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Vulnerability of the nigrostriatal dopaminergic system in alpha-synuclein based animal models

"Molecular Biomedicine"

MSc thesis

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Dedicated to Michalis,

Abstract

Animal models have provided us with a valuable translational tool to understand, diagnose and treat disease for many years. As far as Parkinson's disease (PD) goes, many animal models have been implemented in recent years, in order to elucidate and better understand the pathological mechanisms of degeneration as well as to test novel therapeutic strategies. Since its discovery as a constituent of Lewy Bodies (inclusions found in the surviving substantia nigra neurons of PD patients), α-synuclein (aSyn) has been the focus of countless studies, all aiming to better understand its role in the development of the disease. Thus, a great number of genetic animal models have been generated, most of which, however, fail to fully recapitulate the entire spectrum of pathology and symptoms that are present in PD.

In 2013, a novel study (Nuber et al.,2013) introduced a transgenic animal model (BAC aSyn rat) exhibiting all the features of an ideal model for the study of this disease: aggregation pathology, loss of dopaminergic system intergrity, and non-motor and motor behavioral deficits. However, after establishing a colony in our lab we witnessed a number of phenotypic differences in our animals (GR BAC rats) compared to what the initial study (DE BAC rats) had reported. This phenomenon drove us to investigate the reason behind these differences; from both a genetic and biochemical perspective as far as aSyn goes. Interestingly, differences in both the relative copy number of the human *SNCA (*aSyn gene) and the levels of the protein along the nigrostriatal axis can potentially explain the presence of these differences between the two groups.

Additionally, during the past decade mutations in the *GBA1* gene which encodes for glucocerebrosidase (GCase), a lysosomal enzyme responsible for the breakdown of lipids, have been labeled as the number one risk factor in developing PD. Current literature suggest an interplay between GCase and aSyn, thus our lab generated a novel murine "double-hit" viral model through striatal injection of viral vectors containing a microRNA sequence in order to downregulate GBA while at the same time overexpressing human aSyn. The aim of this project was to evaluate the temporal downregulation of GBA and investigate its effects on the nigrostriatal levels of aSyn. Our results suggest that 8 weeks is an appropriate time point for significant downregulation of GBA. Our findings also report and confirm that the downregulation of GBA leads to subsequent accumulation of human aSyn in both the striatum and the substantia nigra.

Our results indicate the suitability of the BAC aSyn rat as a tool for understanding the pre-motor phase of PD whereas the "double-hit" viral model is better suited for elucidating the mechanisms of degeneration and evaluating therapeutic strategies.

ΠΕΡΙΛΗΨΗ

Τα ζωικά μοντέλα έχουν αποτελέσει χρόνια τώρα καταλυτικό εργαλείο στην προσπάθεια για καλύτερη κατανόηση, διάγνωση και θεραπεία ασθενειών. Στην περίπτωση της νόσου Πάρκινσον (ΝΠ), πολλά διαφορετικά ζωικά μοντέλα έχουν αναπτυχθεί με στόχο την διαλεύκανση των παθολογικών μηχανισμών της νευροεκφύλισης αλλά και της δοκιμασίας νέων θεραπευτικών στρατηγικών. Από την στιγμή που βρέθηκε ότι η α-συνουκλεΐνη αποτελεί ένα από τα κύρια συστατικά των σωμάτων Lewy (πρωτεϊνικά έγκλειστα ευρισκόμενα στους επιζώντες νευρώνες της μέλαινας ουσίας σε ασθενείς ΝΠ μετά θανάτου, έχει μελετηθεί εκτενώς με στόχο την καλύτερη κατανόηση του ρόλου της στην εξέλιξη της ασθένειας. Ένας μεγάλος αριθμός γενετικών ζωικών μοντέλων έχει δημιουργεί με βάση την υπερέκφραση της α-συνουκλεΐνης, παρόλα αυτά η πλήρης αναπαραγωγή όλων των παθολογικών χαρακτηριστικών και συμπτωμάτων της νόσου δεν ήταν επιτυχής μέχρι στιγμής.

Το 2013, μία νέα έρευνα (Nuber et al., 2013) ανέφερε την δημιουργία ενός νέο διαγονιδιακού ζωικού μοντέλου (BAC α-συνουκλεΐνη επίμυες), το οποίο κατείχε όλα τα χαρακτηριστικά ενός ιδανικού μοντέλου για την μελέτη αυτής της ασθένειας όπως συσσώρευση της α-συνουκλεΐνης, μείωση των ντοπαμινεργικών νευρώνων στη μέλαινα ουσία και μη κινητικά και κινητικά ελλείματα . Όμως, κατόπιν δημιουργίας αποικίας στο εργαστήριό μας, υπήρχαν εμφανείς φαινοτυπικές διαφορές ανάμεσα στα ζώα της αποικίας μας και στα ζώα της αρχικής αποικίας, σύμφωνα με τα ευρήματα της έρευνάς μας. Αυτές οι διαφορές μας ώθησαν να διερευνησουμε το λόγο ύπαρξής τους, κυρίως από την γενετική και βιοχημική σκοπιά σε σχέση με την α-συνουκλεΐνη. Παρατηρήθηκε πως διαφορές στο σχετικό αριθμό αντιγράφων του ανθρώπινου γονιδίου της α-συνουκλεΐνης (*SNCA)* αλλα και στο φορτίο της πρωτεΐνης κατά μήκος της μελανοραβδωτής οδού, μπορούν να εξηγήσουν εν μέρει την ύπαρξη αυτών των διαφορών.

Στην συνέχεια, διερεύνησαμε ένα νέο μοντέλο συνουκλεϊνοπάθειας. Την τελευταία δεκαετία, μεταλλάξεις στο γονίδιο *GBA*1*,* που κωδικοποιοεί για μία λυσοσωμική πρωτεΐνη, τη γλυκοσερεβροσιδάση (GCase), υπεύθυνη για τον καταβολισμό σφιγγολιπιδίων, έχουν χαρακτηριστεί ώς ο κορυφαίος παράγοντας ρίσκου για την ανάπτυξη ΝΠ. Η πρόσφατη βιβλιογραφία υποθέτει την αλληλεπίδραση της λυσοσωμικής αυτής πρωτεΐνης (GCase) με την α-συνουκλεΐνη και για αυτόν τον λόγο ένα καινοτόμο ιικό μοντέλο “διπλού χτυπήματος” δημιουργήθηκε στο εργαστήριό μας, το οποίο μέσω της έγχυσης ιικών φορέων που φέρουν μία ακολουθία μίκροRNA, διαθέτει μειωμένη έκφραση του GBA και ταυτόχρονη υπερέκφραση της ανθρώπινης α-συνουκλεΐνης. Στόχος της έρευνας μας ήταν η χρονική εκτίμηση της μείωσης έκφρασης της GCase καθώς και των επακόλουθων αποτελεσμάτων της μείωσης αυτής στα επίπεδα της α-συνουκλεΐνης στην μελανοραβδωτή οδό. Τα αποτελέσματα μας δείχνουν

το διάστημα των 8 εβδομάδων ως κατάλληλο για ικανοποιητική μείωση της έκφρασης του GCase και πως η μείωση αποτελεί παράγοντας που οδηγεί στη συσσώρευση της α-συνουκλεΐνης τόσο στο ραβδωτό όσο και στην μέλανα ουσία.

Συμπερασματικά, το ζωικό μοντέλο BAC aSyn αποτελεί ένα πολύτιμο εργαλείο για την μελέτη της προ-κινητικής φάσης της ΝΠ, ενώ το ιικό μοντέλο “διπλού χτυπήματος” είναι ιδανικό για την κατανόηση των μηχανισμών της εκφύλισης αλλά και την δοκιμασία και αξιολόγηση νέων θεραπευτικών στρατηγικών.

Contents

1. Introduction

1.1.1 Parkinson's disease epidemiology

Parkinson's disease (PD) is a progressive, neurodegenerative disorder, first described by Dr. James Parkinson in his work "An essay on the shaking palsy", in 1817. It is the second most frequent neurodegenerative disorder following Alzheimer's disease and it is currently estimated that 1-2 people per 1000 are affected by it. The risk of developing PD increases with age, as it is understood that approximately 1% of people above the age of 60 are affected by it (Tysnes et al., 2017), while for people over the age of 85 the percentage rises up to 3-5% (Alves et al.,2008; Gasser et al., 2009a). Moreover, it is predicted that due to the increase of the average life span, as stated by the World Health Organization, prevalence of PD is expected to rise (Cardoso et al., 2005). As of yet, no neuroprotective, disease-modifying treatments are available for patients.

1.1.2 Clinical symptoms of Parkinson's disease

The symptons of PD are classified into two categories: motor and non-motor symptoms. The hallmark features of the disease are the motor symptoms which include bradykinesia, resting tremor, rigidity and postural instability. Other motor symptoms include various postural abnormalities (such as camptorormia and Pisa syndrome), freezing of gait, festination, micrographia, hypomimia and more. These motor symptoms have been well characterized and current treatments aim to either slow down or ameliorate these motor symptoms with the use of drugs that stimulate the dopaminergic system.

The non-motor symptoms may not only precede the manifestation of motor symptoms, but also have not been characterized as extensively as the motor symptoms. Such symptoms include depression, sleep disorders, autonomous nervous system disorders and sensory organ disorders. The prevalence of these non-motor symptoms varies from patient to patient. However, given the fact that they often precede the onset of the disease, sometimes by 10 or even 15 years, the elucidation of their underlying neurobiology is of the outmost importance for early diagnosis as well as the employment of potential neuroprotective treatments at an early stage of the disease. A table with the most important non-motor symptom follows:

Neuropsychiatric disorders	Sleep disorders	Autonomous nervous system disorders	Sensory organ disorders
Depression, apathy, anxiety	RBD (REM sleep behaviour disorder)	Heart: Orthostatic hypotension, bradycardia, arrhythmia	Olfactory deficit
Obsessive compulsive disorder	Insomnia	Digestive system: Salivation, dysfagia, nausea, constipation, stool incontinence	Pain
Attention disorder	Sleepiness during the day	Urinal system: urine incontinence, nocturia	Fatigue
Hallucinations/Delusions	Periodic limb movement	Reproductive system: sexual dysfunction, erectile dysfunction, hypersexuality	Weight fluctuations
Panic attacks	Nightmares	Thermoregulation: sweating, mouth dryness, intolerance to cold/hot temperatures	Paraesthesia
Dementia	Sleep apnea		

Table 1: Non-motor symptoms of Parkinson's disease.

1.1.3 Parkinson's disease pathology

The most characteristic pathological feature of PD is the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Forno et al., 1996). These neurons project axons to the striatum (i.e. the *putamen* and *caudate nucleus).* As a result of the neurodegeneration of these neurons, the nigrostriatal pathway is immediately affected, leading to the motor symptoms associated with PD (Jancovic et al., 2008, Johnson et al., 2009). Another significant pathological feature is the presence of cellular inclusions

called Lewy bodies or Lewy neurites. These inclusions consist mainly of misfolded insoluble α-synuclein fibrils (Spillantini et al., 1997) and are considered the hallmark pathological feature of PD. The link between α-synuclein and PD has been proven genetically (Polymeropoulos et al., 1997; Kruger et al., 1998; Singleton et al., 2003; Zarranz et al., 2004; Nalls et al., 2011), pathologically (Spillantini et al., 1997; Muntane et al.,2012) and biochemically (Cookson et al., 2005; Vekrellis et al., 2009, 2011) Specifically, duplication or triplacation of the *SNCA* locus has been found to directly lead to PD, as reported in Klein and Schlossmacher, 2006. The triplication cases in particular have been associated with an earlier age of onset, a more severe phenotype as well as rapid disease progression (Fuchs et al., 2007). In addition, a number of missense mutations have been identified- of which A53T is the most common one, as reported by Polymeroloulos et al., 1997.

Since α-synuclein is localized intracellularly, much research has focused on studying potential pathogenic mechanisms within the cell (Pacheco et al., 2012). However, recent data suggest that α-synuclein can also be secreted into the extracellular space (El-Agnaf, 2003; El-Agnaf, 2006, Emmanouilidou 2011). This finding suggests that α-synuclein may exhibit paracrine functions in PD pathology (Luk et al., 2009). Additionally, α-synuclein is known to easily form aggregates, which are stabilized through β-pleated sheet-like bonding (Uvensky et al., 2003). Initially, these aggregates are called oligomers but quite rapidly form heavily insoluble polymers which are termed fibrils. These aberrant forms of α-synuclein, and in particular the soluble oligomeric forms, were identified as the main culprit for the eventual cell death observed in Vekrellis et al., 2009; one of many reports supporting the fundamental role α-synuclein misfolding possesses in the pathogenesis and progression of PD.

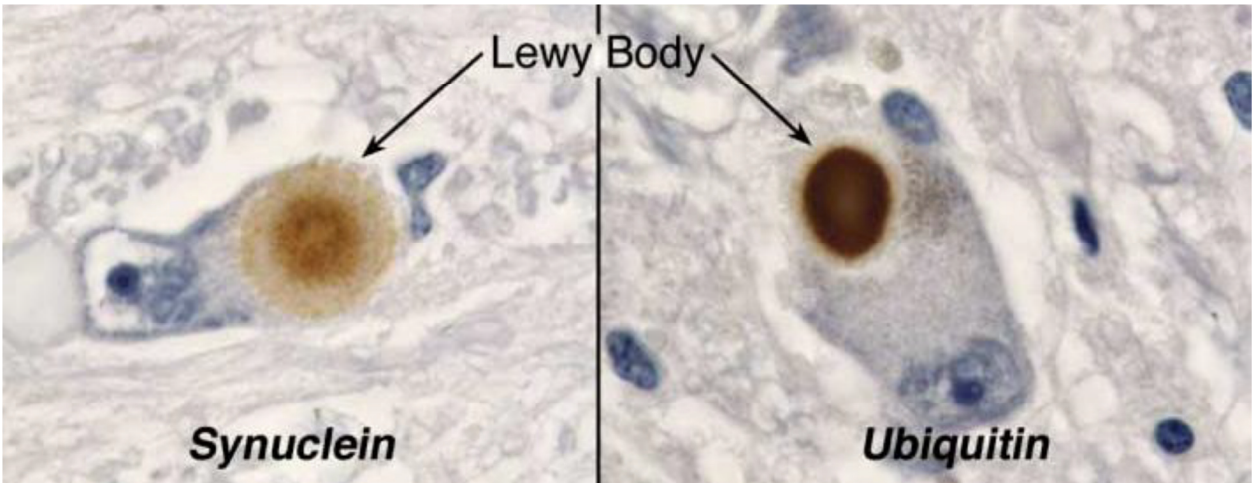


Figure 1: Immunohistochemistry against α-synuclein (left) and ubiquitin (right) on a dopaminergic neuron of the substantia nigra pars compacta. The abundance of α-synuclein is clearly visible. (Image taken from Dauer and Przedborski et al., 2013)

As of yet, no direct correlation has been made regarding the etiology of PD, though ample evidence points towards both environment and genetic factors (Gasser et al., 2009b). There are two forms of PD: familial (approximately 5-10% of all cases have been reported as familial PD) and sporadic. Focusing on the genetics, α-synuclein (*SNCA)* was the first gene to ever be associated with the development of PD. Following this, many more genes have been linked to the disease, and some examples include: parkin (PARK2, Kitada et al., 1998), PTEN-induced kinase protein 1 (PINK1, Valente et al., 2004), DJ-1 (Bonifati et al., 2003) and LRRK2 (Corti et al., 2011). Heterozygous mutations in the gene encoding β-glucocerebrosidase (GBA) are considered the greatest genetic risk factor for developing PD (Sidransky et al., 2005; Sidransky and Lopez, 2012).

