutes. Mixtures separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA is found entirely in the upper aqueous phase. A pipette was used to remove this upper aqueous phase and transfer the solution to new microcentrifuge tubes. For larger samples at least 0.4mL of solution was collected and for smaller samples at least 0.2mL of solution was collected. The remaining lower and middle phases were stored at -20°C. Isopropanol was added to each sample to stimulate RNA precipitation. The amount of isopropanol used equaled the average amount of supernatant recovered. Samples were centrifuged at 10,000 rpm at 4°C for 10 minutes. Supernatant was discarded leaving only the gel-like RNA pellet found on the bottom side
of the tube. Pellets were washed with 1mL 70% ethanol for larger samples and 0.5mL 70% ethanol for smaller samples. Samples were vortexed briefly and then centrifuged a final time at 14,000 rpm at 4°C for 10 minutes. The supernatant was removed completely leaving only the white RNA pellet found on the bottom side of the tube. Pellets dried at room temperature for 10-15 minutes. Once all remaining ethanol had evaporated off, 25-60uL of RNase-free water was used to resuspend the pellets. Samples were vortexed briefly and spun down before being incubated in a heat block at 65°C for 5 minutes. If samples were not immediately used in downstream applications they were stored at -20°C.

**Adherent Cells:** Medium was carefully vacuumed off of cells grown in 12-well plates. Cells were washed with phosphate-buffered saline (DPBS) and 1.0mL of TRIzol® Reagent was added to each well. Plates were shaken at room temperature for 20 minutes. Solution was transferred to microcentrifuge tubes and steps for tissues samples were followed from this point on, using 0.2mL chloroform and 1.0mL ethanol.

**Protein Extraction**

Protein lysis buffer was made using the following formula: 320mM sucrose + 1uM EGTA + 5mM NaF + 1mM Tris + 1mM EDTA. Protein was extracted from substantia nigra tissue from C57BL/6 mice. 100uL of cold extraction buffer was added to each sample and homogenized by hand. After remaining on ice for ten minutes, the samples were at centrifuged at 10,000rpm at 4°C for 10 minutes. Supernatant was transferred to new tubes and then centrifuged again at 10,000rpm at 4°C for 10 minutes. The final
supernatant containing the protein was transferred to new tubes and measured by enzyme-linked immunosorbent assay (ELISA) and MSD protein estimation.

**Transfection with siRNA**

Approximately $2 \times 10^5$ SK-N-MC neuroblastoma cells per well were plated in 12-well culture plates and allowed to grow for 24 hours. Cells were transfected with either target specific human *ADRB2* siRNA (20nmol) or non-target human siRNA (40nmol) to make 80nmol concentrations. Lipofectamine 2000 reagent was used to introduce the siRNA into the neuroblastoma cells. Cells were returned to incubator. After 24 hours cells were treated with clenbuterol (10um). DMSO was used as a control. Cells were grown for 48 more hours then washed with DPBS. Culture plates were frozen at -80°C until mRNA extraction and protein extraction were performed.

**Statistical Analysis**

All values in the figures were expressed as a mean ± SEM. Values were compared by two-group student t-tests in order to determine statistical significance. If p-values were less than 0.05 the differences were considered significant.
Previous Data

The drug screening completed before my arrival resulted in six drugs being further tested for their affect on SNCA gene expression. A primary screening of specialized drug library containing 1,126 compounds resulted in 75 preliminary hits that modulate SNCA gene expression. Of these compounds 41 acted as repressors and 34 acted as trans-activators. The repressors lowered endogenous neuronal SNCA transcript by approximately 40%, and the trans-activators elevated SNCA mRNA abundance by approximately 40% as well. The adrenergic drug class contained both agonists that acted as repressors and antagonists that acted as activators, mutually pointing to the implicated regulatory target. A replicate assay was used to verify the results and rule out false positives. Ten of the 41 preliminary repressors were confirmed. Clenbuterol and Salbutamol were grandfathered in prior to a secondary assay because they belonged to the adrenergic drug class. In the secondary assay six hits were confirmed to be statistically significant with p<0.005. Four of these drugs are FDA-approved with well-established pharmokinetics and suitable toxicity profiles. Two of these four are known to penetrate the blood brain barrier. Importantly, four of the six hits verified hits were $\beta_2$AR agonists. The six identified compounds are summarized in Table 1. The effect each compound had on SNCA mRNA abundance during the screening process is summarized in Figure 1. The repression of SNCA mRNA in the presence of metaproterenol was
verified using an independent, digital gene expression platform based on Nanostring technology.

