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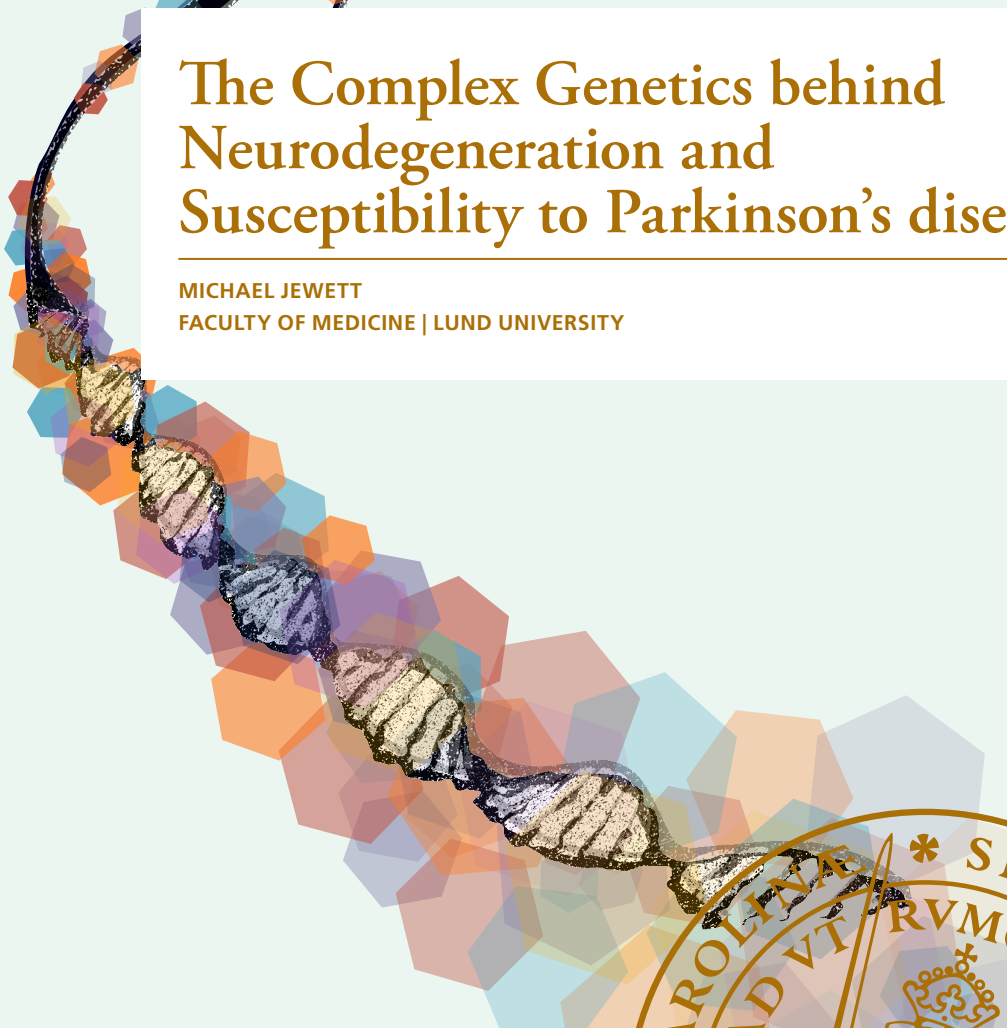

The Complex Genetics behind Neurodegeneration and Susceptibility to Parkinson's disease

DOCTORAL DISSERTATION

The Complex Genetics behind Neurodegeneration and Susceptibility to Parkinson's disease

Michael Jewett

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Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder and affects over 1% of people above the age of 65. This progressive and debilitating disease is usually thought of as a motor disease, with symptoms such as muscle rigidity, slowness of movement, and tremor at rest.

PD can be familial, where a single inherited gene mutation causes the disease, but most cases of PD (90%) are idiopathic and complex, with both genetic and environmental components contributing to disease etiology. Both forms are characterized by degeneration of dopaminergic neurons in the midbrain and by the accumulation of a protein called alpha-synuclein (α-syn) inside neurons. The complexity of idiopathic PD makes it challenging to have a full understanding of its possible causes. Current treatments can only temporarily alleviate symptoms by compensating for the loss of dopamine, but do nothing to slow the progression of the disease. Therefor, there is a need for new therapeutic strategies that can halt, or even prevent disease progression. In order to achieve this, a better understanding of the genetic risk factors contributing to PD is necessary.

This thesis is aimed at reaching this goal by investigating genetic susceptibility to neurodegeneration in three different rodent models modeling idiopathic PD, with naturally-occurring variation as a key factor.

We first explored differences between six rat strains after exposure to PD-like conditions produced by overexpression of α-syn in the substantia nigra *pars compacta* to determine strain-dependent susceptibility to neurodegeneration. Our results do indeed show differences among strains in response to this model, both in terms of dopaminergic cell loss and in terms of movement behavior. We can therefor conclude that there are genetic risk factors involved in the susceptibility to α-syn accumulation in these rats, and further genetic analyses can be used to determine such factors.

Then we applied a method called linkage analysis to determine which loci are responsible for the phenotypic difference between two mouse strains that have a partial knockout of *Engrailed 1*, a gene important for dopaminergic neuron survival. We were able to find several quantitative trait loci (QTLs) determining susceptibility to this model, and will be able to further investigate these loci to find candidate genes.

Finally, we used a congenic rat strain to study whether a specific QTL (*Vra1*), which had been discovered in previous studies as being protective after nerve injury, could protect rats from dopaminergic neurodegeneration induced by two different PD models: the toxin-induced neurodegenerative model, and the α-syn overexpression model. Our results show that the congenic strain suffers less dopaminergic cell loss in both these models, indicating that *Vra1* is protective. We also found higher expression levels of the *Gsta4* gene in the congenics, suggesting that one or more polymorphisms within and near this gene are likely regulating susceptibility to neurodegeneration.

Overall, the results gathered in this thesis have given us enough information to pursue translational studies investigating PD patient and control cohorts that are part of the biobank at Lund University (Sweden).

Key words: Parkinson's disease; α-synuclein; dopaminergic neurons; susceptibility; Gsta4; Vra1; rAAV; 6-OHDA; En1; linkage analysis; congenic strains; axonal swellings; QTLs; risk factors; neuroprotection; astrocytes

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Summary

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease and affects over 1% of people above the age of 65. While this progressive and debilitating disease is usually thought of as a motor disease, with symptoms such as muscle rigidity, slowness of movement, and tremor at rest, non-motor functions like sleep, cognition, and gastrointestinal function are also affected. These symptoms usually precede the motor symptoms and are often overlooked.

PD can be familial, where a single inherited gene mutation causes the disease, but most cases of PD (90%) are idiopathic and complex, with both genetic and environmental components contributing to disease etiology. Both forms are characterized by degeneration of dopaminergic neurons in the midbrain and by the accumulation of a protein called alpha-synuclein (α-syn) inside neurons. These clumps of protein are called Lewy bodies and evidence suggests they lead to these neurons not functioning properly and ultimately losing their ability to produce dopamine, a key neurochemical necessary for producing body movement. The complexity of idiopathic PD makes it challenging to have a full understanding of its possible causes. In terms of environmental components known for contributing to PD, some, such as exposure to pesticides, are well established. Meanwhile, regarding genetic factors, so far 41 different genetic regions (loci) have been identified as increasing disease risk. Current treatments can only temporarily alleviate symptoms by compensating for the loss of dopamine, but do nothing to slow the progression. Therefor, there is a need for new therapeutic strategies that can halt, or even prevent the progression of this disease. In order to achieve this, a better understanding of the genetic risk factors contributing to PD is necessary.

This thesis is aimed at reaching this goal by investigating genetic susceptibility to neurodegeneration in three different rodent models modeling idiopathic PD, with naturally-occurring variation as a key factor. We first explored differences between six rat strains after exposure to PD-like conditions produced by overexpression of α-syn in the substantia nigra *pars compacta* to determine strain-dependent susceptibility to neurodegeneration. Our results do indeed show differences among strains in response to this model, both in terms of dopaminergic cell loss and in terms of movement behavior. We can therefor conclude that there

are genetic risk factors involved in the susceptibility to α-syn accumulation in these rats, and further genetic analyses can be used to determine such factors.

The next step in genetic risk factor discovery involves taking information from strain difference studies and digging deeper into the genetics determining such differences. We applied a method called linkage analysis to determine which loci are responsible for phenotypic difference between two mouse strains that have a partial impairment of Engrailed 1 (*En1*), a gene important for dopaminergic neuron survival. We were able to find several loci determining susceptibility to the partial loss of *En1*, and will be able to further investigate these loci to find candidate genes.

Linkage analysis identifies genetic regions of interest, but other methods are necessary for gaining more knowledge about the identity of causative effects. The use of congenic strains (strains generated to isolate loci found through linkage analysis) is one such tool. We used a congenic rat strain to study whether a specific locus, which had been discovered in previous studies as being protective after nerve injury, could protect rats from dopaminergic neurodegeneration induced by two different PD models: the toxin-induced neurodegenerative model, and the α-syn overexpression model. Our results show that the congenic strain suffers less dopaminergic cell loss in both these models, suggesting that this locus is protective. We also found higher expression levels of the *Gsta4* gene in the congenics, suggesting that one or more polymorphisms within and near this gene are likely regulating susceptibility to neurodegeneration.

The translation from animal models to human samples is a key step in determining genetic risk factors for PD. Overall, the results gathered in this thesis have given us enough information to pursue translational studies investigating PD patient and control cohorts that are part of the biobank at Lund University (Sweden).

Populärvetenskaplig Sammanfattning

Parkinsons sjukdom (PS) är den näst vanligaste neurodegenerativa sjukdomen efter Alzheimers sjukdom och drabbar över 1 % av personer över 65 år. Även om denna progressiva och försvagande sjukdom brukar anses vara en motorsjukdom, med symtom som stelhet, långsamma rörelser och vilotremor, så påverkas också icke-motoriska funktioner som sömn, kognition och mag-tarmfunktion. Dessa symtom förekommer vanligen innan de motoriska symptomen och är ofta förbisedda.