1.2 α-Synuclein

α-Synuclein is a member of the synuclein family, which also consists of β-Synuclein, γ-Synuclein and synoretin. Their sequence is highly conserved, however as of yet, their physiological role hasn't been completely elucidated. Current literature reports these proteins as having a role in the regulation of synaptic function (Cheng et al., 2011; Burré et al., 2015). On the contrary, α-synuclein's role in the pathogenesis of several neurodegenerative diseases has been established; namely, PD, Multiple System Atrophy (MSA) and Dementia with Lew Bodies (DLB) (Dauer and Przedborski et al., 2003; McCann et al., 2014). As far as their structure goes, all members of the synuclein

family share a common amino terminal, while their carboxyl terminal varies (Tofaris and Spillantini et al., 2005).

Focusing on α-synuclein, the locus of the gene (SNCA) is 4q21.3-q22 (Spillantini et al., 1996). The α-synuclein protein consists of 140 amino acids (George et al., 2002) it can be subdivided into 3 distinct sections: a conserved amphiphilic N-terminal, a hydrophobic central part and a final C-terminal. The amphiphilic N-terminal (residues 1-60) is highly conserved and can be found in either no particular conformation or as an α-helix, under specific conditions (George et al., 2002). Within this region, the motif KTKEGV is found seven times. This motif has been linked with the region's ability to form an α-helix as well as its ability to bind to lipids and synaptic vesicles. The central part of the protein is most commonly known as the Non Amyloid Component (NAC) (Vekrellis et al., 2004) and spans from residues 61-95. This name was given to this region of the protein because of its ability to change its confromation into a β-sheet, leading to the creation of fibrilar forms, resembling those of β-amyloid in Alzheimer's disease (Conway et al., 1998). Finally, the C-terminal, spanning from residues 96-140, has been associated with the protein's ability to act as a chaperone, thanks to the abundance of proline, glutamic and aspartic acid (Cookson et al., 2005).

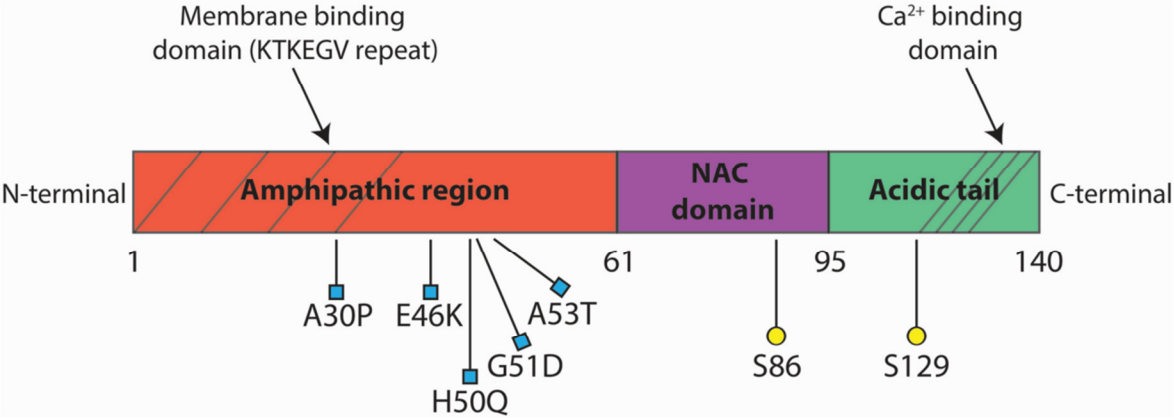


Figure 2: Representation of the structure of α-synuclein and its three regions. On the left, the amphiphilic N-terminal can be seen, with the KTKEGV repeat. The NAC domain is illustrated with purple, in the middle, followed by the proline, glutamic and aspartic acid rich C-terminal. Below the structure, several residues have been marked. Mutations in these residues have been reported to increase the risk of developing PD (taken from Emanuel and Chieregatti, 2015)

The exact conformation of α-synuclein still remains an unanswered question. One study suggested that α-synuclein in vivo forms tetramers (Bartels et al., 2011). However, it has also been suggested that α-synuclein inside the Lewy bodies, present in patients

with PD, forms oligomers and aggregates. These oligomers and the various protein-protein interactions that may occur, are thought to be involved in the pathogenesis of PD (Lashuel et al., 2013).

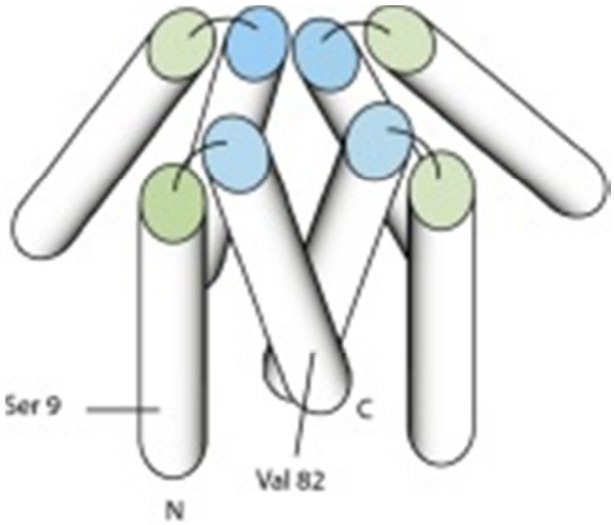


Figure 3: Suggested conformation of α-synuclein. It is evident that in this form, the protein exists as a tetramer. A-helices are represented as cylinders, with the first α-helix of each separate protein labeled with green and the second one labeled with blue. (Wang et al., 2011)

1.3 PD Animal Models

In order to better understand this complex neurodegenerative disease and its pathogenic mechanisms, many different animal models have been created over the last four decades.

The animal models that have been used in PD research can be divided into two groups: those using neurotoxins as a way to recapitulate the pathology and symptoms of the disease and transgenic animals whose DNA has been altered with either mutations or other DNA modifications, based on which loci have been associated with the risk of developing PD.

1.3.1.1 PD Animal Models – Neurotoxin models

Some examples of neurotoxins that have been used on animals in order to understand PD are MPTP (1-methyl-4-phenyl-1,2,3,5-tetrahydropyridine, Przedborski et al., 2001),

6-ODHA (6-hydroxydopamine, Lane et al., 2006), rotenone (Gao et al., 2003) and paraquat (Thiruchelvam et al., 2000). These compounds are used because of their ability to cause degeneration along the nigrostriatal axis, strongly resembling the neurodegeneration observed in PD. However, despite their many advantages, they fail to reproduce several key features that are essential in order to comprehensively understand the mechanisms of PD. One such example is the inability of 6-ODHA, when administered striatally in the brain of rodents to lead to the creation of Lewy bodies (Blandini et al., 2008). However, despite the fact that most of the neurotoxin models have been able to, even partially exhibit most of the parkinsonian symptoms, and predict the neuroprotective capabilities of several compounds used in their studies, the same findings were not observed when these compounds were tested in clinical trials (Athuada and Foltynie, 2015).

1.3.1.2 PD Animal Models – Transgenic Animals

In an effort to overcome the disadvantages of the neurotoxin animal models, several scientific groups created transgenic animals that carry DNA alterations either linked to familial or sporadic cases of PD or overexpressing proteins that have been linked to the risk of developing PD. These animal models were established in the hopes of recapitulating fully the pathology and symptoms of PD i.e. Lewy Body formation, motor symptoms, and non-motor symptoms. According to the literature, more than 18 loci have been associated with PD, based on several GWAS studies (Sutherland et al., 2009). Briefly, some of the loci that have been used in animal models include α-synculein, leukine-rich repeate kinase 2 (LRRK2), DJ-1, parkin, phosphatase and tensin-homologue (PTEN)- induced novel kinase 1 (PINK1).

1.3.1.2 α-synuclein transgenic animal models

Point-mutations or multiplications of the α-synculein gene (mostly duplications or triplications) have been observed and are known to lead to PD by an autosomal dominant manner. Because of the strong link between α-synuclein and PD, a great number of animal models have been created. It is essential to underline that various strategies of delivery have been employed. The integration of the transgene using molecular genetics has been a popular delivery method, however many groups have administered the transgene using viruses (adeno-associated viruses, lentiviruses etc) in order to infect a specific brain region.

Masliah et al., 2000 generated a mouse model overexpressing wild-type α-synuclein, leading to Lewy Body inclusions in the substantia nigra pars compacta, moderate dopaminergic cell death, and motor deficits. Since then, several other studies have used this animal model. The A53T mutation has also been extensively researched. Mice expressing α-synuclein with this mutation were found to have motor dysfunction as well as neurodegeneration, which was the result of filamentous inclusions found in the brains of these mice (Giasson et al., 2002). Another frequently used mutation in animal models is the A30P mutation. Mice carrying this mutation demonstrate neurodegeneration, however, it is limited only to non-dopaminergic regions. This animal model has also failed to reproduce the emergence of Lewy Body inclusions under the Thy1 promoter, whereas when inserted with the PrP promoter, Lewy Bodies as well as mitochondrial dysfunction are evident (Martin et al., 2006). α-synuclein deletion in animal models hasn't given us any further insight into the role that it may play in the development of PD (Abeliovich et al., 2000) leading to research steering away from this approach. Finally, viruses have been employed in animal models to deliver the α-synuclein transgene (Kirik et al., 2002 and Klein et al., 2002). For example, Kirik et al. (2002) administered recombinant adeno-associated viruses (rAAVs) unilaterally to the substantia nigra of Sprague Dawley rats, carrying either the full-length wild type α-synuclein cDNA or the A53T mutated α-synuclein cDNA, both under the CMV/CBA promoter. Klein et al., 2002, used the A30P transgene, delivered using rAAVs to the midbrain of rats, again using the CMV/CBA promoter. In both cases, progressive neurodegeneration was observed in the dopaminergic neurons of the substantia nigra as well as accumulation of α-synuclein. However, both of these studies concluded that the observed neurodegeneration was not sufficient to trigger robust motor and behavioral deficits that mimic symptoms of PD. More recently, a group in Japan attempted to create a transgenic murine animal model, containing the entire α-synuclein gene along with the flanking regions, utilizing the Bacterial Artificial Chromosome (BAC) as a vector (Yamakado et al., 2012). BAC tg mice exhibited reduced anxiety compared to their wild type littermates, however no motor or cognitive impairments were observed. In fact, these mice displayed hyperlocomotion during various behavioral tests, confirming the decreased anxiety that these animals have. This issue is the case for almost all of the animal models that have been used in PD reasearch. As stated by Kirik et al., 2002, Yamada et al., 2004 and Lo Bianco et al., 2004, most of the animal models fail to fully and accurately recapitulate all of the pathological features present in PD, as well as the motor and non-motor symptoms observed. The need for a model that will exhibit both robust expression as well as age-related aggregation of α-synuclein along with all the symptoms is greater than ever.

1.4 BAC ASYN Rats

In 2013, Nuber et al. published a new rat model that was unique compared to all previous animal models created. This animal model has integrated in its genome the entire human α-synuclein locus, along with the 30kb upstream regulatory sequences and 50kb downstream flanking region. In order for this effort to succeed, they employed the Bacterial Artificial Chromosome (BAC), a vector that can integrate inserts 150-350 kb in size. After a comprehensive analysis of the pathological features of these rats as well as their phenotype, it was concluded that this animal model showed strong age-related increased levels of insoluble full length α-synuclein in the dopaminergic synapse-rich striatum, increased insoluble C-terminal truncated α-synuclein, specifically in the brains of aged rats, comparable to findings in PD patients, decreased levels of striatal dopamine, hypolocomotion and an olfaction deficit as well as various other behavioral problems resembling parkinsonian non-motor symptoms.

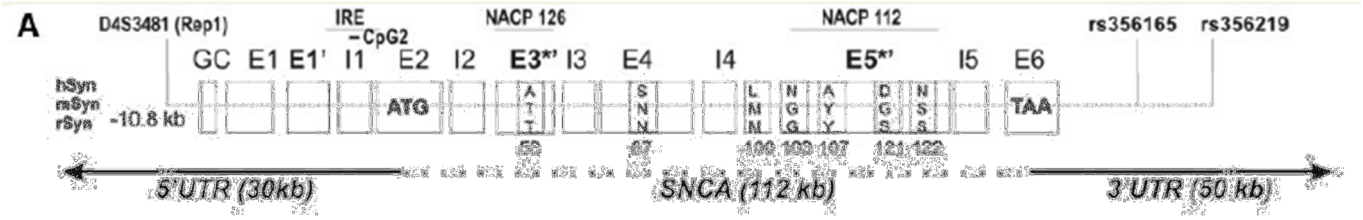


Figure 4: Representation of the human α-synuclein structure, used for the created of the BAC ASYN rats. All introns and exons have been included (Introns: I1-5, exons: E1-6), along with the 5' and 3' UTR regions. Marked on the top are several genetic variants that have been identified as risk factors for PD. (taken from Nuber et al., 2013)

Because of the accurate recapitulation of parkinsonian pathology and phenotype exhibited by this animal model, we were interested in further characterizing their phenotypical and biochemical profile. This effort led to unexpected results. Interestingly, our analysis revealed age-related aggregation of insoluble α-synuclein as well as an olfaction deficit. However, our rats neither demonstrated dopaminergic nigrostriatal neurodegeneration nor did they show any reduction of striatal dopamine levels. In addition, we did not observe motor deficits up until 18 mo. On the contrary,, we observed hyperactivity and increased striatal domapine levels in these rats (Table 2 summarizes the differences in our findings).