**Table 1. Six hits were discovered to repress SNCA expression.** This table identifies and describes the six hits found during the high-throughput endogenous SNCA gene expression assay that measured the levels of endogenous human SNCA in situ in neuronal cells in 384-well plates. Four of the compounds were β2-agonists. Adapted from Mittal et al., 2015 (unpublished).

<table>
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<tr>
<th>Name</th>
<th>Class</th>
<th>Structure</th>
<th>FDA approved</th>
<th>Blood-brain penetrant</th>
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<td>2. Clenbuterol</td>
<td>β1- and β2-Adrenoreceptor Agonist</td>
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<td>Yes</td>
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<td>3. Salbutamol</td>
<td>β2-Adrenoreceptor Agonist</td>
<td><img src="image3" alt="Structure" /></td>
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<td>Yes</td>
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<tr>
<td>4. Dobutamine</td>
<td>β1- and β2-Adrenoreceptor Agonist</td>
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<td>No</td>
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<tr>
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<td>No</td>
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<tr>
<td>6. Riluzole</td>
<td>Norepinephrine transporter, NMDA receptor antagonist</td>
<td><img src="image6" alt="Structure" /></td>
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</tr>
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</table>
Figure 1. Endogenous SNCA expression was repressed by six compounds throughout the screening process. During the screening process SK-N-MC neuroblastoma cells were treated with each compound at a 1μM concentration for 48 hours. Metaproterenol sulphate, dobutamine hydrochloride, betulinic acid, and riluzole chloride showed consistently lower SNCA mRNA abundance in the primary, replicate, and secondary assays compared to control cells treated with DMSO. Clenbuterol hydrochloride increased SNCA mRNA abundance during the first screening, but was included in the replicate assay because of the implicated regulatory effect of its drug class. Salbutamol was not originally in the specialized drug library screened, but was included in the replicate assay for the same reason as clenbuterol. The secondary assay demonstrated the similar repression of SNCA mRNA in the presence of clenbuterol and salbutamol. In the secondary assay all six confirmed hits were significant at $P<0.005$. DMSO = Dimethyl sulphoxide. $N=8$ for drug treatments in the replicate and secondary assay, $N=32$ for DMSO-treated control cells. ** = $P<0.005$. Adapted from Mittal et al., 2015 (unpublished).
Pharmological evidence from the screening indicates that selective β₂AR agonists repress SNCA gene expression in catecholaminergic SK-N-MC cells. The six drugs identified consisted of three selective β₂AR agonists – metaproterenol sulfate, clenbuterol hydrochloride, and salbutamol; one β₁AR agonist with weak β₂AR agonist activity – dobutamine hydrochloride; and two other chemical regulators – betulinic acid and riluzole hydrochloride.