PS kan vara familjär, där en enskild ärftlig genmutation orsakar sjukdomen, men de flesta fall av PS (90 %) är idiopatiska och komplexa, där komponenter från både genetik och miljö bidrar till sjukdomen. Båda formerna kännetecknas av förlust av dopaminerga nervceller i mitthjärnan och av ackumulering av ett protein som kallas alfa-synuclein (α-syn) inuti nervceller. Dessa klumpar av protein kallas Lewy-kroppar och leder troligen till att nervcellerna inte fungerar som de ska och slutligen förlorar sin förmåga att producera dopamin, en signalsubstans som är nödvändig för regleringen av kroppsrörelser. Eftersom idiopatisk PS är komplex är det svårt att få en fullständig förståelse orsakerna till sjukdomen. När det gäller miljökomponenter som ökar risken för PS är vissa, så som exponering för bekämpningsmedel, väl etablerade. När det gäller genetiska faktorer har hittills 41 olika genetiska variationer identifierats som kan öka sjukdomsrisken.

Nuvarande behandlingar kan bara tillfälligt lindra symptom genom att kompensera för förlusten av dopamin, men gör ingenting för att sakta ner det underliggande sjukdomstillståndet. Därför finns det behov av nya terapeutiska strategier som kan stoppa eller till och med förhindra utvecklingen av denna sjukdom. En väg mot att uppnå detta är att få en bättre förståelse för de genetiska riskfaktorerna som bidrar till PS.

Denna avhandling syftar till att uppnå detta mål genom att undersöka genetisk känslighet för neurodegeneration i tre olika djurmodeller som efterliknar idiopatisk PS, med genetisk variation som nyckelfaktor. Att studera genetiska riskfaktorer för sjukdomar i djurmodeller innebär flera steg och metoder. Ett inledande tillvägagångssätt innefattar att definiera fenotypiska skillnader mellan djurstammar under kontrollerade miljöförhållanden, eftersom djur varierar i känslighet för neurodegenerering, precis som människor gör. I denna avhandling undersöker vi skillnaden mellan sex råttstammar efter exponering för PS-liknande tillstånd vilka

produceras genom överuttryck av α-syn i mitthjärnan. Våra resultat visar på skillnader i utfall för denna modell hos de olika stammarna, både när det gäller cellförlust och när det gäller motorisk symptom. Vi kan därför dra slutsatsen att det finns genetiska faktorer som är involverade i mottagligheten för ackumulering av α-syn hos dessa råttor.

Nästa steg i identifieringen av genetiska riskfaktorer innebär att ta information från studier om stamskillnader och gräva djupare in i genetiken som avgör dessa skillnader. Vi tillämpade metoden kopplingsanalys för att bestämma vilka genregioner som är ansvariga för den lägre känsligheten hos C57B16-möss jämfört med SwissOF-möss för att förlora en av två kopior av Engrailed 1 (En1), en gen som är viktig för överlevnad av dopaminerga nervceller. Vi lyckades hitta flera regioner som avgör denna känslighet, och dessa regioner reglerade en eller flera aspekter av PS-lik sjukdom.

Kopplingsanalys identifierar genetiska regioner, men andra metoder är nödvändiga för att studera vilka gener, proteiner och processer som ligger bakom den genetiska kopplingen. Användningen av kongena stammar (stammar som genereras för att isolera loci som hittas genom kopplingsanalys) är ett sådant verktyg. Vi använde en kongen råttstam för att studera huruvida en specifik genetisk region, som tidigare funnits skyddande efter nervskada, skyddar råttor från dopaminerg nervcellsdöd inducerad av två olika PS-modeller: en toxinmodell, och en modell med överuttryck av α-syn. Våra resultat visar att den kongena stammen drabbas av mindre dopaminerg cellförlust i båda dessa modeller, vilket indikerar att denna region är skyddande. Vi hittade också högre uttrycknivåer av en genen Gsta4 hos kongenerna, vilket tyder på att variationer inom eller nära denna gen sannolikt är ansvariga för skillnader i känslighet för PS-lik sjukdom.

Övergången från djurmodeller till humana prover, så kallade translationella studier, är ett viktigt steg för att bestämma genetiska riskfaktorer för PS. Sammantaget har resultaten i denna avhandling givit oss tillräckligt med information för att genomföra translationella studier i en kohort med PS-patienter och kontrollpersoner vilka ingår i biobanken vid Lunds universitet. Från vår musstudie har vi valt kandidatgener från regionerna identifierade med kopplingsanalys; från våra råttstudier har vi identifierat *Gsta4* som en gen av intresse. Förekomsten av variationer i dessa gener kommer jämföras mellan PS-patienter och friska individer för att avgöra om de ökar risken att drabbas av PS.

Abbreviations

ABC	avidin-biotin peroxidase
asf	area-sampling fraction
Bl6	C57Bl/6
BN	Brown Norway
CE	coefficient of error
DA	Dark Agouti
DAB	3,3'-diaminobenzidine-tetrahydrochloride-dihydrate
DAT	dopamine transporter
DBS	deep brain stimulation
DNAJC6	DnaJ heat shock protein family member C6
DJ-1	parkinsonism associated deglycase
EM	expectation-maximization
EN1	Engrailed 1
GBA	acid beta-glucosidase
GFP	green fluorescent protein
GSH	glutathione
GST	glutathione S-transferase
GSTA4	GST alpha 4
GWAS	genome-wide association studies
HNE	4-hydroxyl-2-nonenal
KO	knockout
L-DOPA	levodopa
LE	Long Evans
LEW	Lewis
LID	L-DOPA-induced dyskinesia
LOD	logarithm of odds
LRRK2	leucine-rich repeat kinase 2
m	smoothness factor
MAPT	microtubule-associated protein tau
MFB	medial forebrain bundle
MPP+	1-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3-tetrahydropyridine
N	next generation
NDS	normal donkey serum

O.D.	optical density
O/N	overnight
paraquat	1,1′-dimethyl-4,4′-bypyridinium
PARK2	Parkin
PD	Parkinson's disease
PFA	paraformaldehyde
PINK1	PTEN-induced putative kinase 1
PSEN2	presenilin 2
PVG.1AV1	Piebald Virol Glaxo
r	Pearson correlation coefficient
RT	room temperature
qPCR	quantitative polymerase chain reaction
QTLs	quantitative trait loci
rAAV	recombinant lentiviruses or adeno-associated viruses
ROI	region of interest
ROS	reactive oxygen species
SD	Sprague Dawley
SNCA	synuclein alpha
SNpc	substantia nigra pars compacta
SNPs	single nucleotide polymorphisms
SW	SwissOF1
TBI	traumatic brain injury
TH	tyrosine hydroxylase
tsf	thickness-sampling fraction
VMAT2	vesicular monoamine transporter 2
VPS35	vacuolar protein sorting-associated protein 35
VRA	ventral root avulsion
WPRE	woodchuck hepatitis virus post-transcriptional regulatory element
WT	wildtype
α-syn	alpha-synuclein
6-OHDA	6-hydroxydopamine

Introduction

Parkinson's disease (PD) is a chronic, incurable disease with debilitating effects on the quality of life of patients. It's the second most common neurodegenerative brain disorder, affecting about 6 million people worldwide (Lesage & Brice, 2009). Typical symptoms of PD are motor-related, and they include resting tremor, bradykinesia, rigidity, and postural instability. However, non-motor symptoms such as constipation, sleep and mood disorders, and dementia also affect PD patients, and can occur before or even without any signs of motor symptoms, often leading to misdiagnosis (Reichmann et al., 2016).

The main pathophysiological hallmarks of PD are the degeneration of dopaminergic neurons in a subregion of the substantia nigra, the substantia nigra pars compacta (SNpc), the formation of cytoplasmic alpha-synuclein (α-syn) inclusions called Lewy bodies in several regions of the brain, and widespread neuroinflammation (Braak et al., 2003; Forno, 1996). All of these processes can be tied to oxidative stress mechanisms, which are related to aging, the one factor that most strongly relates to PD onset (Schapira & Jenner, 2011). However, the underlying cause of these processes is still unknown, which makes it extremely challenging to bring forward new therapeutic strategies to stop or even prevent the progression of the disease.

One way of enhancing our understanding of PD etiology is through learning more about the genetics of PD pathophysiology. A small fraction of PD cases is *familial*, where etiology is defined by specific genetic mutations. Several gene mutations have been identified as causative, and their discovery has greatly improved our knowledge of PD (Nuytemans et al., 2010). However, the rest of cases are considered *idiopathic,* where etiology is complex, or multifactorial, with a combination of environmental and genetic risk factors involved in causation (Kruger et al., 1999). While some of the risk factors for these types of cases have been discovered, unidentified genetic factors that increase susceptibility to acquiring PD still account for as much as 40% of the variation in disease risk (Hamza & Payami, 2010).

Uncovering new genetic risk factors is essential for gaining insight on PD pathophysiology and possibly lead to identifying new therapeutic targets or potential biomarkers that could diagnose the disease at earlier stages. This thesis tackles the challenge head-on using genetic approaches in PD animal models to

discover candidate genes linked to susceptibility to neurodegeneration. These candidate genes will then be studied in control and PD patient cohorts in a translational effort to find genetic factors determining PD susceptibility.

PD pathophysiology and current treatments

Dopaminergic cell death

One of the main features affecting PD patients includes difficulty in initiating voluntary movement (a mixture of bradykinesia and akinesia) with a progressive reduction in speed and amplitude of sequential motor tasks (Dickson et al., 2009). These symptoms are largely due to a decrease in dopamine in the striatum, the region of the brain that is part of the basal ganglia, which also includes the globus pallidus, substantia nigra, and subthalamic nucleus (Hikosaka et al., 2000). Thanks to studies by Anden et al., it was shown that the substantia nigra was the main source of dopaminergic input to the striatum, more specifically the dorsal striatum (Anden et al., 1965). More detailed studies of PD pathology pinpointed SNpc as the most affected by cell loss (Greenfield & Bosanquet, 1953; Hirsch et al., 1988). In healthy individuals, a stable fluctuation of dopamine levels within the striatum created by the direct and indirect pathways of the basal ganglia helps generate smooth controlled bodily movement. The loss of dopamine disrupts the balance within these pathways, making movement control almost impossible, thus making patients prone to the motor symptoms already mentioned (Calabresi et al., 2014).

The first motor symptoms are usually evident when approximately 80% of striatal dopamine and 50-70% of nigral neurons are lost. The nigral cell loss is concentrated in ventrolateral and caudal portions of the SNpc (Fearnley & Lees, 1991; Kordower et al., 2013b). Overall, the degree of dopaminergic terminal loss in the striatum appears to be more pronounced than that of SNpc neuron loss (Bernheimer et al., 1973), suggesting that the degenerative process begins in the striatal nerve terminals and then protract to the respective dopaminergic neurons, as was observed in 1-methyl-4-phenyl-1,2,3-tetrahydropyridine (MPTP)-treated monkeys (Herkenham et al., 1991), and in MPTP-treated mice (Wu et al., 2003).