German BAC ASYN rats (DE rats)	Greek BAC ASYN rats
Strong, Age-related insoluble α-synuclein accumulation	Strong, Age-related insoluble α-synuclein accumulation
Decrease of striatal dopamine levels	Increase of striatal dopamine levels
Neurodegeneration – Loss of dopaminergic neurons in the substantia nigra pars compacta	No neurodegeneration – Unchanged numbers of dopaminergic neurons in the substantia nigra pars compacta
Locomotion deficit	Hyperactivity
Olfaction deficit	Olfaction deficit

Table 2: Summary of the phenotypical differences observed between the BAC ASYN rats from the originating colony (German BAC ASYN rats (DE rats)) and our colony (Greek BAC ASYN rats (GR rats)) . Similarities are limited to the age-related aggregation of insoluble α-synuclein and the olfaction deficit, while all other phenotypic features do not coincide between the two colonies.

The discrepancy between the two groups was the driving force behind the first part of this study. We wanted to explore the reasons behind these differences that made this animal model display varying phenotypes when present in two different environments. For this purpose, we focused on a comparative analysis of α-synuclein expression in the nigrostriatal dopaminergic system.

Because mutations in the *GBA* gene are the greatest genetic risk factor for developing PD, we sought to explore the role of α-synuclein and its effects along the nigrostriatal dopaminergic pathway at a deeper level by examining its interaction with β-glucocerebrosidase.

1.5.1 β-glucocerebrosidase

β-glucocerebrosidase (GCase, Fig 5) is a lysosomal enzyme responsible for the conversion of glucocerebroside to glucose and ceramide (Qi and Grabowski et al., 2001). The human *GBA* gene is located at 1q21 and encodes the 497 amino acid GCase protein (Shafit-Zagardo et al., 1981).The rodent homologue is comprised of 515 amino acids. GBA has been associated with Gaucher disease, which is an autosomal dominant lysosomal storage disease caused by loss-of-function mutations of the GBA gene that leads to subsequent accumulation of glucocerebrosides in the lysosome. The protein can be divided into 3 domains: Domain I, an anti-parallel β-sheet, Domain II, a triose phosphate barrel containing the active centre of the enzyme and Domain III, an 8-

stranded β-barrel. It is known that transcription of GBA is dependent on the trascription factor EB (TFEB) which plays a role in the regulation of genes related to lysosome-mediated degradation. Once translated, GCase is translocated to the ER where it undergoes several post-translational modifications and then binds to the LIMP-2 receptor. The GCase-LIMP-2 complex traverses through the trans-Golgi network, reaching the membrane of the lysosome where the two molecules are dissociated in a pH-dependent manner. Finally, activation of GCase in the lysosome is found to be mediated by Saposin-C (Fig. 6 D) (Smith et al., 2017).

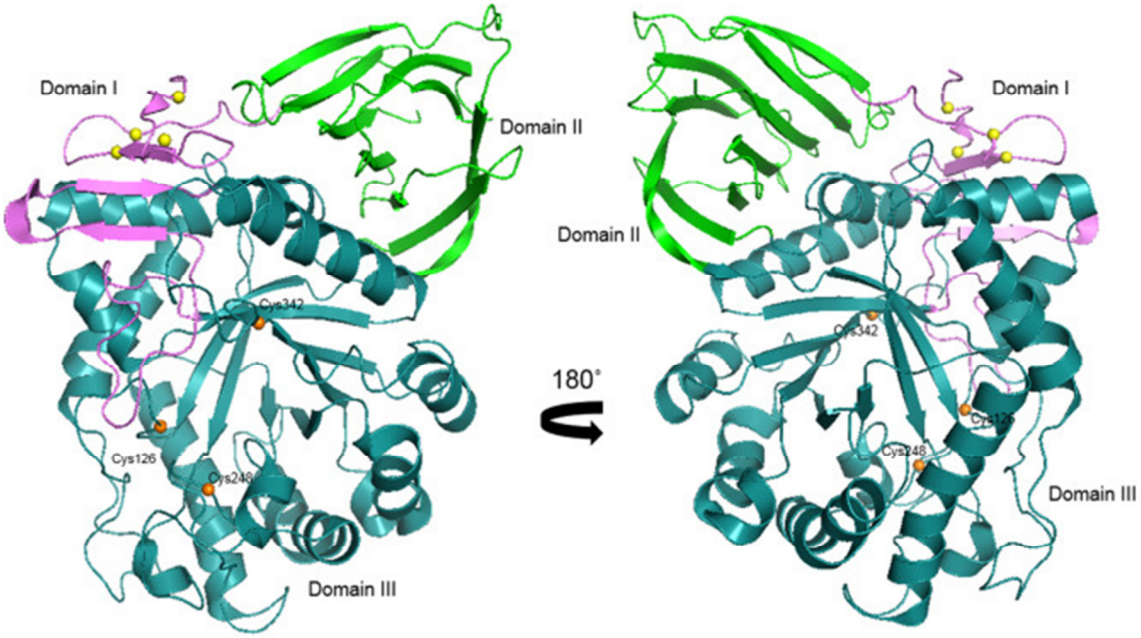


Figure 5: Visual representation of the spatial conformation of β-glucocerebrosidase. The 3 distinct domains of the protein are clearly visible. (Domain I: pink, Domain II: green, Domain III: blue).

1.5.2 The link between PD and GBA

The first evidence pointing towards a link between GBA and PD was observed by a study that correlated mutations in GBA to the chance of developing Parkinsonism (Goker-Alpan et al., 2004). However, in 2009 Sidransky and colleagues established this link when they discovered GBA mutations in a sub-group of PD patients. It was later concluded that about 5-25% of all PD patients carry GBA mutations (Neumann et al., 2009; Schapira et al., 2015). Furthermore, even PD patients that do not have a GBA mutation, demonstrate reduced GCase activity in the striatum and substantia nigra in

conjunction with accumulation of α-synuclein, alterations in lysosomal autophagy, CMA and lipid metabolism (Gegg et al., 2012). Today, mutations in the GBA gene are considered to be the number one genetic risk factor in PD.

Studies have shown that although PD patients and GBA-PD patients seem almost indistinguishable, the progression of motor symptoms is faster in GBA-PD patients (Cilia et al., 2016; Koros et al., 2017). Non-motor symptoms are affected as well with GBA-PD patients being more prone to devoloping symptoms such as cognitive impairment, REM sleep behavior disorder, anosmia and autonomic dysfunction.

1.5.3 Mechanisms through which GBA may contribute to PD

The exact mechanism through which β-glucocerebrosidase contributes to the manifestation of PD has not yet been fully elucidated. Despite this, several studies have proposed different mechanisms. It has been shown that the accumulation of glucocerebroside can lead to accumulation of α-synuclein via stabilization of its toxic forms (α-synuclein oligomers), which in turn can cause a further decrease of GCase activity, leading to a bidirectional positive feedback loop effect as proposed by the work of Mazzulli et al., 2011. Furthermore, it is possible that the interaction between GCase and α-synuclein could potentially be facilitated under acidic conditions, proposed by Yap et al., 2011. The study concluded that interactions between α-synuclein and GCase are not possible in the cytoplasm, which normally has a neutral pH, but only in acidic conditions, thus the lysosome appears to be the appropriate environment for the in vivo interaction of these two molecules. Immunofluorescence has confirmed this as both of these proteins co-localize in the lysosome. Moreover, α-synuclein accumulation was found to be triggered by GCase deficiency as impairment of GCase activity can lead to reduction in the ability of the lysosome to degrade proteins (Mazzulli et al., 2011). Furthermore, GBA PD patients exhibit autophagic and lysosomal dysfunction (Gegg et al., 2012; Schondorf et al., 2014).

1.5.4 GBA and PD animal models

Point mutations in the GBA gene have been used as a way to study the disease in animal models. Examples of point mutations include $GBA^{L444P/L444P}$, $GBA^{V394L/V394L}$, $GBA^{D409H/D409H}$ and $GBA^{D409V/D409V}$. Mice carrying the $GBA^{D409V/D409V}$ were found to have increased α-synuclein aggregation (especially in the hippocampal region), while at the same time exhibit cognitive dysfunction (Sardi et al., 2011). The α-synuclein aggregates

were characterized as Lewy Bodies and Lewy Neurites, as they were Proteinase-K resistant. Another comprehensive study, utilizing a large number of mutated *GBA* animal models, also confirmed that GBA point mutations can lead to α-synuclein aggregation in the brain (Xu et al., 2011). In Cullen et al., 2011, mice with GBA mutations (D409H, D409V, V394L), also exhibited an age-dependent accumulation of α-synuclein. Inhibition of GCase activity with conduritol B epoxide (CBE) leads to a significant increase of α-synuclein toxicity in vitro (Noelker et al., 2015). However, CBE inhibition of GCase in differentiated SH-SY5Y cells and primary neuronal cultures demonstrated no alterations in α-synuclein levels, its clearance or formation of its oligomeric species (Dermentzaki et al. 2013) but GCase deficiency may potentiate cell to cell transmission of pathogenic α-synuclein (Bae et al., 2014). Moreover, CBE inhibition has been shown to lead to accumulation of α-synuclein in nigral cells of wild-type mice (Manning-Bog et al., 2009) and increased striatal levels of oligomeric α-synuclein species in A53T mice (Papadopoulos et al., 2018). Similar results were observed in PrP-A53T SNCA mice when CBE was again used to partially inhibit the GCase activity (Rockenstein et al., 2016). These mice not only exhibited impaired motor functions (both fine and gross), compared to their wild-type littermates, but also demonstrated exacerbated non motor symptoms (contextual memory) along with a huge increase of soluble α-synuclein levels. Altogether, these data support an interplay between α-synuclein and GCase in PD pathology. Subsequently, studies have successfully ameliorated neurodegenerative phenotypes in murine synucleinopathy models by either enhancing GCase activity in the central nervous system (Sardi et al., 2013) or by targeting the pathway of GCase indirectly (via inhibition of glycosylceramide synthase, Sardi et al., 2017). In the first case, improvement of all cognitive deficits was observed along with decreased levels of soluble α-synuclein, when the hippocampus of $GBA^{D409V/D409V}$ mice was injected with AAVs overexpressing the normal GBA gene. Additionally, when these AAVs were injected in the hippocampus of mice expressing the A53T α-synuclein, reduction of the accumulation of soluble α-synuclein was observed. In the second case, targeting of the glycosylceramide synthase led to decreased accumulation of α-synuclein in the hippocampus of A53T-SNCA overexpressing mice as well as $GBA^{D409V/D409V}$. This indirect targeting approach also led to long term benefits as well, such as the amelioration of cognitive deficits exhibited by these two animal models (Sardi et al., 2017).

Based on these studies, we created a murine model using striatal viral injections of microRNAs, targeting the mRNA sequence of GBA. Through this downregulation of GBA, our aim was to utilize this model to further study this interplay between GBA loss of function and α-synuclein accumulation in the nigrostriatal system, a system that, despite its importance in PD pathogenesis, has not been the focus of these previous studies..

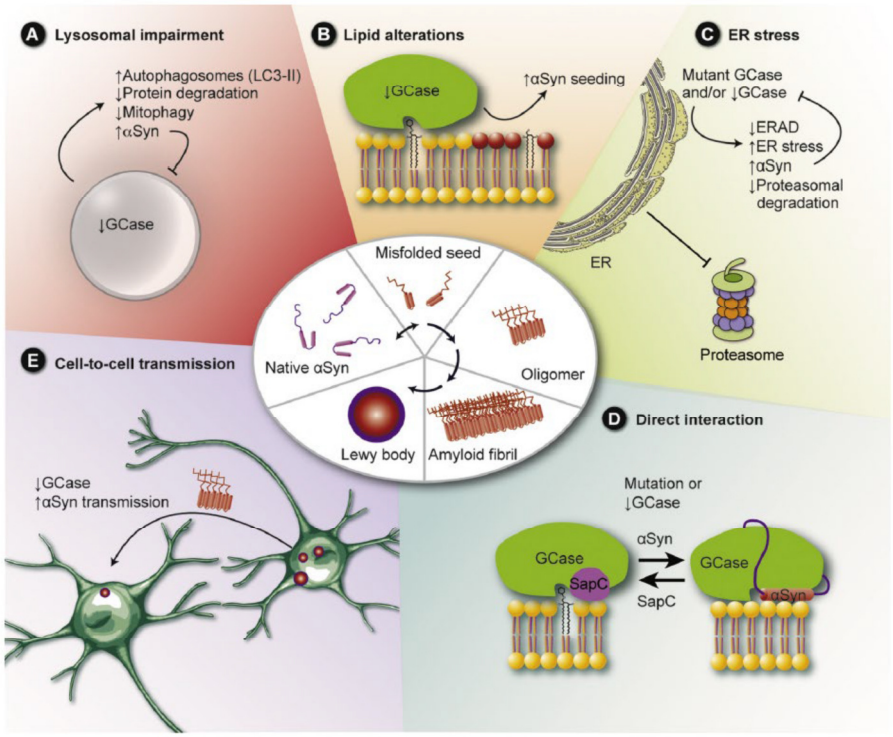


Figure 6: Figure displaying the potential roles that GBA could have in the development of synucleinopathies. A. Impairment of GCase activity can lead to accumulation of α-synuclein and overall decreased protein degradation. B. Alterations in the structure of lipid membranes may be a result of decreased GCase, leading to seeding of α-synuclein. C. Alterations in the abundance of GCase can disrupt normal ER function which in turn leads to ER stress, decreased function of the proteasome and accumulation of α-synuclein. D. Displacement of Sapocin-C due to mutations or reduction of GCase can lead to further reduced GCase activity. E. α-synuclein cell to cell transmission may occur under conditions of reduced GCase activity, leading to propagation of pathological α-synuclein. Figure taken from Sardi et al., 2015.