The six confirmed hits were evaluated for biochemical evidence of cellular toxicity and characterized for target specificity. None of the compounds were found to compromise cellular viability and revealed no off-target effects. Clenbuterol was prioritized because it can penetrate the blood-brain barrier. A Diversity profile assay was performed for the drug to check further for potential off-target effects at the concentration of 10μM. Clenbuterol did not show significant off-target effects. The assay demonstrated that clenbuterol targets both β₁- and β₂-adrenergic receptors. The results of the assay can be seen in Figure 2. In order to see if the six hits regulated SNCA alone or all members of the synuclein family, β- and γ-synuclein gene expression was assayed as well. Only the β₂AR agonists showed a marked transcriptional effect on the entire synuclein gene family.
Clenbuterol showed no off-target effects in the diversity profile assay. The hits were screened for 71 binding and 27 enzyme assays with roughly an equal number of selective, central, and peripheral therapeutically relevant targets at 10uM in a counter screen. Clenbuterol was found to target both β1- and β2-adrenergic receptors. Adapted from Mittal et al., 2015 (unpublished).

The MSD Platform was used to quantify total (unfolded and folded) α-synuclein protein levels in SK-N-MC neuroblastoma cells treated with metaproterenol (10uM), clenbuterol (5uM), salbutamol (5uM), dobutamine (10uM), betulinic acid (5uM), and riluzole (1uM) for two days. In contrast to SNCA mRNA expression, protein levels did not show significant reduction. Protein levels were checked again after administering the drugs for three days. Four of the six compounds – metaproterenol, clenbuterol, salbutamol, and riluzole – significantly reduced the endogenous α-synuclein protein levels in SK-N-MC.
When the prioritized hit, clenbuterol was administered in a time-dependent manner for four days at a concentration of 10uM, longer duration of drug administration was correlated with greater reduction of α-synuclein protein levels. This was observed simultaneously in \( SNCA \) mRNA levels as well. When clenbuterol was administered in a dose-dependent manner at concentrations of 5uM, 10uM, and 20uM, a reduction in protein level correlated with an increase in dosage. \( SNCA \) mRNA levels were observed simultaneously again. It was observed that mRNA expression stabilizes at a dose of 10uM clenbuterol. These results are summarized in Figure 3.

The \( \beta_2 \)AR specific effect on \( SNCA \) expression was further confirmed by systematically probing the adrenoreceptor class with compounds selective for each \( \alpha_1 \)-, \( \alpha_2 \)-, \( \beta_1 \)-, \( \beta_2 \)-, and \( \beta_3 \)-receptors. These results are summarized in Figure 4. Most of the \( \beta_2 \)AR agonists repressed \( SNCA \) expression at a concentration of 1uM. The inactive isomer R-(+)-atenolol, a control compound, had no effect on \( SNCA \) expression. The \( \beta \)-blockers atenolol and alprenolol acted as trans-activators of \( SNCA \) expression.
Figure 3. The effect of clenbuterol on α-synuclein protein expression was tested in both a time-dependent and dose-dependent manner. A) Human neuroblastoma cells were treated with 10uM Clenbuterol for 4 days. Alpha-synuclein protein levels and SNCA mRNA levels were measured following 2, 3, and 4 days of drug administration. B) Human neuroblastoma cells were treated with 5uM, 10uM, and 20uM clenbuterol for 4 days. After 48 hours the medium was changed and the drug was re-administered. Alpha-synuclein protein levels and SNCA mRNA levels were quantified after 4 days. Data presented are corrected to controls. * = P<0.05, ** = P<0.001. Adapted from Mittal et al., 2015 (unpublished).