Formation of α-syn inclusions – Lewy bodies

Another key pathological feature of PD is the presence of intracellular protein inclusions containing α-syn in affected neurons. These protein aggregates presumably form with a conformational shift of the monomeric protein, followed

by progression into larger multimeric proteins. Evidence suggests that soluble oligomeric forms of α-syn are the most neurotoxic. These can act as seeds for the formation of additional aggregates to potentiate pathology (Ingelsson, 2016). The protein clusters form insoluble complex structures called Lewy bodies and Lewy neuritis: spherical in shape, cytoplasmic, and insoluble protein aggregates composed of numerous proteins, including not only α-syn, but also ubiquitin and neurofilaments, found in the cell bodies and throughout their axons, respectively (Braak et al., 1999; Spillantini et al., 1998). It should be noted that Lewy bodies are not specific to PD, and can, in fact, also be a characteristic of Alzheimer's disease and dementia with Lewy bodies (Gibb & Lees, 1988). This creates a challenge at the clinical level when diagnosing patients because of the overlap of Lewy body pathology between these diseases. Nevertheless, it also creates an opportunity, at the experimental level: since the mechanisms of protein aggregation, which include also amyloid plaques and intracellular neurofibrillary tangles, are very similar within each disease, findings relating to one type of aggregation can be essential for other types as well.

Although PD neuropathology is typically characterized by dopaminergic neuron loss, neurodegeneration and Lewy body pathology, other neurotransmitter systems (noradrenergic, serotonergic, and cholinergic) are affected, resulting in non-motor symptoms (Corti et al., 2011; Forno, 1996). However, the timeline of damage to specific neurotransmitter systems is not well established. For example, some patients develop depression months or even years before the onset of motor symptoms, which could be due to early involvement of non-dopaminergic pathways (Halliday et al., 1990; Hornykiewicz & Kish, 1987). Meanwhile, clinical dementia occurs in approximately one-half of PD patients, usually many years after the onset of motor symptoms, and correlation studies have shown that it is associated with cortical Lewy bodies (Hurtig et al., 2000).

Oxidative stress, cellular dysfunction, and *Gsta4*

Oxidative stress is defined as disequilibrium between the levels of reactive oxygen species (ROS), also called free radicals (Betteridge, 2000), produced within cells and the ability of these cells to detoxify and clear the ROS through anti-oxidants. This disequilibrium creates a toxic environment that eventually leads to cellular damage (Dias et al., 2013). Oxidative stress is considered to be the main cause of aging, and aging is the most important risk factor in PD etiology. Therefor, unsurprisingly, it is becoming increasingly clear that oxidative stress plays a major role in the cellular dysfunction and degenerative mechanisms relative to PD (Schapira & Jenner, 2011; Yoritaka et al., 1996; Zhang et al., 1999). Evidence suggests mitochondrial dysfunction plays a major role in oxidative stress, leading to degeneration of these dopaminergic neurons (Beal, 2005; Schapira & Jenner,

2011; Zhu & Chu, 2010). Low levels of glutathione (GSH) (Jenner & Olanow, 2006), which is usually transported to the mitochondria to act as an anti-oxidant, and high concentrations of polyunsaturated fatty acids, which result in lipid peroxidation and the production of toxic byproducts like 4-hydroxyl-2-nonenal (HNE) are major sources of ROS under oxidative stress conditions (Liu et al., 2008).

GSH is a tripeptide molecule that is synthesized in the cytoplasm but is then transported to the mitochondria to act as an antioxidant (Jones & Go, 2010; Wadey et al., 2009). The reduction of GSH in PD brains has been related the impairment of Complex I in the mitochondria of SNpc cells, and as in a feedback loop mechanism, low levels of GSH then cause a further decrease of Complex I (Chinta et al., 2007; Genestra, 2007). Complex I is a key component of cell survival mechanisms; in fact, low levels of this molecule lead to cell death by apoptosis (Schapira et al., 1990).

Lipid peroxidation and its byproduct HNE have shown to result in low levels of GSH in umbilical chord blood samples (Schmidt et al., 1996), and have been shown to induce apoptosis in human T lymphoma Jurkat cells (Liu et al., 2000) and in primary rat hippocampal neurons (Kruman et al., 1997). Therefor, fatty acid propensity for peroxidation under oxidative stress conditions can result in neuronal damage and contribute to PD progression. Evidence of this has been given by the fact that HNE is elevated in the SNpc (Montine et al., 2004), and in the cerebrospinal fluid (Selley, 1998; Yoritaka et al., 1996) of PD patients. However, the cause and consequences for these elevated HNE levels have not been defined yet. Furthermore, HNE has been shown to modify mitochondrial Complex I subunits (Wu et al., 2015), further implicating the role of mitochondria in PD.

Cellular dysfunction

Due to the finding that misfolded α-syn protein aggregates and forms Lewy bodies, several studies have been conducted on protein degradation defects. Normal balance between the formation and degradation of cellular proteins is required for cell survival, and two mechanisms in particular work to keep this balance: the ubiquitin proteasome system and autophagy-lysosome pathway. Both of these mechanisms have been shown to be impaired during PD (Ciechanover, 2005; Rubinsztein, 2006), and this leads to unwanted protein accumulation resulting in cellular toxicity and neurodegeneration (Pan et al., 2008). Both of these cellular pathways are very susceptible to oxidative stress, Complex I inhibition in particular (Dias et al., 2013; Pan et al., 2008).

A critical process that leads to the progressive neuropathology in PD is the prion-like trans-neuronal propagation of α-syn (Brundin et al., 2010). Work in primary human fetal enteric neurons has shown that endocytosis of α-syn into neurons

triggers abnormal protein aggregation leading to a cytotoxic signal cascade that ends in mitochondrial dysfunction and then cell death (Braidy et al., 2014). Notably, HNE induces α-syn oligomerization, enhances its translocation to vesicles and its release from cells, and hence promotes α-syn oligomers to be transferred across cells (Bae et al., 2013).

Gsta4

A gene that relates to both GSH and HNE is *Gsta4* (glutathione S-transferase alpha 4). GSTA4 is part of a large family of glutathione S-transferase (GST) isoenzymes, which are important for cellular detoxification (Martinez-Lara et al., 2003). GSTs are divided in distantly related subgroups (class alpha, mu, pi, sigma, and theta), and alpha class GSTs are among the most abundant in mammals (Hayes & Pulford, 1995). GSTA4 is an alpha class GST and is involved in oxidative stress by clearing toxic lipid peroxidation by-products such as HNE through their conjugation to GSH (Hubatsch et al., 1998; Malone & Hernandez, 2007). Since oxidative stress mechanisms, including lipid peroxidation, are heavily implicated in several neurodegenerative diseases, including PD, *Gsta4* is an interesting target for PD research.

Current therapeutic strategies for PD

Levodopa (L-DOPA), a dopamine precursor, remains the most effective therapeutic agent for controlling motor-related PD symptoms (Jankovic & Aguilar, 2008). However, after prolonged use (already at 4-6 years), many patients develop L-DOPA-induced dyskinesia (LID), which progressively worsen over time as the dosage increases (Manson et al., 2012). A meta-analysis of L-DOPA-controlled trials has shown that initial treatment with dopamine agonists helps delay LID (Chondrogiorgi et al., 2014). Non-dopaminergic treatments are also available, especially for treating non-motor symptoms (Jankovic & Aguilar, 2008). Surgical treatments, such as deep brain stimulation (DBS), can be very effective in treating tremor and dyskinesia for some patients. DBS is a form of electrical stimulation given through implanted electrodes in the ventral intermediate nucleus, the subthalamic nucleus, or the globus pallidus (Volkmann, 2004). All of these treatments are important, but come with side effects and are only able to manage symptoms. Moreover, current clinical therapies are started when symptoms first present themselves, which is when more than 50% of SNpc neurons are lost (Cheng et al., 2010). Therefor, treatments able to slow down or even halt disease progression should be aimed for, and there is a necessity for biomarkers that can help diagnose PD at presymptomatic stages. To reach this aim, better knowledge of cellular mechanisms relating to cell death, protein aggregation, and neuroinflammation is needed.

Genetics of PD

The genetic component characterizing PD is vast, and very important, especially for better understanding some of the cellular mechanisms involved in the disease. PD is generally subdivided in two types: familial and idiopathic, depending on weight genetics plays in disease causation. Most PD cases, the idiopathic cases, are caused by the combination and interaction of several genetic variants with environmental and lifestyle factors (Fig. 1). The DNA sequences linked to the disease range from either very rare variants with very high penetrance or common variants (polymorphisms) within the general population that exert only modest effects (Figure 1) (Lill, 2016).

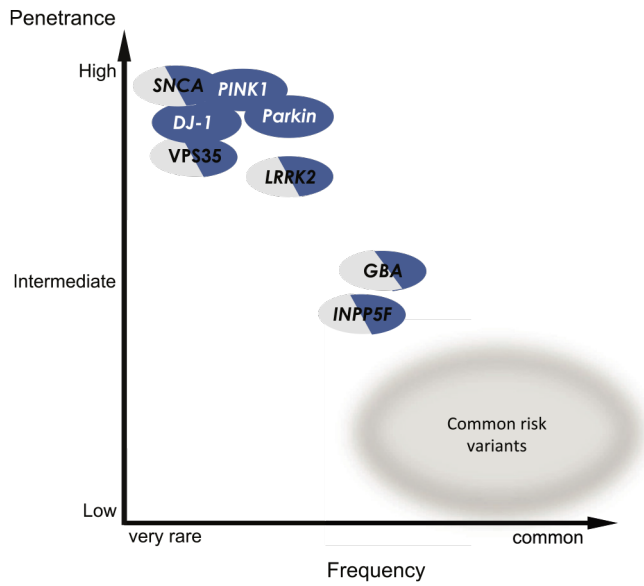

Figure 1. Schematic overview of penetrance related to frequency of genetic variants in known Parkinson's disease genes.
Rare variants (monogenic traits) tend to have high penetrance, while more common variants (polygenic traits) tend to have lower penetrance. Figure adapted from Lill, 2016.

Familial – inherited mutations

Until the discovery of the first autosomal dominant *SNCA* (synuclein alpha) mutation (A53T) associated with PD 20 years ago (Polymeropoulos et al., 1997), the disease was thought to be of sporadic nature and caused mainly by exposure to

environmental factors such as drugs or toxins (Langston et al., 1983). That, of course, was a major finding; one that lead to the development of anti-α-syn antibodies and then to the discovery of Lewy bodies (Spillantini et al., 1998). Moreover, PD genetic research at the time showed low concordance rates in monozygotic and dizygotic twins (Duvoisin et al., 1981; Marttila et al., 1988; Ward et al., 1983), thus creating more doubt about the familial characteristics of PD.