1.6 Aim of the study

This study aimed to assess nigrostriatal dopaminergic system integrity in two different α-synuclein-based animal models, that of BAC human wildtype α-synuclein overexpression in the rat, and a murine viral model of simultaneous microRNA GBA downregulation and human wildtype α-synuclein overexpression.

1.6.1 Part I: A comparative analysis of genotype-phenotype in a human alpha synuclein overexpression model

The phenotypic differences observed between the BAC rats from the originating colony in Germany and the BAC rats breeded here in Greece, led us to attempt to elucidate the reasons behind these differences while at the same time try to better understand the pathology of α-synuclein and manifestation of symptoms or lack there of (e.g.a) lack of neurodegeneration and b) hyperactivity in the AS BAC rats of our colony). To this end, we performed a comparative genetic and biochemical analysis between the two groups.

1.6.2 Part II: A novel "double-hit" model of GBA1 micro-RNA-mediated downregulation and human α-synuclein overexpression

Due to the fact that both α-synuclein and GCase have strongly been linked to the risk of developing PD, we wanted to investigate how the nigrostriatal dopaminergic pathway is affected in an animal model where we induce downregulation of *GBA* and overexpression of α-synuclein, a condition that appears to mimic the human pathogenic state. Immunohistochemical analysis was performed in order to assess the degree of GCase downregulation and potential alterations in levels of α-synuclein along the nigrostriatal dopaminergic pathway following application of the different combinations of viral injections. These studies derive from the fact that prior work in the lab has shown that the combined expression of haSyn with GCase downregulation confers a toxic effect to the nigrostriatal aaxis. Beyond confirming that miRGBA decreases levels of its target GCase, the question therfore was whether aSyn and GCase would reciprocally influence each other's levels, so as to reach to this effect.

2. Materials & Methods

2.1 aSyn BAC RAT Comparative Analysis

1. Dissection

Our collaborators from Germany provided us with brain tissues from 4 WT and 5 BAC animals from their colony. Specifically, the tissues we received were olfactory bulb (OB), frontal cortex (FC), striatum (STR), hippocampus (HIP) and ear biopsies as well. These animals were 12 months old, both male and female. In order to have comparable groups, we sacrificed 5 12 month old animals from our colony by decapitation. Subsequently, we dissected the areas of interest from the brains of these animals, snap froze them on dry ice and stored them at -80^{o}C until analysis.

2. Tissue Homogenization and cytosolic and membrane soluble protein extraction

In order to extract the cytosolic and membrane soluble proteins from the collected tissues, we first homogenized the tissues using 600 ul of STET buffer (50 mM Tris-base pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% Triton-X-100) and a glass Teflon homogenizer. The lysate was then ultracentrifuged at 150.000 g for 1 hour at 4 ^{o}C. The supernatant was transferred to a clean tube (Triton-X soluble fraction). The pellet was washed two times with 1X PBS and centrifuged at 13.000 rpm at 4^{o}C. The lysate was sonicated at 30% amplitude for 3-4 seconds in RIPA buffer + 2% SDS (50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton-X-100, 0.5% Na-deoxycholate) and then centrifuged at 50.000g for 1 hour at 4^{o}C. The supernatant was transferred to a clean tube (SDS soluble fraction). Protein concentration was determined using the Bradford assay (BIORAD).

3. DNA isolation and Real time polymerase chain reaction

DNA was isolated from ear biopsies. Tissues were incubated overnight with 400 ul of TNES buffer (50 mM Tris-base pH 7.6, 0.4 M NaCl, 100 mM EDTA, 0.5% SDS) and 4 ul of Proteinase K (20 ug/ul). 120 ul of NaCl 5M were added the next day and the samples were shook vigorously for 15 seconds and then centrifuged at 13.000 g for 30 minutes at 4^{o}C. Supernatant was transferred to a new eppendorf tube and equal volume of ice cold 95% Ethanol was added and tubes were gently inverted. Samples were again centrifuged at 13.000 g for 30 minutes at 4^{o}C and the supernatant was removed, leaving only the DNA pellet. The pellet was briefly washed with 70% and air dried before being resuspended in ddH_2O. DNA concentration was measured by spectrometry (2 ul of DNA sample + 198ul of ddH_2O).

For the real time polymerase chain reaction, samples were appropriately diluted so that total amount of DNA was 8 ng. Each sample was in duplicate, in a qPCR plate (Roche). For each reaction we used 7.5 ul of SYBR green master mix, 0.5 ul of forward and reverse primers and 4.5 ul of DNase-free H_2O.

Oligo Name	Sequence (5' -> 3')
BAC-SNCARev	CCTCTTTCCACGCCACTATC
BAC-SNCARevPCR	TCCCCGCGGGGACCTCTAGCCTGTCGTCGAAT
BAC-SNCAFor	CCGCTCGAGCGGTAGGACCGCTTGTTTTAGAC
Rat b-actin For	TGGCTCCTAGCACCATGA
Rat b-actin Rev	CCACCAATCCACACAGAG

Table 3: List of the primers used for the DNA insert comparison.

4. Western Blot Analysis

Protein levels of various α-synuclein species were determined using Western immunoblotting. For detection of phosphorylated α-synuclein (paSyn; pS129). we loaded 20μg of the brain lysates whereas for the detection of human α-synuclein (4B12 antibody), rodent and human α-synuclein (Syn1; C20) or for tyrosine hydroxylase (anti-TH) we loaded 10μg- all on 13% sodium dodecyl sulfate polyacrylamide gels. We mixed the protein samples with 4X sample buffer (20% β-mercaptoethanol) and ran the gels. Following this, the proteins were transferred onto a nitrocellulose membrane and blocked using 5% milk in 1X TBST. The blots were probed with antibodies against: paS129 (1:1000; Abcam), 4B12 (1:1000; GeneTex), Syn1 (1:1000; BD transduction), TH (1:1000; Millipore), γ-Tubulin (1:2000; Acris). Blots were probed with horseradish peroxidase-conjugated secondary antibodies (HRP; mouse and rabbit) and then incubated with ECL solution for 3 minutes. Finally, the blots were developed on Super RX film (Fuji film). The resulting films were analyzed and quantified using ImageJ. γ-Tubulin was used as a loading control with which we normalized all values. Re-probing of the membranes with a different antibody was performed after mild stripping (2ml Glycine 2M, 2ml NaCl 5M, adjust pH at 2.6) of the membrane for 20 minutes at room temperature.

2.2 Assessment of nigrostriatal dopaminergic system integrity in a "double hit" model of GBA1 microRNA-mediated downregulation and human α-synuclein overexpression

1. Animals

Eight-week-old male wildtype C57B/6 mice (27-33 g) were housed (5-6 animals/cage) with free access to food and water under a 12-h light/dark cycle. All experimental procedures performed were approved by the Ethical Committee for Use of Laboratory Animals in the Biomedical Research Foundation of Athens.

2. Adeno-associated viruses (AAVs)

Adeno-associated viral vectors, kindly provided by Dr. P. Sardi (Sanofi), were used to downregulate GCase expression with a small hairpin microRNA vector targeting GBA1 (miR GBA), control vector with a scrambled microRNA (miR control) or co-transduced with GFP, where indicated (miR control-GFP), rescue vector expressing a miRNA-resistant GBA (i.e. ectopic expression of the wild type GBA1 gene) and vector overexpressing human α-synuclein (aSyn). Subjects were assigned to one of 6 treatment groups: miR control +/- aSyn, miR GBA +/- aSyn and miR rescue GBA +/- aSyn (see table 1 for the list of viruses and titers).

Virus	Virus	Titer
miR control	AAV2/1 CBA-miR-Control	$2{,}80 \times 10^{12}$
miR control-GFP		
miR GBA	AAV2/1 EGFP-miRNA-gba1 B	$3{,}30 \times 10^{13}$
miR rescue	AAV2/1 scGusB-GC*-miR gbaB	$3{,}40 \times 10^{12}$
aSyn	AAV1-CBA-aSynuclein	$6{,}30 \times 10^{12}$

Table 4: A table containing the viral load injected into the striatum of the animals used for our experiments.

Six experimental groups were created for the purposes of our methodology, which are as follows:

Treatment	Abbreviation
microRNA control (GFP) – human aSyn	mirCTL – aSyn
microRNA GBA (GFP) – human aSyn	mirGBA – aSyn

microRNA rescue – human aSyn	mirRescue – aSyn
microRNA control (GFP) + human aSyn	mirCTL + aSyn
microRNA GBA (GFP) + human aSyn	mirGBA + aSyn
microRNA rescue + human aSyn	mirRescue + aSyn

Table 5: Table containing the six different experimental groups created for the purposes of our experiments. Noted on the right side the abbreviations used for each group.

3. Surgical Procedures

All surgical procedures were performed under isoflurane (Abbott, B506) anesthesia. Animals were given i.p. carpofen (dose) for analgesia. After placing the animal into a stereotaxic frame (Kopf Instruments, USA), 2 µl of AAV solution (with final titer of 3,3E13 gc/mL) and 1 µl of DPBS or a-synuclein was injected unilaterally into the right striatum using the following coordinates: –0.5 mm anteroposterior, +1.6 mm mediolateral from the bregma, and –3.4 mm dorsoventral from the skull, according to the mouse stereotaxic atlas (Paxinos, The mouse brain in stereotaxic coordinates). Injection was performed using a pulled glass capillary (diameter of approximately 60–80 µm) attached to a Hamilton syringe with a 22s gauge needle. After delivery of the viral vector using an injection rate of 0.1 µL/15 sec the capillary was held in place for 5 min, retracted 0.1 µm, and, after 2 min, was slowly withdrawn from the brain.

4. Intracardiac perfusion

4 or 8 weeks post surgery (WPS), animals were perfused under isoflurane anesthesia. The perfusion was performed through the ascending aorta using approximately 30 ml of 1X PBS for blood clearance and then 50 ml of 4% paraformaldehyde (PFA) for tissue fixation. The brains were removed and post-fixated overnight in 4% PFA, then transferred to 15% sucrose overnight followed by 30% sucrose overnight. Following this, the brains were frozen using isopentane on ice (-55^{o}C) and stored at -80^{o}C until analysis.

5. Cryotome

The perfused brains were cryosectioned through the coronal plane in 30 µm increments. We collected sections from the striatum and substantia nigra. The sections were

collected free floating in anti-freeze buffer (NaH_2PO_4 ; Na_2HPO_4 ;Etylen glycol;Glycerol; H_2O) and kept in storage at -20^oC until analysis.

6. Immunohistochemistry

For immunohistochemichal analysis of the obtained sections, we selected striatal and nigral sections (for the substantia nigra, planes 4-7 were ideal for maximum TH+ cell somata). The sections were washed with PBS, followed by antigen retrieval with 10mM citrate buffer at 80^oC for 20 minutes then placed on ice for an additional 20 minutes. The sections were then blocked using 5% normal goat serum (NGS) and 0.1% Triton-X for 1 hour at room temperature. Subsequently, the sections were incubated for 48 hours at 4^oC with the following antibodies: GBA (1:500;), human α-synuclein 211 (1:10.000;), GFP (1:2000;), TH (1:2000; Millipore), Dopamine transporter (DAT; 1:2000;). Finally, the sections were incubated with the following secondary antibodies: rabbit red (1:2000), chicken green (1:2000), rat red (1:2000), mouse far red (1:500), DAPI (1:2000).

7. Total RNA isolation and reverse transcription

Midbrain tissues were dissected from mouse brains and total RNA was isolated. For this purpose, 1 ml of TRIZOL was used per 50-100 mg of tissue, followed by centrifugation at 12.000xg for 10 minutes at 4^oC. The supernatant was transferred to a new tube and incubated for 5 minutes at room temperature. Afterwards, 0.2 ml of chloroform was added per 1 ml of TRIZOL reagent. The tubes were shook vigorously for 15 seconds and incubated again at room temperature for a further 3 minutes, followed by centrifugation at 12.000xg for 15 minutes at 4^oC. The mixture is separated into a lower red, phenol-chloroform phase, an interphase and a colorless upper aqueous phase, which contains the RNA. The aqueous phase was transferred into a new eppendorf tube and the RNA was precipitated using 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL. Samples were incubated overnight at -20^oC, in order to increase the yeald. Following this, the samples were centrifuged again at 12.000xg for 10 minutes at 4^oC

and the supernatant was then completely removed, leaving only the RNA pellet which was washed using 75% ethanol (1 ml per 1 ml of TRIZOL). After another centrifugation at 7.500 g for 5 minutes at 4^oC, the pellets were air dried and dissolved in RNase-free H_2O. Concentration was measured using spectrometry and RNA integrity was assessed by running 500 ng of each sample on a 1% agarose gel.

Following this, we proceeded to perform reverse transcription in order to create cDNAs from these samples. DNase treatment was then performed (RQ1 10x buffer, RQ1 DNase by Promega, 30 minutes at 37^{o}C) and stopped by adding 1 ul of DNase Stop Solution (Promega) at the sample and incubating for 10 minutes at 65^{o}C. Finally, 1 ul of oligo dT per sample was added and the samples were incubated for 5 minutes at 70^{o}C. For the cDNA synthesis we used 5 ul of 5X RT buffer (Promega), 5 ul of dNTPs, 1 ul of RNaseOUT (Promega) and 1 ul of reverse transcriptase (M-MLV RT, Promega) and incubated the samples for 1 hour at 37^{o}C. qPCR was then performed.

8. Confocal microscopy and analysis

Images from the stained sections were obtained using confocal microscopy (Leica SP5 mark II with conventional photon-multiplier tube, at 23°C using the Leica Advanced Fluorescence v2.7 acquisition software (Leica Microsystems, Wetzlar, Germany)). After acquiring representative images, quantification of the stained proteins was done using Imaris, a 3D & 4D Interactive Microscopy Vizualization Software (Version 8.0), for GBA or ImageJ, for aSyn. For each animal, two sections per brain area were used and a total of 3 images per section were obtained.