β2AR agonist-induced effect was reversed pharmacologically

Pharmacological abrogation of the drug-induced affect on SNCA mRNA levels was demonstrated when SK-N-MC neuroblastoma cells were treated for two days both independently and collectively with three β2AR agonists – metaproterenol, clenbuterol, salbutamol – and with propranolol, a β2AR antagonist. As shown in Figure 4., propranolol significantly abrogated the β2AR agonist-induced repression of SNCA mRNA levels and protein levels. This finding was supported when cells were treated for four days both independently and collectively with clenbuterol and propranolol. When the β2AR antagonist was administered alone protein levels were elevated.
β2AR antagonists had the opposite effect of β2AR agonists on SNCA mRNA expression levels and abrogated β2AR agonist-induced repression when combined. A) A chemical genomics screen to verify β2AR specific effect was performed with a commercially available library. Selective compounds for β2AR were screened at a concentration of 1μM for 48 hours and SNCA expression was quantified by qPCR. The inactive isomer R(+)-atenolol included in the chemical genomics screen functioned as a control. β-blockers atenolol and alprenolol increased relative SNCA mRNA abundance. B) Human neuroblastoma cells were independently treated with the β2AR agonists metaproterenol (10μM), clenbuterol (10μM), and salbutamol (5μM) and the β2AR antagonist propranolol for 48 hours. Cells were also treated with each β2AR agonist and propranolol (100μM) together. SNCA expression was quantified by qPCR. C) Humane neuroblastoma cells were independently treated with clenbuterol (10μM) and propranolol (100μM) and with both compounds together for 4 days. After 48 hours the medium was changed and the drugs were re-administered. SCNA protein levels were measured by MSD and ELISA. Data presented are corrected to controls. * = P<0.05, ** = P<0.001. Adapted from Mittal et al., 2015 (unpublished).

β2AR agonists repress endogenous SNCA mRNA and protein expression in rat primary cortical neurons and PD-relevant substantia nigra of wild type mice

The effect of β2AR agonists was further confirmed on endogenous SNCA mRNA expression levels in rat primary cortical neurons derived from E18 rat embryos. Results can be seen in Figure 5a. When treated with metaproterenol (5μM) and clenbuterol
(20um) for 48 hours, a significant decrease in the SNCA mRNA and protein levels was observed.

Wild-type mice with intraperitoneally administered clenbuterol showed significant repression in endogenous SNCA mRNA and protein levels as compared to a vehicle-treated control group. Results can be seen in Figure 5b.

A pharmokinetic study using various doses of intraperitoneally administered clenbuterol, analyzed plasma and brain samples for drug concentrations by LC-MS/MS at each concentration. Brain penetrance of clenbuterol was confirmed. The repression of SNCA mRNA expression levels was brain to plasma ratio dependent.

Figure 5. β2AR agonists repressed endogenous SNCA gene expression in rat primary cortical neurons, and clenbuterol’s effect on SNCA expression was further confirmed in the PD-vulnerable substantia nigra of wild-type mice. a) Rat primary cortical neuron derived from E18 rat embryos were cultured for 10 days and subsequently treated with metaproterenol (5um) and clenbuterol (20um) for 48 hours. SNCA mRNA and protein levels were quantified by qPCR and ELISA. b) A single dose of clenbuterol (10uM) was administered intraperitoneally to wild-type mice. Endogenous SNCA mRNA and protein expression in the substantia nigra were quantified 24 hours later. The SNCA expression from ten age and sex matched vehicle mice were quantified as well. * = P<0.05, ** = P<0.001. Adapted from Mittal et al., 2015 (unpublished).
Histone modulation of SNCA transcription by β2AR activation

We found that clenbuterol treatment of neuroblastoma cells decreased the acetylation of histone H3. We also discovered that clenbuterol’s effect on histone acetylation was inhibited by propranolol. A quantitative chromatin immunoprecipitation (ChIP) assay was used to identify the acetylation site affected by clenbuterol. We found a significant decrease in histone H3 acetylation on Lysine 27. A decrease in total H3 acetylation on several of the predicted H3K27 acetylation sites on the SNCA transcript was found as well. The observed regulation of SNCA transcription by clenbuterol, a β2AR agonist, is described in Figure 6. The ChIP assay confirmed that the acetylation of H3 in the promoter region of SNCA was significantly decreased by clenbuterol and increased by valproic acid, a histone deacetylase inhibitor.