Today we know that approximately 10% of PD cases are familial, where disease etiology is explained by rare genetic mutations that are highly penetrant. To this day, 7 genes have been strongly linked to the disease (Lill, 2016). Mutations in *SNCA* (Polymeropoulos et al., 1997), leucine-rich repeat kinase 2 (*LRRK2*) (Zimprich et al., 2004), and vacuolar protein sorting-associated protein 35 (*VPS35*) (Vilarino-Guell et al., 2011; Zimprich et al., 2011) are linked to autosomal dominant forms of inheritance; PTEN-induced putative kinase 1 (*PINK1*) (Valente et al., 2004), Parkin (*PARK2*) (Kitada et al., 1998), DnaJ heat shock protein family member C6 (*DNAJC6*) (Edvardson et al., 2012) and parkinsonism associated deglycase (*DJ-1*) (Bonifati et al., 2003) are linked with recessive forms of familial PD.

In regards to *SNCA*, the genetics linking it to familial PD is abundant. After the discovery of the missense A53T mutation, more research on *SNCA* followed with several duplications and triplications of *SNCA* being reported (Klein & Westenberger, 2012). Missense mutations in *SNCA* have been suggested to result in up to 85% penetrance (Polymeropoulos et al., 1997), while duplications and triplications have much lower penetrance (around 30%) (Nishioka et al., 2006). Moreover, the discovery of duplications and triplications in *SNCA* has lead to the suggestion that *SNCA* is linked to PD in a dose-dependent manner, meaning that an increased number of copies is associated with earlier disease onset, quicker progression, and a higher severity of symptoms (Fuchs et al., 2007; Ross et al., 2008).

Idiopathic PD – a complex disease

The remaining cases of PD (roughly 90%) are idiopathic and considered complex. A complex disease is one in which a combination of environmental and genetic risk factors influence susceptibility for disease. Therefor, in such cases, an individual's genetic setup interacts with environmental factors such as diet, high levels of inflammation due to infections or injury, or exposure to toxic chemicals or pollutants to cause the disease. This means that there will be heterogeneous causation for patients with the same diagnosis. In terms of genetic risk factors for

complex diseases, genetic variants such as single nucleotide polymorphisms (SNPs) are mainly discussed.

Genetic risk factors

SNPs associated to idiopathic PD, can also be found in healthy individuals, so it is not easy to quantify the effects of these variants, especially since these risk alleles can interact with each other as well as with the environment. Therefor, a person will develop PD only if the combination of risk factors reaches a specific threshold, according to the threshold model (Smith, 1971).

Genetic association studies have been instrumental in genetic susceptibility factor discovery. So far, a meta-analysis of PD genome-wide association studies (GWAS) has identified 41 genetic risk loci for PD, including *SNCA*, *LRRK*, microtubule-associated protein tau (*MAPT*), and acid beta-glucosidase (*GBA*) (Chang et al., 2017; Nalls et al., 2014), all of which provide insight into PD pathogenesis (Hardy, 2010). Several of these loci overlap with the ones described for monogenic forms of PD (Lill, 2016). *SNCA* illustrates such a case, as its mutations have been shown to cause familial PD (Polymeropoulos et al., 1997) and common variants of this gene are associated with idiopathic PD (Chang et al., 2017; Kruger et al., 1999). This makes it clear that α-syn is implicated in PD etiology, and is therefore widely used in PD animal models: from transgenic rodent models (Harvey et al., 2008)to viral vector-mediated models (Blesa & Przedborski, 2014), with the latter being able to deliver a more consistent and progressive PD-like phenotype (Visanji et al., 2016).

Environmental risk factors

Aging is by far the most common risk factor for PD susceptibility. Not only does the risk of idiopathic PD increase with age, but the penetrance of some monogenic forms increases with age as well (Horowitz & Greenamyre, 2010). However, after the fluke discovery of MPTP, a by-product of illicit heroin synthesis, leading to parkinsonism (Langston et al., 1983), environmental risk factors for PD have been studied extensively, environmental toxins in particular (Gao & Hong, 2011). For example, paraquat (1,1′-dimethyl-4,4′-bypyridinium), a common herbicide, and 1-methyl-4-phenylpyridinium ion (MPP+), the active metabolite of MPTP, are structurally similar. This prompted speculation that paraquat might be a dopaminergic neurotoxicant and exposure to paraquat may be related to the development of PD. Epidemiological studies have reported that exposure to pesticides including paraquat correlates with increased incidence of PD (Ascherio et al., 2006; Berry et al., 2010; Liou et al., 1996). Other pesticides and toxins and other environmental factors such as brain trauma and inflammation have been associated with PD as well (Gao & Hong, 2011).

The discovery of toxins as environmental risk factors for PD has helped launch some of these toxins as tools for PD rodent models. The most common being MPTP, but paraquat and retonone are commonly used as well (Gao & Hong, 2011). All of these are effective in generating dopaminergic cell death through different molecular pathways (Heikkila et al., 1985; Jackson-Lewis & Przedborski, 2007; Liou et al., 1996), and can upregulate α-syn or lead to α-syn aggregation (Manning-Bog et al., 2002; Sherer et al., 2003).

Strategies for genetic risk factor discovery

Finding new genetic risk factors for idiopathic PD is challenging since genetic analysis of complex traits may be complicated by various factors such as incomplete penetrance, multiple disease susceptibility loci, variable age of onset, gene-gene and gene-environment interactions (Nöthen et al., 1993). Two main genetic approaches, association and linkage, are typically used for identifying genetic risk factors underlying susceptibility. Both approaches work to localize genes underlying the clinical phenotypes of a disease based on their correlation with polymorphic markers. This is done without the need for a prior hypothesis about function and localization of such genes (Klein & Westenberger, 2012) making it unbiased.

Linkage analysis

Linkage analysis is based on the tendency of quantitative disease traits to be inherited together with genetic markers near the specific loci regulating the trait As a consequence of their physical proximity on a single chromosome, the disease and the marker are rarely separated by recombination events (Klein & Westenberger, 2012). Linkage can be performed in large families to identify disease-causing genetic regions, or in crosses of experimental animals that model a trait or disease of interest A quantitative trait has phenotypic variation that can be measured thanks to genetic and/or environmental factors that influence it (CTC, 2003), and linkage analysis is meant to identify quantitative trait loci (QTLs), related to a specific phenotype (Miles & Wayne, 2008). Then QTLs represent the likelihood that one or more causative polymorphisms is genetically linked to a specific marker, and this probability is called the LOD (logarithm of odds) score (Klein & Westenberger, 2012). Overall, this strategy has many advantages in terms of taking into account features relating to Mendelian traits, such as reduced penetrance, and it provides statistically based evidence of the involvement of variants in disease etiology (Ott et al., 2015).

Animal models can be very useful for studying mechanisms behind complex traits. This is usually done through the use of inbred rodent strains, which are homozygous throughout their genome, meaning they are identical within their strain. So each inbred displays stain-specific traits or phenotypes, and differences between these strains are useful for the search of genetic elements regulating each trait. Despite large sample sizes being required, linkage analysis thus becomes an essential tool for this search.

Some studies in PD research using rodent models have taken advantage of the linkage analysis approach to identify QTLs linked to midbrain neurodegeneration, particularly in toxin-induced mouse models. Two of these studies performed linkage analysis in N2 generations obtained from intercrossing and subsequent backcrossing of C57Bl/6J (Bl6) to Swiss-Webster mice. One study identified *Mptp1*, a locus on chromosome 1 containing 66 known genes including presenilin 2 (*Psen2*) as conferring strain sensitivity to MPTP (Cook et al., 2003). In another study, two QTLs were identified, one on chromosome 5 and one on chromosome 14 as linked to susceptibility to paraquat (Jiao et al., 2012).

Genetic association studies

GWAS, a type of association analysis, have proven very useful for PD genetic risk factor discovery. GWAS involves the analysis of hundreds of thousands or even millions of different SNPs covering the entire genome (Kalinderi et al., 2016) in large groups (usually a few thousand) of idiopathic PD patients and healthy individuals, and comparing frequencies of SNPs between the two groups. After the analysis, variations found more frequently in PD patients are termed “associated” with the disease (Klein & Westenberger, 2012). Therefor, these types of association studies are able to systematically map naturally occurring DNA variants (common and rare) on a genome-wide scale (Lill, 2016).

As powerful an approach as GWAS is, risk loci detected by most GWAS are associated with small effects and do not have a large affect on disease phenotypes (Simón-Sánchez & Singleton, 2008). In fact, although a large number of genetic variants have been associated with sporadic PD through GWAS, they only account for approximately a 1.5 times increase in the risk of developing PD (Kalinderi et al., 2016). Furthermore, they generally explain only a small portion of phenotypic variance due to additive genetic factors (Manolio et al., 2009), and have low power in determining rare genetic variants (Auer & Lettre, 2015). Therefore, other strategies are required to complement the GWAS.

Congenic strains

Congenic breeding is a strategy based on the transfer of genes or genomic segments from one inbred strain (the donor strain) to another (the recipient strain). The new strain created with this strategy is called a congenic strain. This genetic procedure is common for studying a genetic region of interest (ROI) or a QTL discovered through linkage analysis, and is therefor a key method for candidate gene discovery. One of the limitations of this strategy is the fact that the random assortment of parental alleles is lost, thus limiting the effects of interactions between alleles of the different parental strains (Wakeland et al., 1997).

The classic approach to generating a congenic strain is through traditional backcrossing. Offspring of the first generation backcross are genotyped for the markers flanking a QTL or a region of interest and then bred back to the same parental strain, thus diluting the original genetic background by 50% for each backcross generation. After ten generations, less than 1% of the genetic material from the original background strain remains. This approach is effective, but requires 2-4 years. The speed congenics approach, on the other hand, is a form of marker-assisted selection of breeders, and requires only 5 backcross generations to generate a congenic strains with less than 0.1% of its parental background (Wakeland et al., 1997; Wong, 2002).