For GBA quantification (N=5 for 4WPI, N=3 for 8WPI), the surface function of the Imaris software was used to select only GFP positive cells by appropriately adjusting the number of voxels as well as the intensity of the GFP channel in the threshold tab. The intensity of GBA was then calculated by masking the GBA channel within the aforementioned selected surfaces.

For the quantification of human aSyn (N=4-6) in the striatum and substantia nigra, the entire surface using DAPI intensity or TH positive somata, respectively, was selected and the threshold was adjusted appropriately, after converting the image to 8-bit. The selected area was assigned as the region of interest (ROI) and using the measure tool, we measured the intensity of hu aSyn staining within that ROI.

3. Results

3.1 A comparative analysis of genotype-phenotype in a human alpha synuclein overexpression model

3.1.1 Genetic analysis

In order to assess whether there are genetic differences between the animals of the German (DE) and the Greek (GR) colony, quantitative real-time PCR was performed on 5 samples from each group. As a loading control for the normalization of the results, rat β-actin was used. The results are shown in the figure below.

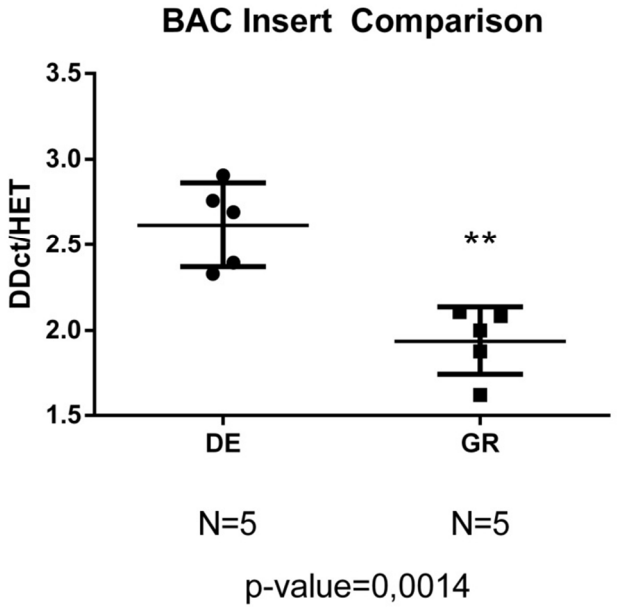


Figure 7: Genetic analysis with qPCR between the DE and the GR colony. These results indicate that the BAC rats from the DE colony have a greater relative copy number of the human α-synuclein gene than the GR colony. ** P≤0.01

From this analysis, it is evident that there is a statistically significant difference between the two colonies, with the DE colony having higher normalized levels of human α-synuclein gene expression compared to the GR colony (Fig. 1).

3.1.2 Biochemical analysis

Following the genetic analysis, we wanted to identify whether any differences can be observed in the protein level of various aSyn species, along the nigrostriatal axis.

As far as the midbrain tissue samples go, Western blot analysis revealed very low levels of paSyn in the Triton-X soluble fractions (Fig. 2, 3A). Moreover, no differences were observed between colonies when human and total aSyn levels were compared (Fig. 2, 3B, C). As far as TH levels go, decreased levels were observed in the DE midbrain tissue samples, suggesting that neurodegeneration of dopaminergic neurons in the midbrain of these animals is significantly greater when compared to the animals from the GR colony (Fig. 2, 3D). Results for the SDS soluble fraction revealed elevated levels of paSyn in the midbrain of the DE BAC rats (Fig. 2, 3E). However, levels of human and total aSyn in the SDS soluble fraction are decreased in the DE BAC rats (Fig. 2, 3F, G).

Comparison of striatal tissue samples of DE and GR BAC rats revealed increased levels of paSyn, human aSyn and total aSyn in both Triton-X-soluble and Triton-X -insoluble (SDS) fractions (Fig. 4, 5A-C, E-G). There was no difference in cytosolic TH levels (TX-soluble fraction only) (Fig. 4, 5D).

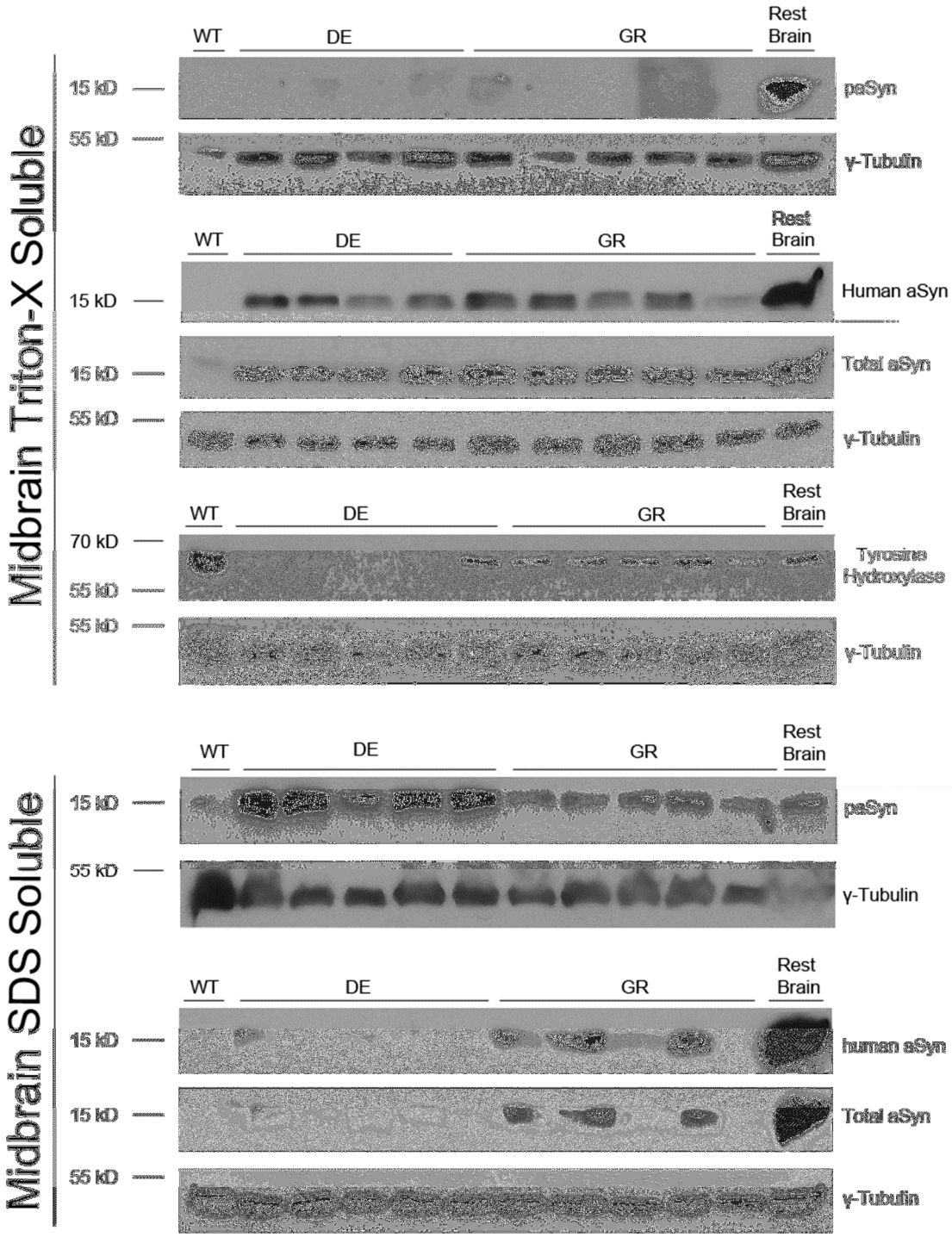


Figure 8: Western immunobloting analysis of the midbrain tissue samples of the DE and GR BAC rats. Midbrain tissue from wild type (WT) rats was used as negative control, whereas the remaining brain after dissection of the brain regions of interest was used as positive control (Rest brain). BAC rats from both the originating lab (Germany, denoted as DE) and our lab (Greece, denoted as GR), were lysed and two protein fractions were prepared – Triton-X 100 soluble (top panel) and SDS soluble (bottom panel). Membranes were probed using antibodies against phosphorylated aSyn (paSyn), human aSyn (4B12), total aSyn (Syn1) and tyrosine hydroxylase (TH). Relative density for the monomeric aSyn species (15 kDa) as well as TH (60 kDa) was normalized to the intensity of the γ-tubulin band (40 kDa).

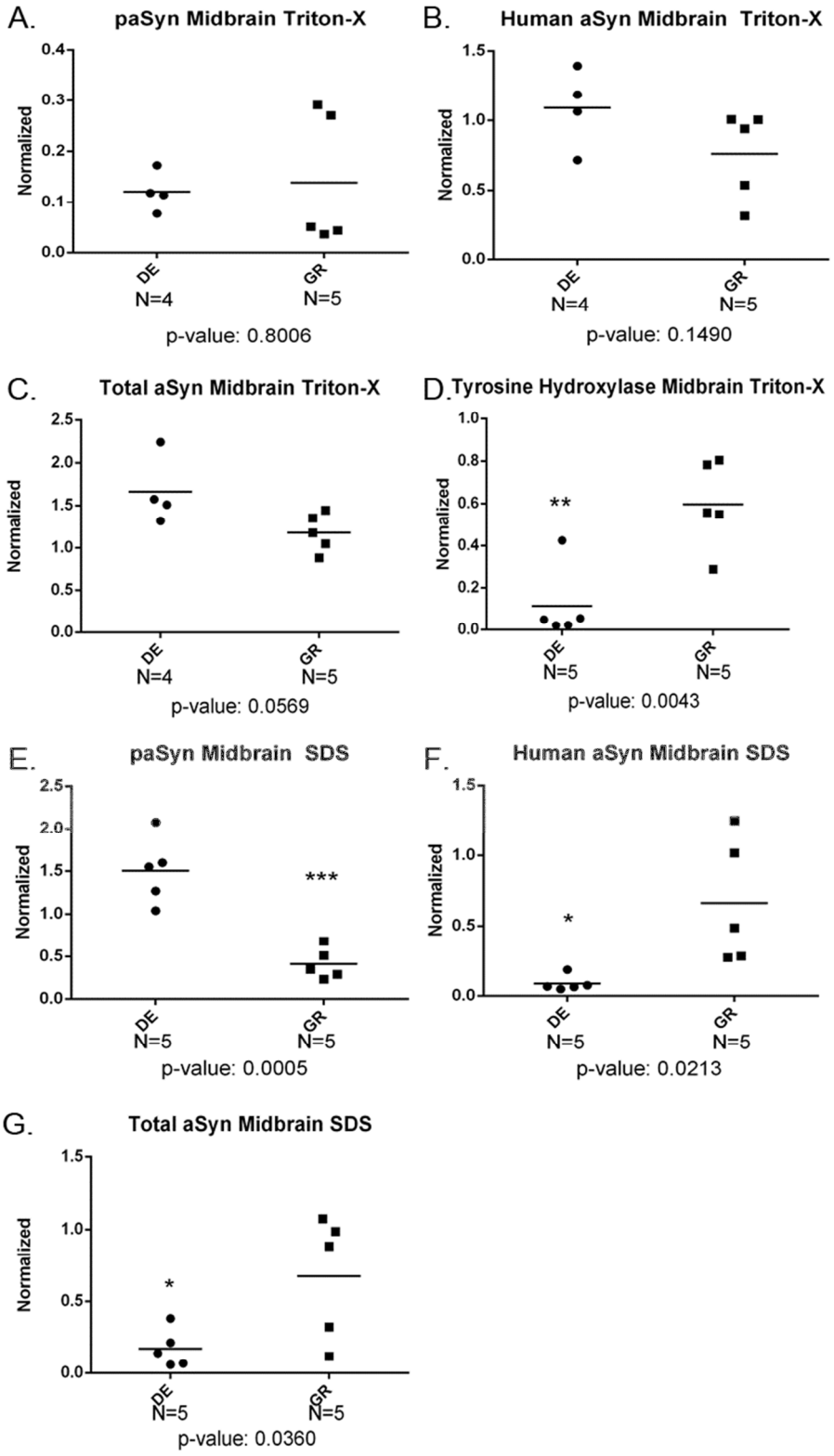


Figure 3: Grouped scatter plots of the Western blot protein quantification from the midbrain samples from the two colonies, regarding the Triton-X and SDS soluble fractions. The two groups are denoted below the plots (DE for the German BAC rats, GR for the Greek BAC rats). The number of samples used for each blot is also denoted below each plot. No significant differences are displayed between the two colonies, regarding the levels of paSyn (A), human (B) and total aSyn (C). Tyrosine hydroxylase levels are decreased in the German BAC rats (D), whereas levels of paSyn (E), human (F) and total (G) aSyn are elevated in the SDS soluble fraction. All values are expressed as normalized protein of interest vs. γ-tubulin. *p≤0.05, **p≤ 0.01, ***p≤0.001