Figure 6. Clenbuterol regulates SNCA transcription by histone modulation. a) Top, effects of clenbuterol and propranolol on SNCA mRNA expression levels. Valproic acid acted as a negative control. Bottom, effects of clenbuterol, propranolol, and valproic acid on acetylation of histone H3 (Ac-H3). b) Chromatin Immunoprecipitation (ChIP) assay for the presence of acetylated H3 and H3K27 in the promoter region and two predicted acetylation regions of SNCA in neuroblastoma cells. The ratios of immunoprecipitated product to input qPCR product quantities were calculated as relative ChIP. * = \( P<0.05 \), ** = \( P<0.001 \). Adapted from Mittal et al., 2015 (unpublished).
Endogenous SNCA levels rise when β2ARs are silenced

Silencing of β2ARs resulted in a significant increase of SNCA mRNA abundance in neuroblastoma cells as seen in Figure 7. Cells transfected with non-target siRNA experienced repression of SNCA expression when treated with clenbuterol, a β2-agonist. The non-target siRNA did not silence β2ARs. Transfection with ADRB2 siRNA only resulted in the silencing of approximately 70% of ADRB2 mRNA, so cells transfected with ADRB2 siRNA still demonstrated a slight repression of SNCA expression when treated with clenbuterol.

![Figure 7. Silencing of β2-adrenergic receptors increased SNCA expression. SNCA mRNA abundance was increased when SK-N-MC cells were transfected with ADRB2 siRNA compared to non-target siRNA. SNCA expression was repressed when both cells expressing non-target siRNA and cells expressing ADRB2 siRNA were treated with clenbuterol. ADRB2 siRNA was only 70% effective. ** = P<0.001. Adapted from unpublished, confidential work from Mittal et al., 2015. Adapted from Mittal et al., 2015 (unpublished).](image)
DISCUSSION

The innovative gene expression high-throughput screening method conducted by my laboratory was successful in identifying small molecules that repress \textit{SNCA} gene expression in neuroblastoma cells. The results did not only reveal six compounds that repress \textit{SNCA} expression, but also that a specific class of drugs has the potential to modulate \textit{SNCA} gene expression. From this we discovered that $\beta_2$-adrenoreceptors might be a regulatory target for \textit{SNCA}. We identified $\beta_2$AR agonists, specifically clenbuterol, as potent \textit{SNCA} expression-lowering drugs.

Betulinic acid and riluzole were the two verified chemical regulators of \textit{SNCA} expression that were not adrenoreceptor agonists. Though these compounds were not our primary focus, research on their ability to repress \textit{SNCA} expression could be furthered to find the mechanism by which these chemicals regulate \textit{SNCA}. Not much is known about betulinic acid, but the possible implication for riluzole is significant as the drug is already a well-established treatment for neurodegeneration. It is currently the only disease-modifying compound federally approved in the United States for the treatment of neurodegenerative disease. Riluzole is the common treatment prescribed for Amyotrophic Lateral Sclerosis (ALS; Lou Gehrig’s disease). The drug is already being considered as a potential treatment for Parkinson’s disease. In the 6-hydroxydopamine rat model of PD, some attenuation of dopamine neuron degeneration due to riluzole treatment was demonstrated, suggesting a possible neuroprotective effect (Carbone, Duty, & Rattray, 2012). A blind pilot study on human subjects did not produce significant evidence of
symptomatic effects, but did importantly show that riluzole was tolerated well by PD patients (Jankovic & Hunter, 2002).

No effect on cell viability and no material transcription off-target effects observed in the gene expression assays allows for further investigation into the means by which the six confirmed compounds repress \textit{SNCA} gene expression. It also helps confirm that the compounds are regulating synuclein itself, and not many different gene families resulting in a complex and indirect variation of expression. Although the $\beta_2$AR agonists were found to repress the entire synuclein gene family, this is unlikely to have negative repercussions. Alpha- and beta-synuclein were discovered to have some redundant functions when transcriptional effect of various synuclein deficiencies were analyzed, and a knockout study in mice for $\alpha$-synuclein and $\gamma$-synuclein demonstrated that deficiencies in either or both genes resulted in no apparent phenotype (Kuhn et al., 2007). A double knockout study in mice of $\alpha$-synuclein and $\beta$-synuclein had similar results, concluding that there is likely redundancy between the two types of synuclein and that there is no impairment to brain function or survival when one is knocked down (Chandra et al., 2004). These findings allow us to infer that general repression of the entire synuclein family would not likely present adverse neuronal side effects. Specifically targeting of the entire synuclein gene family by $\beta_2$AR agonists is exciting as it presents insight into the potential mechanism through which \textit{SNCA} mRNA is regulated.