For this thesis, we used the DA.VRA1 congenic striain, which had originally been created to study the susceptibility to neurodegeneration of a specific QTL: *Vra1*. The *Vra1* locus was identified by linkage analysis in a cross between DA and Piebald Virol Glaxo (PVG.1AV1) rats as linked to neurodegeneration after ventral root avulsion (VRA) (Lidman et al., 2003). The PVG.1AV1 strain was backcrossed multiple times to DA to create the DA.VRA1 congenic strain, carrying PVG.1AV1 alleles in the neuroprotective *Vra1* region on a DA strain background. This strain was used to fine map *Vra1*, and several candidate genes were discovered (Swanberg et al., 2009). A later study determined *Gsta4* as the strongest candidate gene regulating neurodegeneration in response to VRA (Strom et al., 2012) and traumatic brain injury (TBI) in DA.VRA1 congenic rats (Al Nimer et al., 2013).

As made clear by the examples above, studying different animal strains can help us learn more about complex traits and uncover genetic risk factors pertaining to a specific disease. This concept is a key factor throughout this thesis.

Animal models of PD

Animal models of PD are essential for studying the mechanisms that produce symptoms relating to the disease. They have been very effective in the search for clues underlying cause of the disease and in the discovery of new treatments for motor symptoms. Yet, no animal model has the ability to thoroughly reproduce all the pathophysiological hallmarks of PD. Therefor, the use of different models is necessary for a comprehensive study. This thesis relies on three animal models for utilizing the genetic approaches mentioned above: Engrailed 1 (En1) partial knockout (KO) in mice, the 6-hydroxydopamine (6-OHDA) striatal lesion in rats, and the recombinant adeno-associated viruses (rAAV)-α-syn overexpression in SNpc of rats

En1 partial KO in mice

Learning more about the genetics of PD has been fruitful not only for better understanding the pathological mechanisms underlying the disease, but also for developing genetic animal models to simulate those mechanisms. For example, transgenic or KO mice have been developed with mutations or deletions of genes that have been linked to or associated with PD. One of the most common transgenic mice used in this capacity are the A53T transgenic mice, which overexpress α-syn in neurons (van der Putten et al., 2000). However, this model is not consistent in overexpressing α-syn in the SNpc or reproducing dopaminergic cell death (Bezard et al., 2013; Rieker et al., 2011).

En1 is a homeobox transcription factor that is crucial for both the development and survival of mesencephalic dopaminergic neurons (Le Pen et al., 2008; Sonnier et al., 2007). Polymorphisms in this gene, have been reported to be associated with PD (Fuchs et al., 2009), and mice that lack one *En1* allele have reduced mitochondrial Complex I activity (Alvarez-Fischer et al., 2011) as well as progressive midbrain dopamine neuron degeneration in adulthood (Sonnier et al., 2007). These are features associated with PD, and such characteristics imply that the *En1* mutant mouse can be a promising PD model (Nordstrom et al., 2015). Recent studies in mice have identified En1 and En2 as survival and regulatory factors for adult dopaminergic neurons (Alvarez-Fischer et al., 2011), and En2 has been shown able to compensate for loss of En1 activity, partly restoring the function and integrity of these neurons (Alvarez-Fischer et al., 2011; Rekaik et al., 2015; Sonnier et al., 2007). The value of En1+/− mice as a model for PD-like degeneration is strengthened with evidence of changes in autophagy and the presence of enlarged circular structures, called axonal swellings, at dopaminergic

striatal terminals in SwissOF1 (SW) mice (Nordstrom et al., 2015; Sonnier et al., 2007).

Studies in mice with the partial KO of *En1* have revealed differences in phenotypes between two strains: SW and Bl6. The SW mice display characteristics similar to those seen in PD patients: progressive degeneration of dopaminergic neurons in the SNpc, reduced storage and release of dopamine in the striatum, and motor deficits similar to akinesia and bradykinesia; meanwhile, the Bl6 mice appear normal and only display similar phenotypes after complete KO of *En1* and the additional partial KO of *En2* (Sgado et al., 2006). These data indicate that genetic variants outside *En1* are responsible for differences in susceptibility to the partial KO of *En1* between the two strains. This is the perfect setup for implementing linkage analysis to find QTLs responsible for protecting dopaminergic neurons and reducing the risk for PD-like pathology, which is one of the topics of this thesis.

rAAV-α-syn overexpression in SNpc of rats

Gene delivery systems through a viral vector, generally recombinant rAAV in rats, which are, sometimes categorized as genetic models thanks to their ability to overexpress α-syn (Blesa & Przedborski, 2014), have shown promising advances in terms of replicating both the progressive neurodegeneration of the nigrostriatal pathway and the build-up of cytoplasmic α-syn inclusions, similar to what is observed in human PD pathology (Kirik et al., 2002; Lo Bianco et al., 2004). The rAAV-α-syn model, which has been broadly studied and reproduced in different animal models, efficiently produces an overexpression of human wildtype (WT) α-syn in nigral dopaminergic neurons (Ulusoy et al., 2010). This overexpression, when studied in Sprague Dawley (SD) rats, can be enhanced using the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and driven by the synapsin-1 promoter. The resulting accumulation and spreading of α-syn within the nigrostriatal pathway is accompanied by dopaminergic neuron cell and fiber loss beginning at around 3 weeks and progressing until reaching a maximum (60-80% degeneration when compared to the respective control) between 8 and 16 weeks. The associated motor deficits also follow starting at about 8 weeks post injection (Decressac et al., 2012).

6-OHDA striatal lesion in rats

As previously mentioned, neurotoxins such as MPTP, rotenone, and paraquat have been associated with PD, and are now commonly used in PD rodent models. Another toxin-based model commonly used in rodent PD models is 6-OHDA. 6-

OHDA is a hydroxylated analogue of dopamine that specifically targets chatecolaminergic cells. Its neurodegenerative effects in the dopaminergic system of the brain were discovered after injections in the SNpc of rats, which lead to anterograde degeneration of the nigrostriatal pathway along with motor disturbances (Ungerstedt, 1968). Dopaminergic neurons are particularly vulnerable to 6-OHDA because their plasma membrane transporters and the dopamine transporter have high affinity for this molecule (Luthman et al., 1989). Once the toxin enters the cells, it is oxidized in the cytoplasm, which leads to the generation of ROS and other oxidative stress mechanisms (Drechsel & Patel, 2008). Since intraparenchymal injection of 6-OHDA to the brain specifically targets the nigrostriatal pathway, it is a widely used neurotoxin in PD research.

This model, which is commonly used in both mice and rats, has two main variants depending on the target of injection into the brain: medial forebrain bundle (MFB), or the dorsolateral striatum. The unilateral MFB lesion has been the most targeted in pre-clinical research due to its strong efficacy in almost completely destroying the nigrostriatal pathway, followed by severe motor deficits (Francardo et al., 2011). This type of lesion creates phenotypes that mirror very late stages of PD in patients (Kirik et al., 1998). To study mild to moderate parkinsonian phenotypes (early stage parkinsonism) and the effect of neuroprotective agents or alleles, a lesion that produces a more progressive degeneration of the nigrostriatal pathway is necessary (Deumens et al., 2002). Kirik et al. have established parameters for such a lesion in striatum of rats. The unilateral distribution of 21µg of 6-OHDA over three sites in the dorsolateral striatum produced partial degeneration of the nigrostriatal pathway(Kirik et al., 1998). This leaves room for studying differences between strains in a controlled environment.

The 6-OHDA model is not able to mimic the cardinal symptoms of PD in their totality; nevertheless, it is a reliable model suitable for reproducing many of the changes that occur in the nigrostriatal pathway of PD patients (Bjorklund et al., 1997; Kirik et al., 1998; Kordower et al., 2013a) and offers a better time-window for the evaluation of neurorestorative interventions (Bjorklund et al., 1997).

Aims of the Thesis

The overall objective of this thesis was to bring us closer to determining genetic risk factors for idiopathic PD. Within four studies, we used three genetic approaches and three different PD animal models to advance our search for candidate genes responsible for susceptibility to dopaminergic neurodegeneration.

The individual aims of the four studies presented in this thesis were to:

Paper I

determine any differences in genetic regulation of phenotypic and behavioral outcomes related to dopaminergic cell death and dysfunction after α-syn overexpression in six phylogenetically diverse rat strains.

Paper II

identify genetic loci responsible for susceptibility to dopaminergic neurodegeneration by performing linkage analysis on an F2 population of *En1* +/- transgenic mice after measuring specific phenotypes related to PD caused by the partial KO of *En1*.

Paper III

(i) study the effects of the *Vra1* locus on susceptibility to neurodegeneration after 6-OHDA striatal injections in the DA.VRA1 congenic rat strain, and (ii) measure expression levels and cellular localization of *Gsta4* within this strain.

Paper IV

(i) study the effects of the *Vra1* locus on susceptibility to neurodegeneration after rAAV-mediated α-syn overexpression in the SNpc of the DA.VRA1 congenic rat strain and (ii) measure expression levels and cellular localization of *Gsta4* within this strain.

Methods

A brief overview of the utilized methods will be given in the following section. More detailed descriptions can be found in the papers constituting this thesis.

Ethical considerations

All animal experiments were conducted in accordance with the ethical guidelines and approved by the ethical committee of Lund University (Sweden). Animals were housed under a 12-h light/dark cycle with *ad libitum* access to food and water. Several precautionary steps were taken to minimize stress and pain to all animals.

Animal models of PD

Three different rodent models of PD were used in this thesis: the En1 hemizygous mouse model **(paper II)**, the 6-OHDA striatal injection rat model **(paper III)**, and the viral vector-mediated α-syn over-expression rat model **(paper I** and **IV)**. While some experimental methods overlap between the different models, the En1 mouse model involved a quite different set of methods compared to the rat models, and these differences will be clearly distinguished.

In vivo experiments

Breeding of transgenic mice and congenic rats

Mice

In **paper II** the En1+/− strain was generated as described by Sonnier et al. (Sonnier et al., 2007). Our first aim was to generate an F2 population of both

En1+/+ and En1+/− mice on a SW background. We crossed Bl6 males with SW-En1+/− females to create the F1 generation, and then En1+/+ males were crossed with En1+/− females to produce the F2 generation. In total, 129 F2-En1+/− and 57 F2-En1+/+ males were sacrificed at 17 weeks of age (Figure 2). Our second aim was to study Bl6 background En1+/− mice. To do so, the disrupted *En1* locus was transferred from SW-En1+/− to Bl6 with a speed congenic approach consisting of repeated backcrossing to Bl6 females. The backcross started with an En1+/− male from the F2 population mentioned above. In each generation, En1+/− male mice were subjected to SNP analysis with the Illumina Golden Gate assay (Fan et al., 2003) to estimate the fraction of Bl6 background in the genome. The En1+/− male with the highest number of Bl6 alleles was kept for backcrossing with wild-type Bl6 females to produce the next (N) generation. The Bl6-N4 generation was phenotyped and had an average of <3% SW alleles outside the En1 locus.