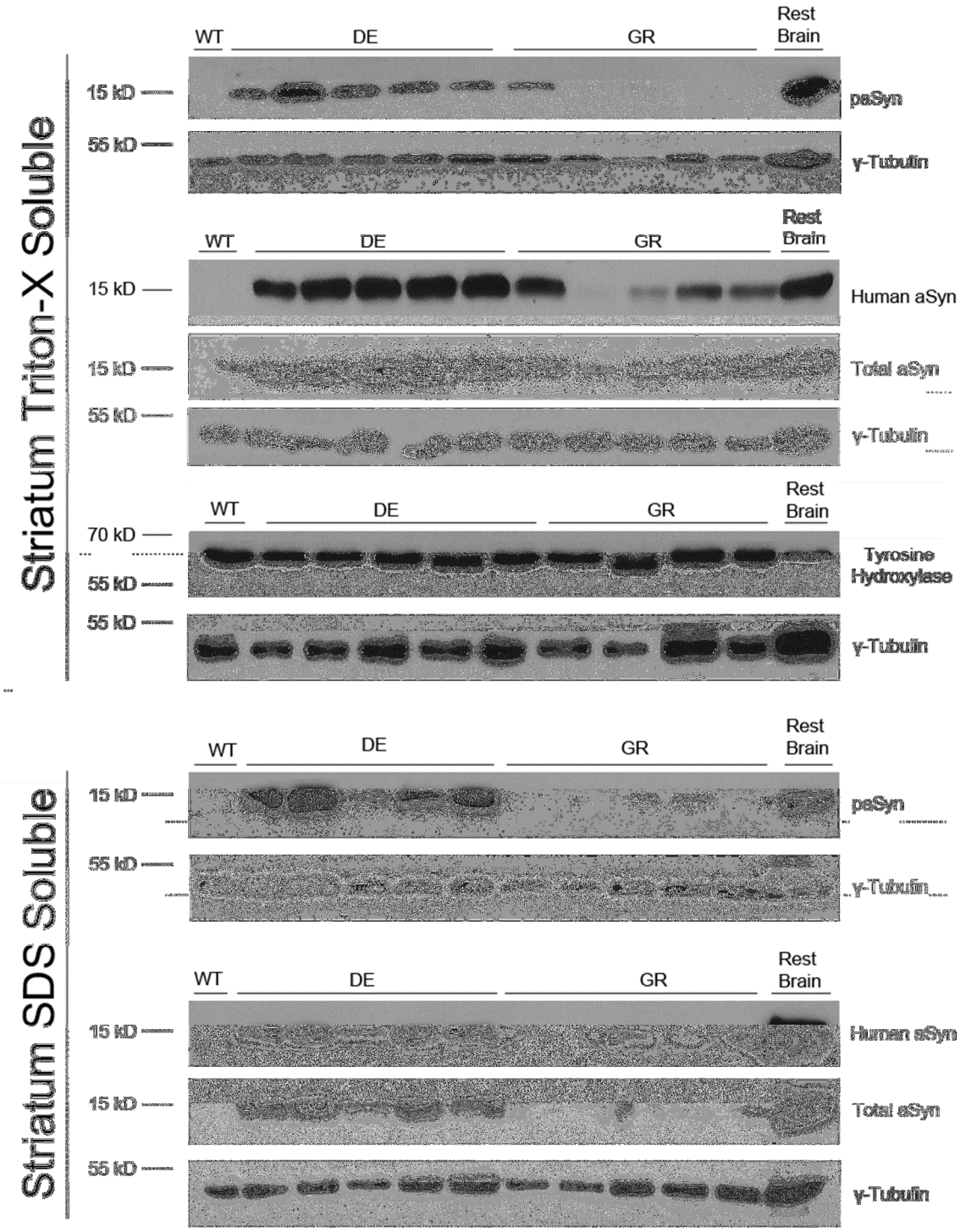


Figure 4: Western immunobloting analysis of the striatal tissue samples of the DE and GR BAC rats. Striatal tissue from wild type (WT) rats was used as negative control, whereas the remaining brain after dissection of the brain regions of interest was used as positive control (Rest brain). BAC rats from both the originating lab (Germany, denoted as DE) and our lab (Greece, denoted as GR), were lysed and two protein fractions were prepared – Triton-X 100 soluble (top panel) and SDS soluble (bottom panel). Membranes were probed using antibodies against phosphorylated aSyn (paSyn), human aSyn (4B12), total aSyn (Syn1) and tyrosine hydroxylase (TH). Relative density for the monomeric aSyn species (15 kDa) as well as TH (60 kDa) was normalized to the intensity of the γ-tubulin band (40 kDa).

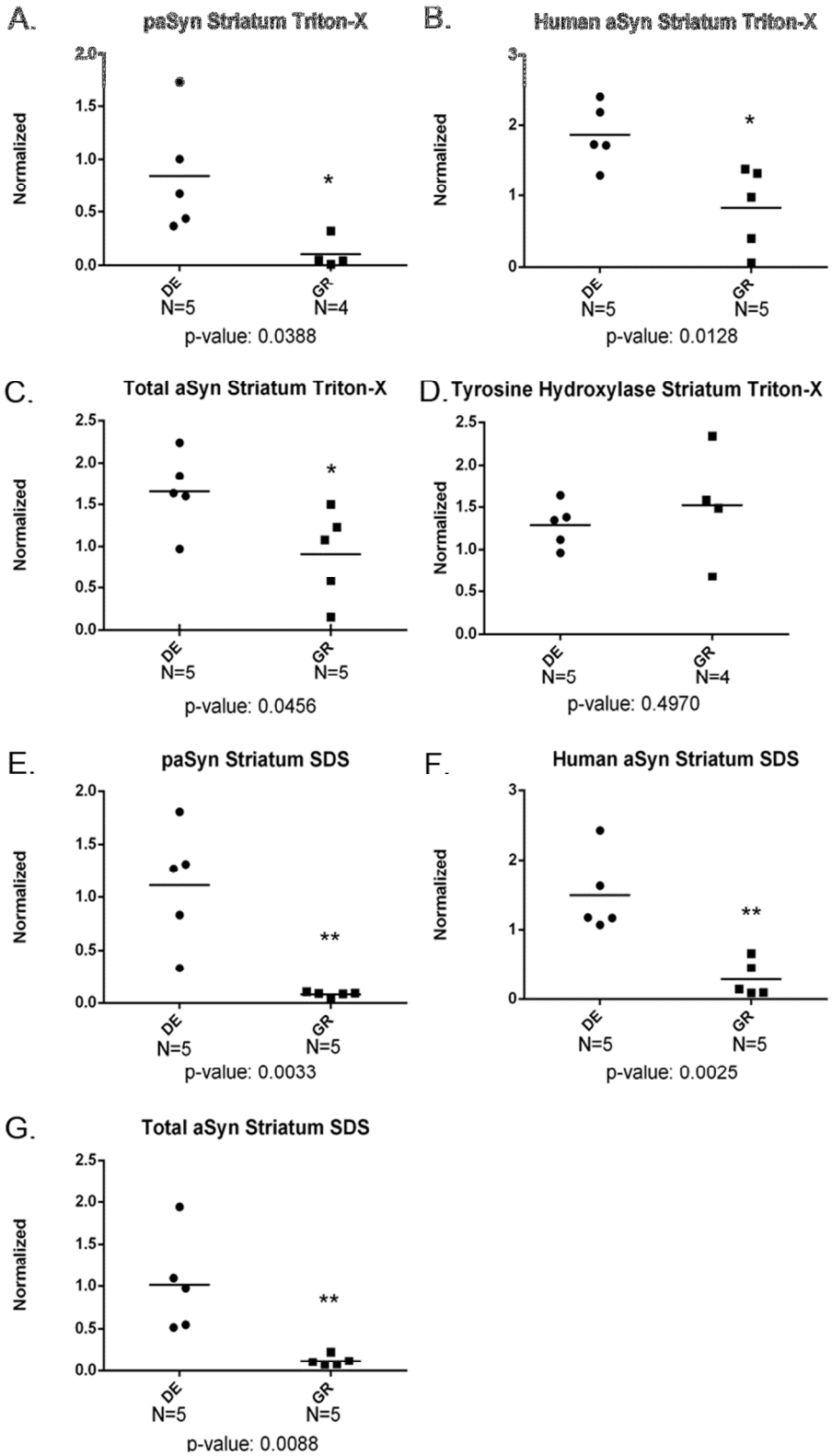
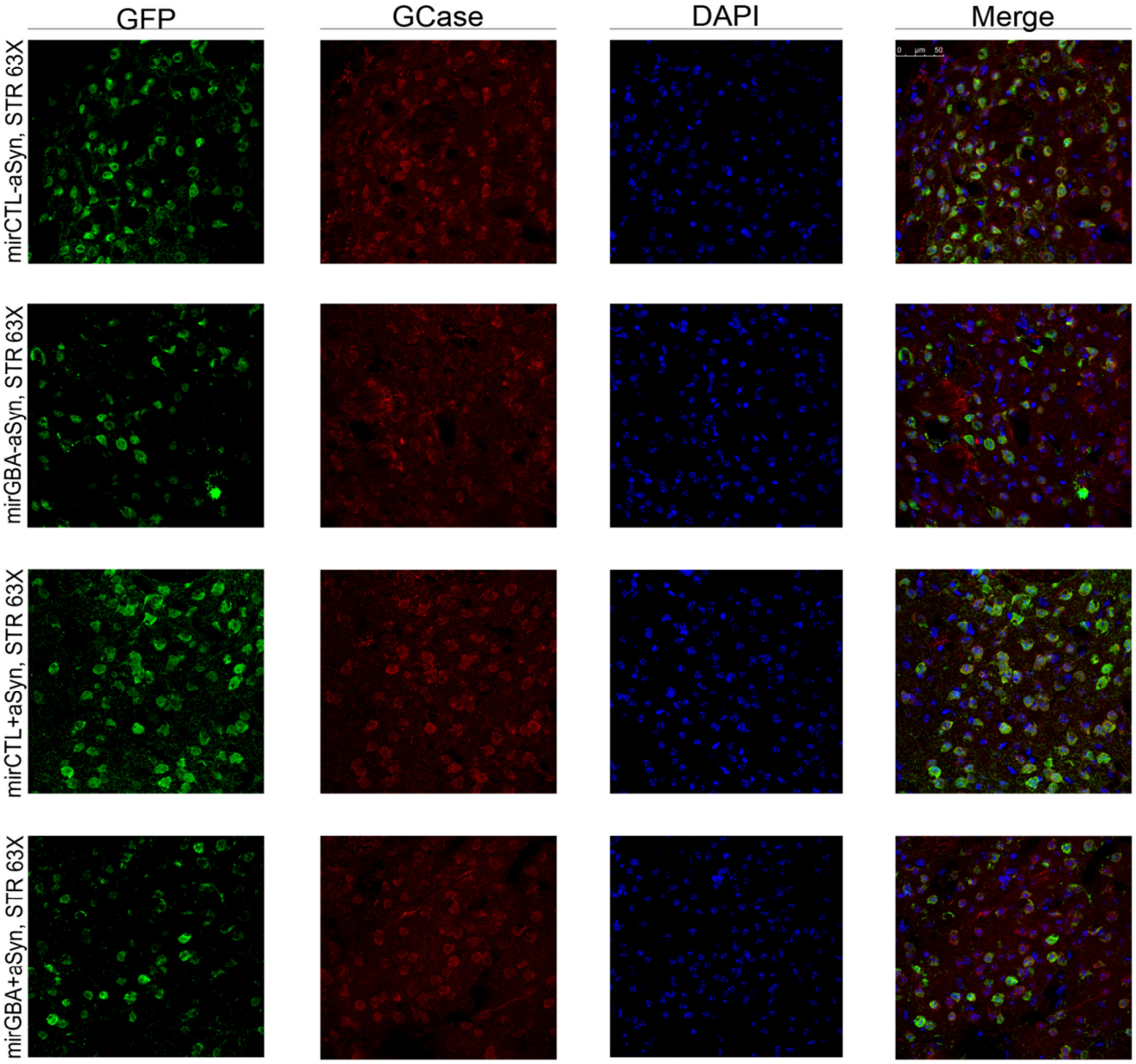


Figure 9: Grouped scatter plots of the western blot results from the Triton-X and SDS soluble fractions of striatal samples from the two colonies. The two groups are denoted below the plots (DE for the German BAC rats, GR for the Greek BAC rats). The number of samples used for each blot is also denoted below each plot. Levels of paSyn (A,E), human (B,F) as well as total aSyn (C,G) are increased in the DE BAC rats compared to the GR BAC rats in both the Triton-X soluble fraction (top panel) as well as the SDS soluble fraction (bottom panel). Tyrosine hydroxylase levels display no significant differences between the two groups. All values are expressed as normalized protein of interest vs. γ-tubulin. *p≤0.05, **p≤ 0.01, ***p≤0.001.

3.2. A novel "double-hit" model of GBA1 micro-RNA-mediated downregulation and human α-synuclein overexpression

3.1.1 GCase downregulation at 4 weeks post surgery

In order to assess the downregulation of GCase 4 weeks post surgery (WPS), coronal striatal sections from the groups mirCTL – aSyn, mirCTL + aSyn, mirGBA – aSyn and mirGBA + aSyn, were stained against GCase, GFP and DAPI. Characteristic images from these groups were obtained using confocal microscopy and the amount of GCase within the GFP-positive stained cells was quantified. Analysis suggests that no significant differences exist between the groups in regards to the intensity of GCase in GFP-positive neurons in the striatum. Representative images are shown in Fig. 6.



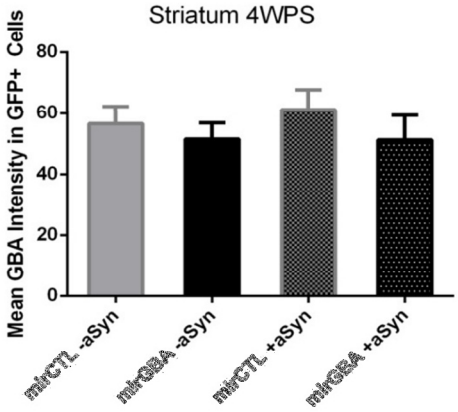


Figure 10: Representative images and bar chart of the analysis of ipsilateral striatal sections stained for GFP, GCase and DAPI 4 weeks post surgery (WPS) in subjects injected with either the mirCTL -/+ aSyn or the mirGBA -/+aSyn . As indicated by the bar chart in the bottom panel, no significant differences were observed between the groups at 4 WPS, though a minor downregulation is observed in the mirGBA – aSyn and mirGBA +aSyn groups.

3.1.2 GCase downregulation at 8 weeks post surgery

Following assessment of the downregulation of GCase 4 WPS, we wanted to investigate the levels of downregulation at 8 WPS (Fig. 7). Characteristic images from these groups were obtained using confocal microscopy and the amount of GCase within the GFP-positive stained cells was quantified, as above. In this case, the available groups included mirCTL-aSyn, mirGBA+aSyn, and mirRescue –aSyn, thus some groups that would make the analysis more meaningful were missing. The intensity of GCase appears to be decreased in the mirGBA+aSyn group, compared to the mirCTL–aSyn, indicating effective downregulation of GCase expression with the miRNA for GBA; however, the aSYN expression in this condition may also play a role in the effective downregulation of GCase. . Moreover, the intensity of the mirRescue –aSyn group looks relatively the same as the mirCTL –aSyn, indicating the rescue of the phenotype in this group. Quantification could not be performed in this case, as the expression vector lacked GFP.