The $\beta$-adrenergic-mediated repression of \textit{SNCA} gene expression by $\beta_2$AR agonists, specifically metaproterenol and clenbuterol, was established in our study. While all six of our confirmed hits markedly repressed \textit{SNCA} mRNA abundance after 48 hours
of treatment, α-synuclein protein levels did not drop in a similar fashion as expected and after 72 hours of treatment only the neuroblastoma cells treated with the β₂AR agonists showed significantly reduced α-synuclein protein levels. This 24-hour delay in protein suppression can by credited to the stability and long half-life of the α-synuclein protein. Betulinic acid and riluzole did not significantly repress protein levels in 72 hours, implying that these two compounds are not as potent SNCA gene repressors as β₂AR agonists.

The selective effect of β₂AR agonists on SNCA gene expression was further verified for metaproterenol and clenbuterol in rat primary cortical neurons. The repressive effect of clenbuterol was demonstrated in vivo in PD-vulnerable substantia nigra of wild-type mice, establishing the future potential of the β₂AR agonist to downregulate α-synuclein expression in vivo in humans. The repressive effect of a drug class meant to mimic the action of catecholamines is not that surprising as growing evidence supports that norepinephrine deficiency may be vital to the pathology of PD and that noradrenergic enhancement in combination with dopaminergic treatments may improve symptomatic relief (Espay, LeWitt, & Kaufmann, 2014).

The opposing effects of β₂AR agonists and β₂AR antagonists on SNCA mRNA abundance support β₂AR as an important regulatory target of SNCA. β₂AR agonists mimic the effect of endogenously occurring catecholamines by stimulating β₂-receptors. A potential problem of using β₂AR agonists to treat α-synuclein overexpression is the autoregulatory receptor desensitization associated with β₂AR activation. Of course the extent of desensitization is based on the degree and duration of the agonist and receptor
interaction and response. Short treatment only results in limited receptor function due to partial uncoupling that will be reversed in minutes. Longer treatment with β2AR agonists could lead to sequestration, which is normally reversed within hours, or even downregulation causing more permanent loss of the receptors (Johnson, 2006). Based on our findings it is plausible to believe that if downregulation occurred and receptors were lost, α-synuclein levels would increase and become overexpressed.

It is interesting that β2AR antagonists abrogated the effects of β2AR agonists when used together, because β2AR antagonists are not actually very competitive for the same receptor state as β2AR agonists. β2AR antagonists bind the inactive, low-energy form of the receptor, and β2AR agonists, while they can directly stimulate the receptor, work more readily by binding to and temporally stabilizing the active, high-energy form of the receptor. In this sense the two drugs are not competitive for the same receptor (Johnson, 2006). With our discovery that β2AR antagonists raise SNCA gene expression in neuroblastoma cells, it is noteworthy that the β2-blocker propranolol, the drug with which we demonstrated this, is a commonly prescribed treatment for tremor, one of the four cardinal Parkinsonism symptoms. It is worth further researching the implications of this to ensure that physicians are not prescribing a drug for symptomatic relief to patients that may in fact worsen their symptoms in the long run by inducing α-synuclein overexpression.