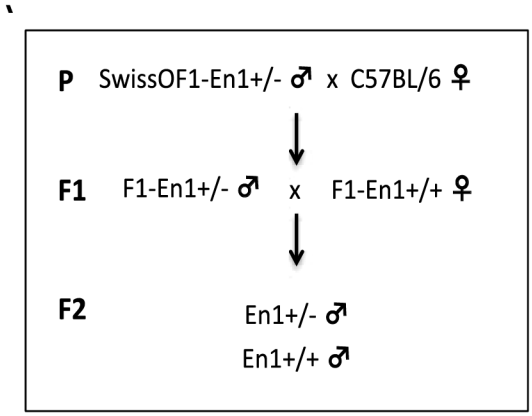

Figure 2. Breeding scheme for generating an F2 population of En1+/+ and En1+/− mice on a SW background. Parental (P) SwissOF1-En1+/- females and C57Bl/6 males were intercrossed to obtain the F1 generation, from which En1+/- females and En1+/+ males were crossed to obtain F2-En1+/- and F2-En1+/+ male mice. A total of 129 F2-En1+/- and 57 F2-En1+/+ male mice were generated. Figure adapted from **paper II;** artwork by Zuzanna Kurowska.

Rats

In **paper I**, all strains are WT; four (LE, LEW, BN, SD) were obtained from a certified breeder (Charles River), DA and PVG from our own breeding facility at the CRC in Malmö. In **papers III** and **IV** we compared the parental DA strain with its respective DA.VRA1 congenic. Both these strains were originally bred and provided by Professor Piehl at the Karolinska Institutet, Stockholm, Sweden. The DA.VRA1 congenic rats were originally bred as previously described (Strom et al., 2012): male rats with PVG.1AV1 alleles in the Vra1 locus on chromosome 8 were repeatedly backcrossed to the DA strain in order shorten the *Vra1* fragment

and reduce the number of DA alleles outside Vra1 to <0.1% of the resulting DA.VRA1 congenic strain genome (Figure 3). For the purpose of our experiments, these rats were maintained at the CRC in Malmö from where they were transferred to Lund University (Sweden).

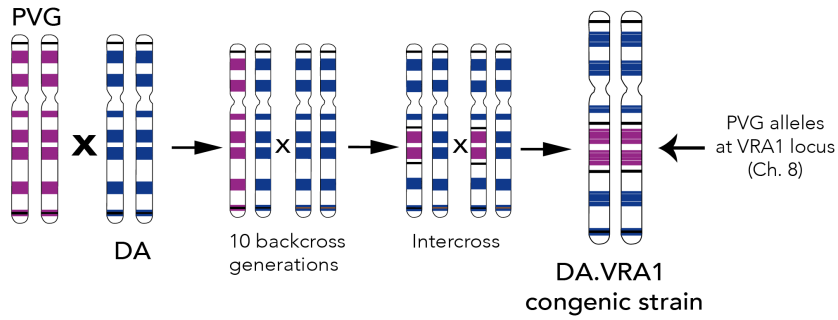

Figure 3. Breeding scheme for generating the DA.VRA1 congenic strain.
PVG.1AV1 was back-crossed to DA 10 times with selection on the *Vra1* region; an intercross generated the homozygous DA.VRA1 congenic strain, with >99.9% DA genome outside the congenic fragment.

6-OHDA injections

For **paper III**, rats were subjected to 6-OHDA injections (Figure 4) at 13 weeks of age, corresponding to a body weight of approximately 220g. After anaesthetization with isofluorane, they were placed in a stereotaxic frame with flat-skull position. Unilateral injections of 6-OHDA (3.5 mg/ml dissolved in a solution of 0.9% saline with 0.02% ascorbic acid) were made in the dorsal striatum using a 10 µl Hamilton syringe fitted with a glass capillary (outer diameter of 250 µm). Three injections of 2 µl each were performed at the following coordinates, given in mm relative to bregma and dural surface (George Paxinos, 2007): (i) AP = +1.0, ML = -3.0, DV = -5.0; (ii) AP = -0.1, ML = -3.7, DV = -5.0; (iii) AP = -1.2, ML = -4.5, DV = -5.0.

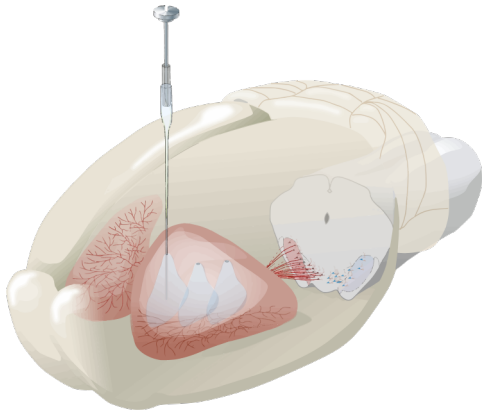

Figure 4. Injection scheme for 6-OHDA lesions.
Three deposits of 2 µl each were made unilaterally in the dorsolateral part of the striatum. Artwork by Bengt Mattsson.

rAAV-α-syn/GFP injections

In **papers I** and **IV**, rats were subjected to unilateral injections of the rAAV6 vector construct (Figure 5) to overexpress human WT α-syn or green fluorescent protein (GFP) in the midbrain at 12 weeks of age, weighing about 250g. The surgical procedure was the same as for 6-OHDA injections, except for stereotactic coordinates, which targeted the SNpc: AP = -5.3, ML = -1.7, DV = -7.2. In **paper I**, six strains were used for the study. Four strains were provided by Charles River: Sprague Dawley (SD), Lewis (LEW), Long Evans (LE), and Brown Norway (BN); two strains were provided by Professor Piehl at Karolinska Institute in Stockholm, Sweden: Piebald Virol Glaxo (PVG) and Dark Agouti (DA). Four of the six strains are inbred (LEW, BN, DA, PVG) and two are outbred (SD and LE). Only SD, LEW, and BN were injected vector-mediated GFP as controls.

For these experiments, the viral vectors were kindly generated and provided by Jenny Johansson at Lund University (Sweden). The expression of the transgenes is lead by the synapsin-1 promoter and enhanced with the WPRE (Decressac et al., 2012b).

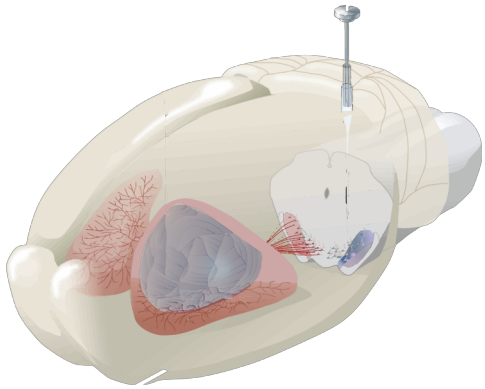

Figure 5. Injection scheme for rAAV-mediated α-syn/GFP transduction.
One deposits of 3 µl each was made unilaterally in the SNpc. Artwork by Bengt Mattsson and Michael Jewett.

Behavioral analysis

In **paper I,** two different assessments of behavior were performed: the *stepping test* and the *cylinder test*.

Stepping test

Forelimb akinesia was assessed performing the stepping test (Olsson et al., 1995) before, 4 weeks, and 8 weeks after vector injection in the SNpc. Steps for each forelimb were recorded and averaged across trials. Results are presented as the average number of adjusting steps made by the forepaw contralateral to the rAAV-α-syn injection.

Cylinder test

Forelimb asymmetry was assessed performing the cylinder test (Alvarez-Fischer et al., 2008; Schallert et al., 2000). In this test the paw movements during natural exploratory behavior were scored. Rats were placed in a glass cylinder, with 2 mirrors placed at an angle of 90° behind to allow for assessment of the subject from all angles. Spontaneous use of the forepaws was video-recorded and analyzed post-hoc. The first 30 touches made were counted. Data are presented as the ratio between average number of left paw touches (contralateral to the injection) and right paws touches (ipsilateral to the site of injection).

Perfusions and brain dissections

Mice

The aim with mouse brains in **paper II** was to keep one hemisphere for histological analysis and the other for molecular analysis. Therefor, after Pentobarbital sedation, all mice were transcardially perfused with ice-cold saline at 17 weeks of age. Brains were extracted, isolated, and placed in a mouse brain slice matrix where they were cut sagitally down the midline with a fine razor blade. The left hemispheres were immediately placed in 10–15 mL paraformaldehyde (PFA) (4%, pH 7.4), post-fixed overnight (O/N), and subsequently cryoprotected in 30% sucrose. The right hemispheres were placed on an ice-cold plate where striatum and midbrain were carefully dissected and snap frozen. The cerebellum was sliced off and post-fixed in PFA (4%, pH 7.4) for 20 min and then transferred to saline for subsequent LacZ staining.

Rats

Histology. All rats in **papers I, III,** and **IV** were transcardially perfused. Most rats underwent a 2-step perfusion at 8 weeks post-injection with room temperature (RT) saline as a first step to flush out the blood and then fixed with PFA for 6 min. The brains were then extracted and post fixed in PFA O/N before being cryoprotected in 30% sucrose.

Gene expression. Some rats in **papers III** and **IV** underwent a single-step perfusion in ice-cold saline before extraction. These brains were placed in a rat brain slice matrix where striatum and midbrain were cut out with fine razor blades and placed on an ice-cold plate. Both right and left hemispheres were dissected resulting in 4 pieces of approximately 30mg each per brain. Each piece was placed in lysing matrix tubes and snap-frozen for subsequent RNA extraction. This procedure was performed at 2 and 7 days post injection for rats in **paper III**, and at 3 weeks post injection for rats in **paper IV**.

Histological analysis

Immunohistochemistry

All animals prepped for histological analysis underwent the same immunohistochemistry protocol with slight variations in antibodies or antibody concentrations (listed in Table 1). Mouse and rat brains were sectioned coronally on a freezing microtome at 40 µm. Immunohistochemical stainings were

performed on free-floating sections at RT. The SNpc sections were given an initial antigen-retrieval incubation in Tris/EDTA when stained for tyrosine hydroxylase (TH). This was performed to enhance the visualization of dopaminergic cells, which was sometimes impaired due to poor fixation. This step often created a challenge in terms of handling the sections during and after the staining process due to the high temperature, which caused the sections to fold and become more fragile. All sections were quenched and then placed in blocking solution with normal serum for 1 hr before O/N primary antibody incubation. The following day, sections were incubated with biotinylated secondary antibody (1:200 concentration) for 1 hr. This was followed by a 30-min incubation with an avidin-biotin peroxidase (ABC) solution. Then the antigen was visualized using 3,3'-diaminobenzidine-tetrahydrochloride-dihydrate (DAB) as a chromogen. Stained sections were mounted on glass slides, dehydrated with increasing concentrations of ethanol and pure xylene, and finally coverslipped using DPX mounting medium.