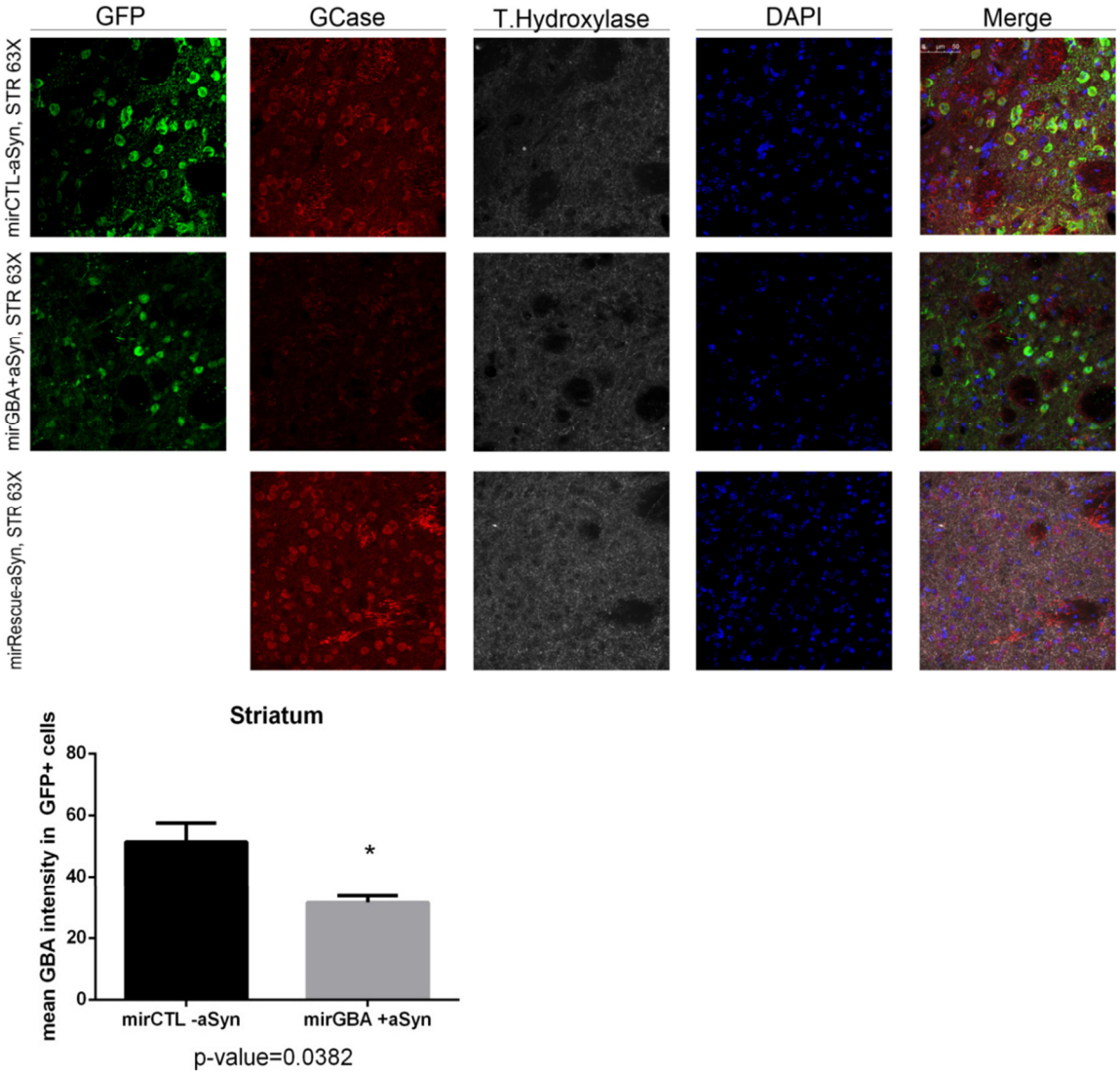


Figure 11: Representative images from ipsilateral striatal sections of injected mice 8 weeks post surgery. GFP is coloured green, GCase red, tyrosine hydroxylase grey and DAPi blue. The intensity of GCase appears to be decreased in the mirGBA +aSyn group, compared to the mirCTL –aSyn. Moreover, the intensity of the mirRescue –aSyn group looks relatively the same as the mirCTL –aSyn, indicating the rescue of the phenotype in this group. Below the images is a bar chart with the quantitative results after the Imaris analysis, indicating that the downregulation is statistically significant at 8 WPS. N=3, * = P≤0.05

3.2.1 Accumulation of human α-synuclein along the nigrostriatal axis at 8 WPS

Our data suggest that we have statistically significant downregulation of GCase at 8 WPS. Therefore, in order to assess the phenotype of these animals as far as the accumulation of human α-synuclein goes, we decided to focus on the 8 WPS time-point. Enhanced accumulation of human α-synuclein is evident in both the striatum and substantia nigra in the mirGBA + aSyn groups, with the intensity in the mirRescue + aSyn group, being comparable to the levels as in the mirCTL+ aSyn (Fig. 8). This is based on a regional analysis of the striatum and the nigra respectively. Of note, the reason for the human aSyn expression in the SN is that there is retrograde and antergograde transfer of the transgenes to the nigra, following the striatal injections.

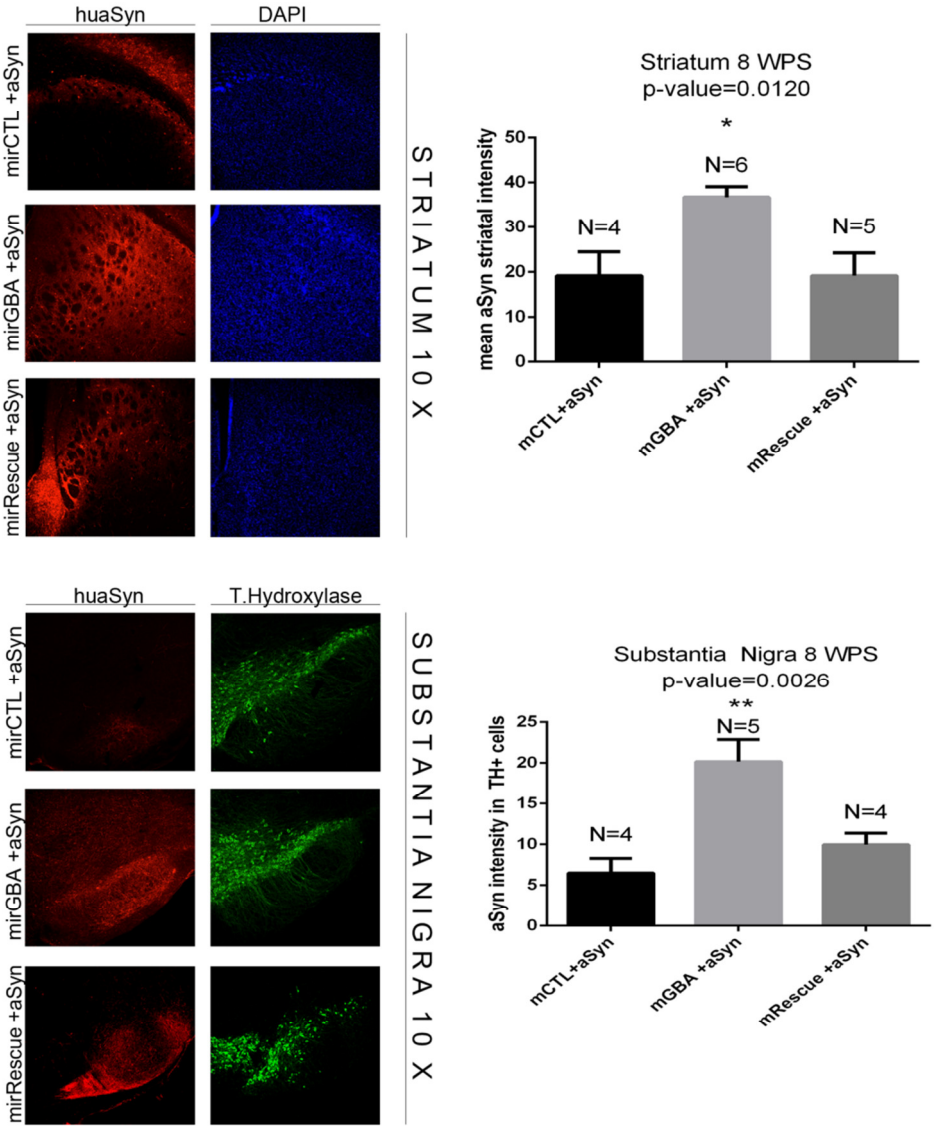


Figure 12: Immunohistochemistry revealing the levels of human α-synuclein in the striatum and substantia nigra of animals 8 WPS. Striatal and nigral coronal sections from animals sacrificed 8 weeks post-surgery were stained against human α-synuclein and TH/DAPI (for the striatum and substantia nigra). Using ImageJ, we calculated the intensity of the human α-synuclein signal in DAPI+ cells. For the nigral sections, we calculated the intensity using the TH+ neurons as reference. On the right, bar charts represent the quantification of human α-synuclein in both these brain regions. Accumulation of human α-synuclein (huAS) is evident in both brain regions in the mirGBA + aSyn groups, with the intensity in the mirRescue + aSyn group, being comparable to that of the mirCTL +aSyn group.

*p≤0.05, **p≤ 0.01

3.2.2 Human α-synuclein cDNA levels

In order to preclude differences in human α-synuclein levels due to the expression of the viral transgene in the various conditions, we quantified the cDNA levels of human α-synuclein expressed in the tissue of these animals. The data from the qPCR analysis revealed no differences in aSyn cDNA expression in the substantia nigra (Fig. 9).

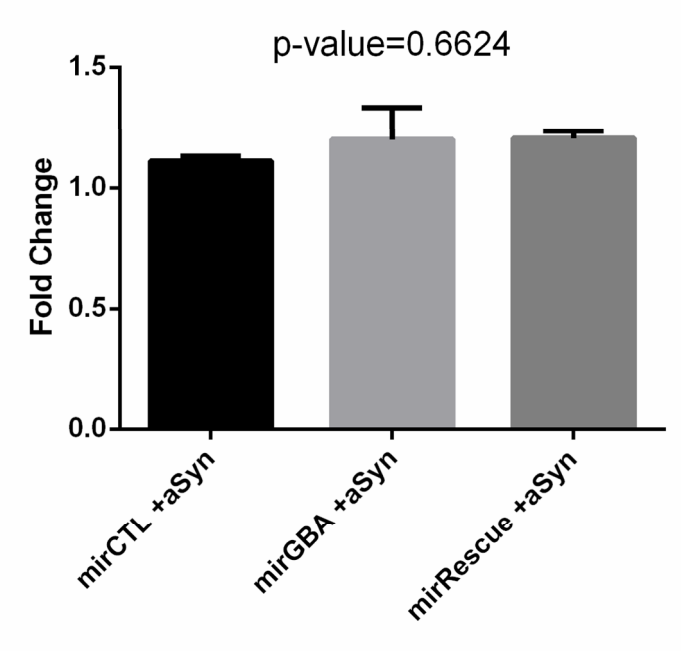


Figure 13: cDNA levels of human α-synuclein reflecting the expression levels of the viral transgene containing the human α-synuclein sequence. No differences are observed between mirCTL + aSYN, miRFBA + aSyn and mirRescue + aSyn. Results are expressed as fold of change in the expression levels of cDNA compared to a reference sample (heterozygote for huaSyn).

3.3 Detection of toxic α-synuclein species

After confirmation of the downregulation of GCase and subsequently observing human α-synuclein accumulation in the mGBA + aSyn-injected animals, we then examined the presence of toxic α-synuclein species. A pilot study was performed (N=1) on coronal striatal and nigral sections that were stained against oxidized/nitrated aSyn (Syn303) and tyrosine hydroxylase. According to our data, miRGBA + aSyn-injected mice appear to have a greater presence of nitrated/oxidized forms of aSyn as compared to mirCTL + aSyn, at least in the striatum (Fig. 10). However, since this is only a pilot study, these results are inconclusive and only serve as an indication of this apparent emergence of toxic aSyn species. Further investigation is mandatory in order to confirm this hypothesis.

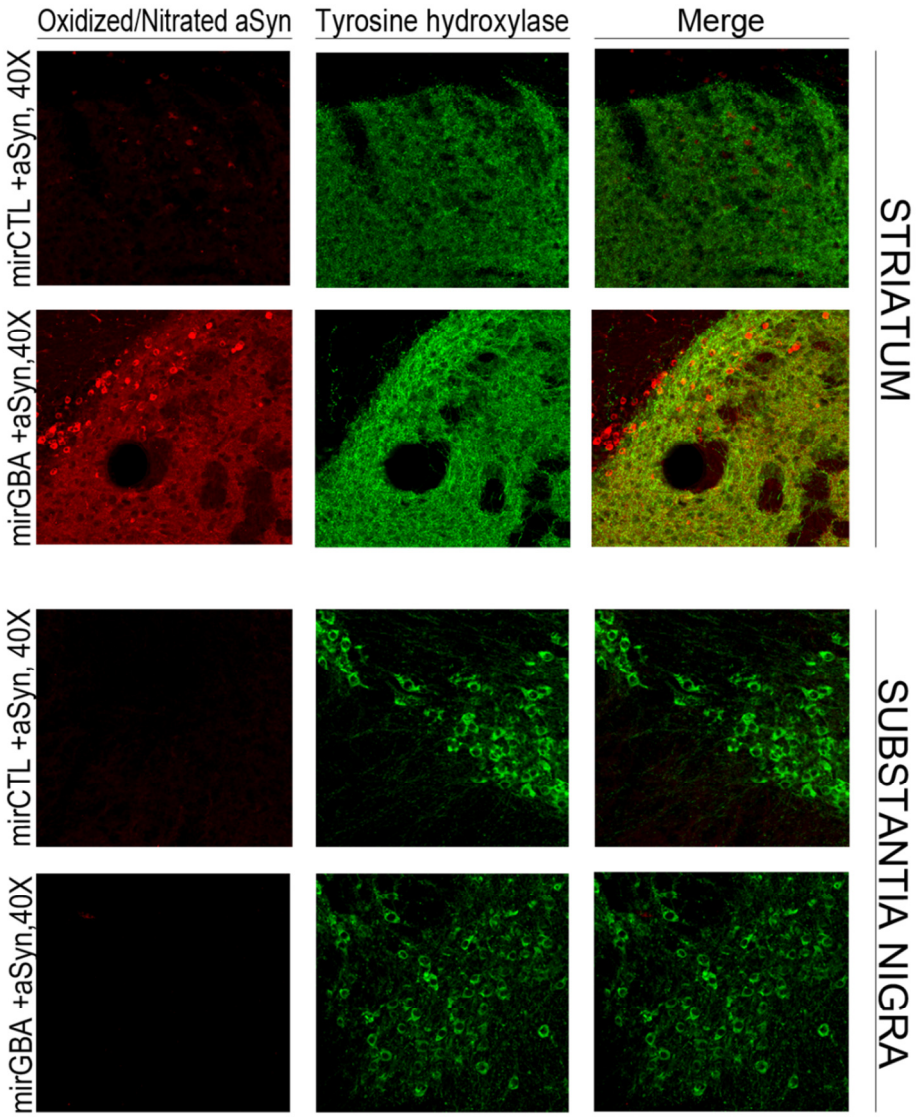


Figure 14: Representative images from the sections stained against the oxidized/nitrated aSyn. From these images, the presence of nitrated and oxidized α-synuclein species is evident. However, these toxic forms are limited to the striatum and do not appear to be present in the substantia nigra at 8 WPS.