Validating the six hits exposed in the screening process has helped elucidate a probable regulatory target that may help research examine the pathways important for regulation of SNCA at the transcriptional level. Our work has shown that stimulation of
 β2ARs leads to downregulation of SNCA, reducing both the mRNA abundance and protein levels. It is vital to continue investigation into this finding and discover the mechanism by which β2AR agonists do this. Chromatin modeling is recognized to be a significant mechanism for regulation of gene expression (Willyard, 2010). Acetylation is one expansively studied histone modification that is thought to be associated with transcription (Camelo et al., 2005). We have demonstrated with the quantitative ChIP Assay that clenbuterol likely represses SNCA expression through inhibition of histone H3 acetylation. This was demonstrated through hypoacetylation in the SNCA promoter region and other predicted H3 acetylation sites. In our test valproic acid induced hyperacetylation of histone H3 leading to an increase in SNCA expression. This coincides with an earlier study that showed valproic acid, a histone deacetylase (HDAC) inhibitor causes time- and concentration-dependent increases in endogenous levels of SNCA mRNA and protein expression in neurons likely through histone H3 hyperacteylation induced by inhibition of HDAC (Leng & Chuang, 2006). Decreased acetylation of histone tails is known to suppress relaxation of chromatin and lead to a disruption of histone-DNA and histone-histone interactions, thought to inhibit gene expression (Camelo et al., 2005). This all supports our finding that lowered histone H3 acetylation, perhaps specifically at H3K27, stimulated by β2AR agonists play an important role in the downregulation of SNCA. Further research is required to definitely prove the possible regulatory pathway and mechanism implied by our finding.

Our experiment with ADBR2 siRNA further supports β2ARs as a regulatory target for SNCA gene expression and the repressive effects of β2-agonists. Silencing of ADBR2
in neuroblastoma cells caused a moderate increase of approximately 20% in SNCA expression. This further supports the idea that β2ARs do play a role in downregulating SNCA gene expression. Cells expressing non-target siRNA showed decreased SNCA mRNA abundance when treated with clenbuterol, once again verifying the repressive effects of the β2-agonist. Silencing of ADBR2 abrogated clenbuterol-induced repression of SNCA transcription.

Experiments to verify the action of β2AR agonists were carried out with clenbuterol primarily because of its ability to cross the blood-brain barrier and its consistently repressed SNCA expression levels. Clenbuterol is prescribed as a bronchodilator outside the United States, but is only approved for the treatment of asthma in cattle in this country. Information on the pharmacokinetics, safety, and efficacy of the compound is known, but it is difficult to know how PD patients would respond without clinical trials. We have shown neuroblastoma cell viability with no evident cytotoxic or off-target effects, but serious side effects of high dosages of clenbuterol misused as performance-enhancing drugs have been exposed (Spiller, James, Scholzen, & Borys, 2013). More studies on dosage effects are necessary before the drug begins any testing as a potential PD treatment in humans.

All six of our confirmed hits, especially the β2AR agonists and particularly clenbuterol, could be further researched and developed into clinical trials as α-synuclein-expression-lowering compounds. Successful pre-clinical animal studies followed by dosage effect studies in humans could be the next steps in creating a novel class of disease-modifying therapeutics for neurodegeneration. These findings have the potential
to act as an acute solution for $\alpha$-synuclein overexpression by directly affecting $SNCA$ gene expression. The implications of $\beta_2$AR agonists for $\alpha$-synuclein could not only be a potential therapy for PD, but also for the related neurodegenerative synucleinopathies such as Dementia with Lewy bodies, Hallervorden-Spatz syndrome, LB variants of Alzheimer’s disease, Shy-Drager syndrome, striatonigral degeneration, and olivopontocerebellar atrophy. Other neurological disorders in which some percentage of cases involve $\alpha$-synuclein aggregation including Gaucher diseases, prion disease, Amyotrophic Lateral Sclerosis, and traumatic brain injuries, could be helped by these potential therapeutics (Ulusoy & Di Monte, 2013; Galvin, Lee, & Trojanowski, 2001) I believe we have discovered an exciting possibility to fight a significant pathology of Parkinson’s disease, a neurodegenerative disorder that desperately needs a cure, at its source with $\beta_2$AR agonists.
REFERENCES