Immunofluorescence

Double immunofluorescence stainings were performed as described above without the antigen retrieval and quenching steps. Blocking and primary antibody incubations were performed with 10% normal donkey serum (NDS) in PBS-T. Sections were incubated with primary antibodies at 4°C, then with Cy-3 and Alexa 488-conjugated secondary antibodies (1:500 concentration) for 1.5 hr at RT. A short 10-minute incubation in Dapi (1:1000) was added as a last step. After mounting sections on glass slides and waiting 15-20 min for the sections to dry, the slides were coverslipped with PVA-DABCO.

TABLE 1.
LIST OF ANTIBODIES

Primary ab	Isotype	Concentration	Provider	Paper
TH	Rabbit (monoclonal)	1:4000	Millipore	II
TH	Mouse (monoclonal)	1:1000	Immunostar	I-IV
Hu. WT α-syn	Mouse (monoclonal)	1:2000	Santa Cruz	I, IV
GFP	Mouse (polyclonal)	1:20000	Abcam	I, IV
GSTA4	Rabbit (polyclonal)	1:100	Antibodies-online	III, IV
NeuN	Mouse (monoclonal)	1:1000	Millipore	III, IV
IBA1	Chicken (polyclonal)	1:500	Synaptic Systems	III, IV
GFAP	Mouse (monoclonal)	1:1000	Santa Cruz	III, IV
Vmat2	Rabbit (polyclonal)	1:4000	Immunostar	IV

Striatal densitometry analysis

In **papers I, III,** and **IV**, rat striatal TH^+ fiber optical density (O.D.) was measured by image densitometry at three coronal levels (+1.60, +0.70, -0.30 mm from bregma) of the striatum using the *ImageJ* software. Each image was analyzed using the O.D. values obtained from the *Rodbard* calibration curve after being transformed into 8-bit images. Corpus callosum O.D. values were used to correct for non-specific background staining. Pictures of the striatum were taken at 2x magnification using a bright field microscope linked to a high-resolution camera and *cellSens Dimension* software. The data is presented relative to the corresponding area from the intact side.

Midbrain stereology analysis

Stereology on all samples was performed according to the optical fractionator principle in order to quantify the total number of TH^+ dopaminergic neurons in the substantia nigra. We used a Leica microscope connected to a digital camera employing the *Stereo Investigator* software. Tracing ROIs was done using the 5X lens, and counting was performed with 100X lens.

*Mice (**paper II**)*

Every third section of the midbrain region (Bregma -2.70 to -3.78) was analyzed, which yielded between 9-11 sections per animal. The thickness-sampling fraction (tsf) was 1. Section thickness was measured at every fourth site while counting

and the area-sampling fraction (asf) was on average 0.08. Dissector volume (h* Aframe) was 60,500 mm^3 on average. A maximal Gundersen coefficient of error (CE) (Gundersen et al., 1999) of 0.08 was accepted, and the smoothness factor (m) of 1 was used. To test for normal distribution of cell numbers among F2 generation, the Shapiro-Wilk normality test in R (3.0.2) was used.

*Rats **(papers III, IV)***

Every fourth section covering the full extent of the substantia nigra was sampled for analysis, yielding 10-12 sections per animal. The tsf was 1. The asf was on average 0.112. Dissector volume (h* Aframe) was 86,400 mm^3 on average. A maximal Gundersen CE of 0.08 was accepted, and the m of 1 was used.

Axonal swelling analysis

For En1 mice in **paper II**, in order to quantify the enlarged TH^+ terminals in the striatum, three to five consecutive sections from each animal were stained and analyzed. High-resolution pictures were taken using the same microscope at 25x, camera and software as for the stereology. Four pictures were taken of precisely delineated ROIs representing the dorso-lateral part of the striatum. A 5-step macro installed in the *ImageJ* software was then used to identify the swellings and calculate their total number, as well as their size, by setting an exclusion threshold for particles <3 μm^2. The experimenter performing the image acquisition and processing was genotype-blind.

Molecular analysis

Gene expression analysis

In **papers III** and **IV**, RNA isolation was performed using the RNeasy Mini kit following the supplier's protocol from steps 4 to 7. The first three steps were substituted by adding 600 µl Trizol to each sample before a 20s homogenization step with a FastPrep homogenizer. The samples were then transferred to a clean eppendorf tube and resuspended in 0.14 ml chloroform, then centrifuged for 15 min at 12,000g (4°C). RNA concentration was determined using NanoDrop. Reverse Transcription was performed using the SuperScript® III First-Strand Synthesis System according to the manufacturer's protocol. Quantitative polymerase chain reaction (qPCR) was conducted using the SSoAdvanced Universal SYBR®green Supermix according to the following protocol: 5µl

Supermix + 1 µl H_20 + 0.5 µl of each primer + 3 µl cDNA for each sample. Amplification was performed with a 3-step PCR protocol (1. 30s at 95°C; 2. 60s at 64°C in **paper III**, 62°C in **paper IV**; 3. 5 min at 68°C). Primers utilized are found in Table 2.

Genotyping analysis

Genotyping in rats

In **papers III** and **IV**, to confirm congenic status of rats, tail DNA was extracted and PCR was performed with primers for flanking *Vra1* markers (D8Rat104; D8Mgh4), listed in Table 2.

Genotyping in mice.

To identify the single allele KO of *En1* in **paper II**, we performed PCR with primers for *LacZ* (Table 2), the gene used for deleting the *En1* allele. We also performed qPCR for genotyping of *LacZ* by following the SsoAdvanced SYBR® Green Supermix protocol and using the same *LacZ* primers.

TABLE 2.
LIST OF PRIMERS

Primer name	Forward Sequence (5'-3')	Reverse sequence (5'-3')	Concentration	Paper
LacZ	TGTATGAACGGTCTGGTCTTTG	AACAGGTATTCGCTGGTCACTT	10 µM	II
Gapdh	CAACTCCCTCAAGATTGTCAGCAA	GGCATGGACTGTGGTCATGA	10 µM	III, IV
β-actin	AAGTCCCTCACCCTCCCAAAAG	AAGCAATGCTGTCACCTTCCC	10 µM	III, IV
GSTA4	GACCGTCCTGAAGTTCTAGTGA	TGCCTCTGGAATGCTCTGT	10 µM	III, IV
D8Rat104	TCCTGAACTCTGACTCATTGCT	TGTGTTCTGCAATGGTGGTT	10 µM	III, IV
D8Mgh4	GAGTTAACCCAACAACTCTAAGCC	CCCAAATGCAGCAGTCTACA	10 µM	III, IV

Linkage analysis

Linkage analysis is an essential tool to identify mutations in familial diseases and also to experimentally map complex traits in crosses of animal strains. It is an unbiased method that links naturally occurring phenotypic variance across inbred rodent strains to QTLs (Miles & Wayne, 2008). In order for this analysis to work, two or more genetically different strains of a particular organism are needed as well as molecular markers, such as SNPs, to distinguish between the parental lines. This analysis was run in **paper II**.

Genome-wide SNP assay

In order to perform genome-wide linkage analysis, we relied on a panel of 377 SNPs as our molecular markers. The SNP&SEQ technology platform at Uppsala University (Sweden) performed the SNP genotyping of our F2 population. To find parental-specific alleles, the genomes of two mouse strains (Bl6 and SW founders) were also genotyped. We needed the SNPs to be parental strain-specific, so they had to vary between the two strains, but not within the population of outbred SW used for intercrossing. Therefor, 114 of the 377 were parental-specific and were utilized for the analysis.

Single QTL analysis

The data were analyzed using R/qtl (Broman et al., 2003) to identify gene regions linked to neurodegeneration. *Scanone* was used for single QTL analysis. For expectation-maximization (EM) and Haley-Knott methods, the genotype probabilities were calculated with 0.5 cM distance and a genotyping error rate set at 0.001. For multiple imputation the genotype was simulated with 1000 simulation replicates, step length of 0.5 cM and error probability of 0.001. Significance thresholds for LOD scores were obtained by permutation test, with 1000 permutations using Haley-Knot-regression.

Multiple QTL analysis

The single-QTL analysis is based on the assumption that there is only one QTL, and the scan is performed at one locus at a time. To increase the power to detect QTLs with additive or epistatic effects, we performed multiple-QTL analysis. The multiple QTL models were fitted starting with the locus with the highest LOD score in the single-QTL model. The models were iteractively built by scanning for interactive and additive loci using *addqtl* with Haley-Knot regression. *Fitqtl* was used to fit the models. Loci and interactions with a p-value (p)<0.05 in the drop-one-term ANOVA were kept in the model and used in scanning for additional loci. Genotype probabilities were calculated with a step length of 0.1 cM and error probability of 0.001. To estimate the positions of QTLs in the model, we calculated the approximate 95% Bayes credible intervals (Manichaikul et al., 2006). Significance thresholds for the multiple QTL models were estimated by permuting randomly-selected positions 5000 times and taking the LOD score at the 95th percentile. This was done for the full model LOD scores as well as for individual QTL LOD scores in the model. The full model significance threshold thus is a measure of significance of the full models, while the QTL LOD score significance threshold gives an estimation of the contribution of a specific QTL to the respective model.

Statistical analysis

All quantitative data were analyzed using GraphPad Prism and $p<0.05$ was considered statistically significant. Data are expressed as mean ± standard deviation except for **paper I** where standard error of the mean was used.

Sample groups of two with parametric data such as O.D. and stereology were subjected to an unpaired Student's *t*-test; sample groups of more than two with parametric data such as axonal swelling quantification or gene expression analysis were subjected to analysis of variance and in significant cases followed by the Bonferroni's or Tukey's multiple comparisons tests.

Correlation analyses were performed using the Pearson correlation coefficient (*r*) using a 95% confidence interval in **papers II** and **III**.

Sample groups with non-parametric data such as for densitometry analysis in **paper I** were subjected to a Kruskal-Wallis with Dunn's multiple comparisons.