4. Discussion

An animal model that can acurately recapitulate the progression of PD as seen in humans is a highly sought after goal in PD research. The purpose of these models is to help us better understand both the mechanisms of pathology underlying this disease, provide us with the ability to develop valuable diagnostic tools and finally, test novel therapeutic strategies in an attempt to better treat the ever increasing number of PD patients globally. However, both transgenic and neurotoxin-induced PD animal models recapitulate some but not all of the complex pathological features and symptoms. aSyn-based models are particularly important for the study of PD as this fascinating protein is implicated in both familial and sporadic forms of the disease. In 2013, Nuber et al. created a rat model that possesses the entire locus of the human aSyn gene, along with the upstream and downstream cis-regulatory elements. This BAC aSyn rat model is an important step for PD research as it was found to possess all the necessary elements of a robust animal model for this disease including aggregation pathology, loss of dopaminergic system intergrity, and non-motor and motor behavioral deficits.

Our biochemical and behavioral characterization of these aSyn BAC rats revealed some similarities with the originating colony such as an early olfactory deficit and aggregation pathology but also yielded some unexpected results. Our colony demonstrated age-independent locomotor hyperactivity rather than hypoactivity and lack of dopaminergic neurodegeneration in the nigrostriatal system up to 12 mo. Thus, we sought out to explore potential underlying genetic and biochemical differences between the originating colony and our colony.

Initial genetic analysis of the relative copy number of the human α-synuclein gene revealed reduced copy number in animals from our colony, the Greek colony (GR), compared to the originating colony in Germany (DE) (Fig. 1). The lower relative copy number of the human αSyn gene could potentially explain the phenotypic differences observed. A lower copy number could result in lower expression of the transgene and in combination with the work of Klein and Schlossmacher in 2006 and Fuchs et al. in 2007 regarding the effects of *SNCA* locus multiplication on the severity of the PD phenotype in patients, we can assume that fewer copy numbers in the GR aSyn BAC rats could potentially lead to a less severe phenotype compared with their DE aSyn BAC counterparts. Additionally, One possible explanation could be that our founders had a lower relative copy number and thus relatively lower gene expression to begin with. This fact along with any effects genetic drift may have had on our colony, could potentially lead to altered relative gene copy number between the two colonies.

Next, a thorough biochemical profile comparison was performed so as to probe for any differences between the two colonies with respect to the expression of various aSyn species at the protein level along the nigrostriatal axis. Western blot analysis was

performed in order to quantify these species both in Triton-X soluble and Triton-X insoluble/SDS-soluble homogenized tissue lysate from the midbrain and striatum of both GR and DE aSyn BAC rats (Fig 2 & 3). With respect to the midbrain, no significant difference was observed in the levels of serine 129 phosphorylated (pS129) aSyn, human-specific and total αSyn in the Triton-X soluble fraction, indicating that both groups share a similar biochemical profile of soluble aSyn species. On the contrary, levels of pS129 aSyn in the Tritox-insoluble fraction were significantly elevated in DE aSyn BAC rats when compared with GR aSyn BAC rats. This is an important finding as serine129 phosphorylated αSyn is postulated to be a toxic species of aSyn and believed to play a role in disease progression. A case in point, pS129 aSyn has been found in abundance inside Lewy Bodies of patients suffering from synucleinopathies (Fujihara et al. 2002). Additionally, it has been shown that the presence of this specific aSyn species, promotes the formation of aSyn fibrils, thus leading to protein aggregation and in vivo expression of pS129 aSyn in the substantia nigra of a lentiviral rat model induced significant loss of neurons while at the same time reducing dopamine and tyrosine hydroxylase (TH) levels, the synthesizing enzyme of dopamine, in the striatum (Gorbatyuk et al., 2008). This leads us to conclude that the elevated levels of pS129 aSyn in the DE aSyn BAC could also contribute to the exacerbated phenotype of these animals. Interestingly, GR aSyn BAC rats demonstrated higher levels of human and total aSyn, a result which doesn't initially seem in accordance with the levels of pS129 aSyn. Importantly however, the TH levels, ie. a marker for dopaminergic neurons, were reduced in the midbrain of DE aSyn BAC rats, compared with GR aSyn BAC rats. This reduction could represent reduced nigral dopaminergic cell expression, indicative of nigral neurodegeneration. In Nuber et al., 2013, pS129 aSyn levels were not examined, however, they reported age-dependent accumulation of insoluble (Urea/5% SDS) full-length aSyn in the substantia nigra that were possibly undetectable in our Triton-X-soluble/SDS-soluble fraction analysis.

Biochemical analysis of the striatum revealed that the Triton-X soluble fraction exhibited a strong increase in the levels of pS129, human-specific and total aSyn in DE aSyn BAC rats (Fig. 4 & 5). This trend is consistent with the Triton-X insoluble fraction, where all probed aSyn species were elevated. These results are in accordance with the reported observations in Nuber et al., 2013 where an increase of aSyn was observed in the striatum of these animals in both the Triton-X soluble and SDS soluble fraction. Importantly, these results are comparable to the mutant A53T aSyn model described in Giasson et al., 2002 where accumulation was evident throughout the nigrostriatal axis. The increase of the pS129 aSyn along with the increase of the insoluble forms of both human and total aSyn (Spillantini et al., 1997; Vekrellis et al., 2009) can also explain the observed discrepancy in the severity of the phenotype between the two groups. However, unlike the midbrain, the TH levels were comparable between the two groups.

Taken together, the fact that the aSyn BAC rats from the originating colony (DE) exhibit higher levels of pS129, human and total aSyn along with increased relative copy

number of the human aSyn insert, can potentially provide an explanation for the phenotypic differences between the two groups. An increased copy number of the human aSyn has previously been found to be causative of the induction of an early PD phenotype (Daher et al., 2009). The main conclusion drawn from these experiments is that the severity of the phenotype and therefore, the neurodegenerative potential of aSyn is dependent on its expression and protein load, as it has been observed with animal models that overexpress aSyn (Dawson et al., 2010) which exhibited different pathology and range of symptoms based on differences that influence the expression of aSyn such as the promoter under which the gene is expressed, the regional expression (for viral models, see Kirik et al. 2002; Klein et al., 2002) or even different forms of mutant aSyn, such as A53T can induce differences in the observed aSyn pathology (Giasson et al. 2002, Lee et al. 2002).

The reason for the decrease in aSyn expression levels in the colony established in BRFAA may include a founder effect, meaning that the animals used for the creation of the colony possessed a low copy number of the human aSyn transgene to begin with, or because of genetic drift, which occurs naturally within a population and thus, regular screening of the genetic profile of the animals is needed (Fahey et al., 2013). However, it is important to understand that other factors could also be playing a role in these phenotypic discrepancies. It has been shown that many environmental factors can influence aSyn aggregation and deposition and nigrostriatal neurodegeneration. For example, an iron-rich diet exacerbates the pathology of A53T mice and leads to neurodegeneration (Jia et al. 2018) and environmental enrichment can lead to an amelioration of nigral cell loss (Jungling et al., 2017). Various other studies have also shown that the diet can affect the microbiome of an organism (Voreades et al., 2014) and many studies have concluded that the microbiome might play a more important role in the manifestation of neurodegenerative disease than we previously thought (Cho et al., 2012). Further insights into this possibility were revealed by Sampson et al., 2016 where it was shown that when the gut microbiome was removed using antibiotics, the motor deficits that were normally observed were no longer present and returned when the gut bacterial colony was restablished. Since the microbiome of animals depends on their environment, region and species, the need to investigate whether our animals are influenced by this factor arises.

The second part of the study focused on examining the interplay between aSyn and GBA in nigrostriatal axis integrity. To this end, we created a novel double-hit viral model of micro-RNA-mediated downregulation of the *GBA1* gene and overexpression of human αSyn in the striatum. First, downregulation of GCase was assessed 4 weeks and 8 weeks post surgery. At 4 weeks, only a small but not statistically significant reduction in the amount of GCase in the striatum was observed in the mirGBA – aSyn and the mirGBA + aSyn groups (Fig. 6). The reason behind this could potentially be the fact the viruses used for this experiments weren't efficient enough in order to reduce the amount of GCase significantly 1 month after the surgery. Current literature suggests that the

specific viral serotype (2/1) is highly infective and are quickly uptaken within a few days, especially by neurons, astrocytes and oligodendrocytes (Bartlett et al., 1998). However, studies ultilizing such viral models perform their experiments at various timepoints ranging from as early as 3-4 weeks after the injection or 8 weeks (Kirik et al., 2002; Gorbatyuk et al., 2010), and sometimes going up to 6 months or even a year after the injection (Gorbatyuk et al., 2010; Klein et al., 2002). On the other hand, at 8 weeks post surgery, an approximate 50% reduction of striatal GCase was observed (Fig. 7). It is important to point out that the comparison was carried out between the mirCTL – aSyn and the mirGBA + aSyn groups, however, aSyn expression in this condition may also play a role in the effective downregulation of GCase. As stated in Mazzulli et al., 2011, the ability of the aggregated aSyn to inhibit the normal trafficking of GBA from the ER to the lysosome as well as its normal lysosomal function and therefore, further reduction in the normal GCase activity could be facilitated in the mirGBA +aSyn group compared to the mirGBA –aSyn and the controls. Accordingly, further analysis needs to be done in future experiments to quantify and compare miR CTL vs. miR GBA in the presence and absence of aSyn. Overall, this finding provides the impetus to continue our exploration of this model at the 8 week time point.

Next, we assessed whether the reduction of GCase leads to an abnormal accumulation of human αSyn. Interestingly, the mirGBA + aSyn group exhibited elevated levels of human aSyn both in the striatum and substantia nigra when compared with miR CTL + aSyn and miR resc + aSyn groups, further supporting a “toxic” interplay caused by the reduction of GCase and the presence of aberrant AS. Both the miR CTL + aSyn and miR resc + aSyn groups appeared to have similar levels of human αSyn expression, showing that reversal of the phenotype and restoration of the normal GCase function by overexpression of the human *GBA1* gene proves enough to prevent the aberrant accumulation of aSyn. We confirmed that this enhanced accumulation of αSyn was due to the downregulation of GBA along the nigrostriatal axis and not due to variance of the viral load delivered in the striatum of the animals as all groups appear to have similar levels of human aSyn mRNA. From our findings, it is apparent that when there is a reduction in the GCase activity of neurons overexpressing aSyn, there is an enhanced accumulation of αSyn. Previous studies support that the accumulation of aSyn could potentially be facilitated by changes in the concentration of GlyCer, due to deficits in the activity of GCase (Ginns et al., 2014; Farfel-Becker et al., 2014). Deficits in normal lysosomal function and thus protein clearance has additionally been proposed (Gegg et al., 2012; Schondorf et al2014). Whether this is the case for our animal model as well, needs to be investigated in the future in order to futher elucidate the mechanisms of neurodegeneration.

Additionally, in a preliminary study, we assessed the presence of nitrated and oxidized aSyn and our results indicated the presence of these toxic species in the striatum of miR GBA + aSyn group compared with mirCTL + aSyn, where they were absent (Fig. 10). Previous studies propose that nitrated aSyn in neurons can elicit an increased

inflammatory response (Chavarria et al., 2013) leading to a cascade of events that exacerbates aSyn pathology, whereas oxidized aSyn facilitates the stabilization of the oligomeric aSyn species (Li et al., 2004). Our finding further supports a “toxic” interplay between GBA and aSyn that possibly leads to either increased synthesis of these toxic species or reduced clearance, but further elucidation is required.

Overall, our findings offer valuable insight with respect to the role of aSyn and its effects on the nigrostriatal axis and its propensity to cause neurodgeneration in two different animal models of PD. However, the two animal models contribute to our knowledge base from a different perspective. On one hand, the aSyn BAC rat is an animal model that provides a unique translational tool to study the pre-motor phase of PD, since the expression and protein levels of αSyn do not lead to the full recapitulation of the motor symptoms but do lead to a plethora of pre-motor symptoms such as olfaction deficit and age-dependent accumulation of aSyn. This model is ideal to assess environmental factors that may lead to phenoconversion to a neurodegenerative phenotype but also to identify potential phenotypes and/or biomarkers that will permit early intervention with neuroprotective or disease-modifying therapeutics for PD. On the other hand, our novel double-hit mouse viral model of micro-RNA-mediated GBA1 downregulation and human αSyn overexpression is a valuable tool for the further investigation into mechanisms of nigrostriatal dopaminergic neurodegeneration. Future studies should focus on assessing the GBA-aSyn interplay in relation to substrate accumulation, autophagic dysfunction and neuroinflammation.

5. References

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