CURRICULUM VITAE

ELIZABETH K. LONG
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eklong@bu.edu
Year of Birth: 1991

Education

Master of Science in Medical Sciences, expected May 2015
Boston University School of Medicine, Boston, MA
• Current GPA: 3.21

Bachelor of Science in Biology, May 2013
The University of Alabama, Tuscaloosa, AL
• Cumulative GPA: 3.32/4.0
• Minor in Spanish
• UA Presidential Scholarship Recipient
• University Honors Program, International Honors Program

Related Course Work

General Biology Human Physiology
Cell Biology General Chemistry
Genetics Organic Chemistry
Microbiology General Physics
Biochemistry Calculus I & II
Histology Pathology
Embryology Biostatistics

Research Experience

Undergraduate Research Assistant, January 2011–May 2012
The University of Alabama – Department of Biological Sciences, Tuscaloosa, AL
The Clark Lab - Dr. John L. Clark

Graduate Research Assistant, August 2014-April 2015
Brigham and Women’s Hospital, Boston, MA
The Neurogenomics Laboratory – Dr. Clemens R. Scherzer
Work Experience

Banquet Event Staff, June 2012-October 2012
The Cypress Inn, Tuscaloosa, AL
• Waitress, caterer, and bar runner for various private and public events

Medical Secretary, May 2010-July 2010 & May 2011-July 2011
Woodlands Medical Specialists – Hematology-Oncology Department, Pensacola, FL
• In charge of the organization of all patient files - this required me to learn about and have a basic understanding of many different medical tests and procedures
• Caught up on backed up paperwork dating back two years and remained up to date; Assisted doctors and nurses in keeping up with patient information

Roger Scott Tennis Center, Pensacola, FL
• Tennis teacher and mentor for children ages 4-13; Created and organized arts and crafts projects

Involvement/Leadership

University Stewards, UA October 2012-May 2013
Pi Beta Phi, UA August 2009-May 2013
• Policy and Standards Board 2012
• Philanthropy Chair 2011-2012
• New Member Mentor 2010-2011

Panhellenic Recruitment Counselor, UA Jan 2011-Aug 2011
SGA Homecoming Committee, UA 2010-2011
Freshman Forum, UA August 2009-2010
• Selection Committee 2010
**Community Service**

<table>
<thead>
<tr>
<th>Activity</th>
<th>Duration</th>
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<tbody>
<tr>
<td>Big Sister Association of Greater Boston</td>
<td>February 2014-present</td>
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<tr>
<td>Champions Are Readers Program (CAR Program) –</td>
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<tr>
<td>Holt Elementary School, Tuscaloosa, AL</td>
<td>August 2009-May 2013</td>
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<tr>
<td>Boys and Girls Clubs of America, Tuscaloosa, AL</td>
<td>August 2009-May 2013</td>
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<tr>
<td>Big Brothers Big Sisters, Tuscaloosa, AL</td>
<td>April 2012-April 2013</td>
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<tr>
<td>Arts n Autism, Tuscaloosa, AL</td>
<td>February 2013-March 2013</td>
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<tr>
<td>Sitters for Service Initiative, UA</td>
<td>January 2010-December 2012</td>
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<tr>
<td>• Pilot Program</td>
<td>January 2010-May 2010</td>
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<tr>
<td>Greek Tornado Relief, UA</td>
<td>April 2011-May 2011</td>
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<tr>
<td>Costa Rica Mission Trip –</td>
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<tr>
<td>Alabama Greek Missions, UA</td>
<td>December 2010</td>
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<tr>
<td>Child Life Volunteer – Nemours Children’s Clinic,</td>
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<tr>
<td>Sacred Heart Hospital, Pensacola, FL</td>
<td>May 2010-July 2010</td>
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**Foreign Language**

Intermediate proficiency in Spanish