Results

Susceptibility to neurodegeneration differs between rat strains

The first step in discovering genetic risk factors for PD when following an experimental linkage-based approach is to analyze if there are any relevant phenotypic differences between strains. Strain differences in certain PD animal models, such as MPTP and En1, have already been measured and in some cases analyzed; however, while these lead to the discovery of QTLs relating to dopaminergic cellular dysfunction and cell death mechanisms, none of these related to α-syn overexpression and accumulation. In order to find such QTLs in an animal model, it is essential to test the susceptibility of different strains to α-syn overexpression. If differences between strains are observed, then linkage analysis can proceed. In **paper I** we aimed for this by injecting a viral vector constructed to overexpress α-syn in the dopaminergic cells of the SNpc of 6 different strains of rats, SD, PVG, LEW, BN, DA, and LE.

Forelimb akinesia tests reveal first strain differences

Immediately before the injections, and at 4 and 8 weeks after injections, we conducted behavioral measurements with the stepping and cylinder tests to determine forelimb akinesia. This is when we saw the first measurable differences between the six strains. After the stepping test, we found that delivery of rAAV-α-syn had a significant effect on motor performance of all strains at 4 weeks post injection when measuring differences between time points within each strain. However, at 8-weeks significant reduction in the percentage of left paw use was significant just for BN ($p<0.05$) and LEW ($p<0.05$) (Figure 6A). Furthermore, when we compared each strain with every other strain, the *post-hoc* test showed a significant reduction in motor performance only between SD and LE at 8 weeks post-injection ($p<0.05$) (Figure 6B).

To evaluate forelimb akinesia during explorative behavior, we performed the cylinder test at two time points, immediately prior and 8 weeks after injections. Delivery of rAAV-α-syn had a significant effect on spontaneous use of the left

paw 8-weeks after injection in all the strains except PVG when measuring differences between time points within each strain (Figure 6C). When measuring differences among strains, the *post hoc* test revealed no significant difference in the percentage of left paw use over time among SD, LEW, and PVG; in contrast, compared to LEW and PVG, LE (p<0.0001), BN (p<0.0001), and DA (p<0.0001) all showed significant reductions (Figure 6D).

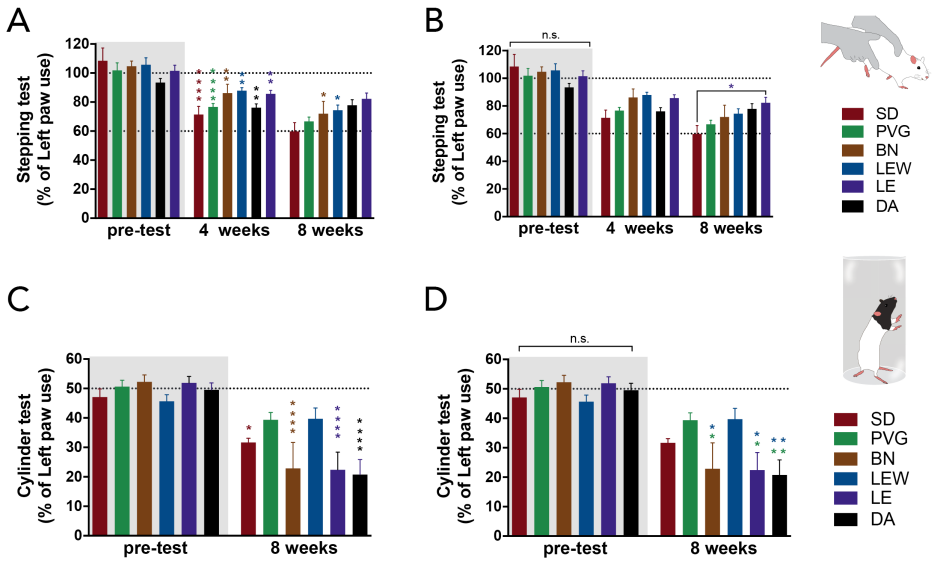

Figure 6. Genetic background regulates level of motor impairment in the stepping and cylinder tests. Forelimb akinesia was assessed using the stepping test (**A, B**) an the cylinder test (**C, D**). **A, B**. Stepping test: all strains were tested for 1-5 days before (pre-test) and 4 and 8 weeks after rAAV-α-syn delivery into the SNpc (SD, n=8; PVG, n=9; BN, n=6; LEW, n=9; DA, n=7; LE, n=9), or rAAV-GFP (SD, n=5; LEW, n=6; LE, n=6). **C, D**. Cylinder test: all strains were tested for 1-5 days before (pre-test) and 8 weeks after rAAV-α-syn delivery in SNpc (SD, n=8; PVG, n=9; BN, n=6; LEW, n=9; DA, n=7; LE, n=9), or rAAV-GFP (SD, n=5; LEW, n=6; LE, n=6). **A**, **C**. Differences indicate behavior changes in time within each strain. **B**, **D**. Differences in behavior observed at 8 weeks when comparing each strain to all other strains. Data are expressed as mean ± SEM. *p<0.05 (2-way ANOVA with Bonferroni *post-hoc* test). SD: Sprague Dawley; PVG: Piebald Virol Glaxo; BN: Brown Norway; LEW: Lewis; DA: Dark Agouti; LE: Long Evans.

Strains that were injected with the rAAV-GFP (Figure 7A) vector did not show significant differences in motor performance at the latest time point, however SD (71.55% ± 7.02) animals did show a reduction in the percentage of left paw use at 4-weeks compared to LE (89.59% ± 2.3, p<0.05) in the stepping test (Figure 7B). No differences among strains in motor performance were observed after the cylinder test (Figure 7C).

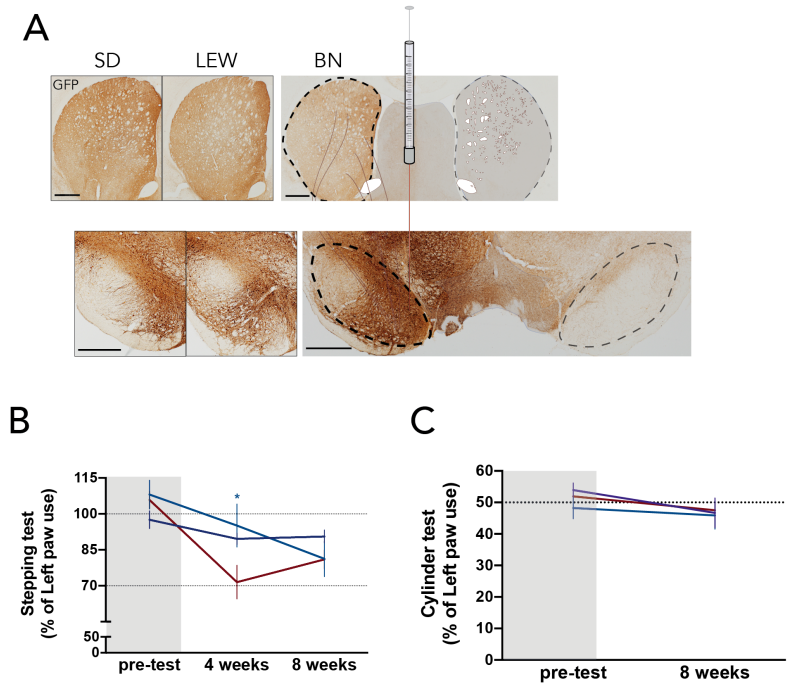

Figure 7. Overexpression of GFP does not induce motor impairments at 8 weeks.
A. DAB immunostainings for GFP show stable expression levels in ipsilateral striatum and substantia nigra (delineated by darker dashed lines in BN examples) of SD, LEW, and BN rats after unilateral injections in the SNpc (as shown by the representative needle). Scale bars = 500µm. SD: Sprague Dawley; LEW: Lewis; BN: Brown Norway. **B**. Behavior differences among strains (SD, LEW, BN): SD rats show a reduction in left paw use at 4 weeks, but recover at 8 weeks. **C**. There are no impairment or behavior differences among strains in the cylinder test. A 1-way ANOVA with Dunnet *post-hoc* test was run for **B** and **C**. Data are expressed as mean ± SEM.

PVG is most susceptible strain to dopaminergic fiber loss after α-syn overexpression

At 8 weeks after the injections, the animals were sacrificed. We stained for α-syn in striatum and SNpc to check that expression of the protein could be visualized throughout the targeted area (Figure 8A). After excluding animals with poor targeting, we stained those same regions for TH in order to measure the amount of dopaminergic fiber loss in the striatum. We found that PVG rats were the most susceptible to fiber loss, and displayed a significantly lower amount of remaining dopaminergic fibers compared to LEW rats (Figure 8B).

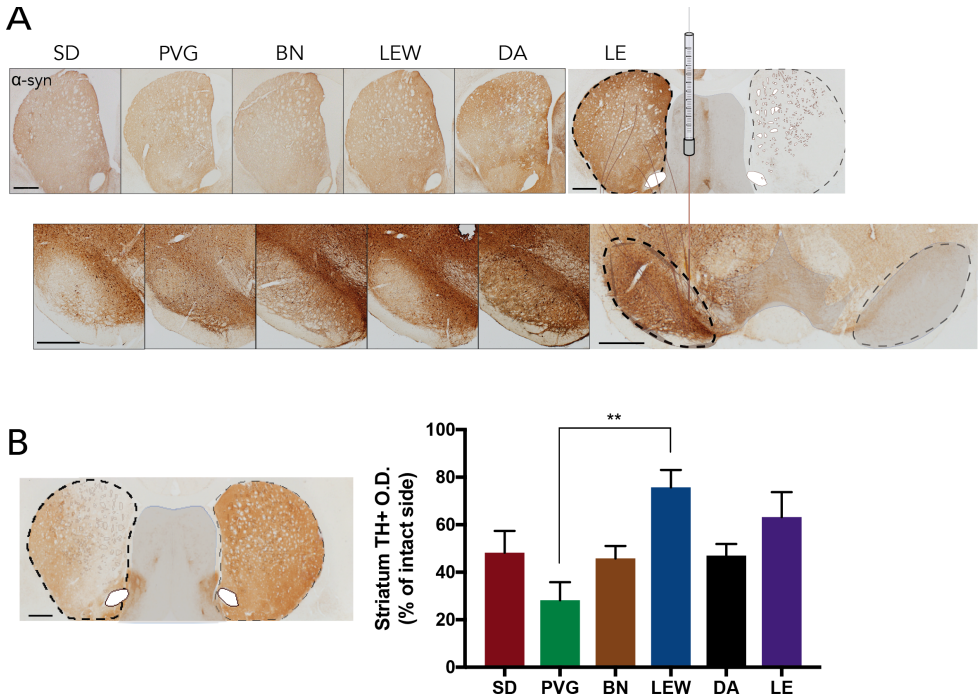

Figure 8. Correct targeting of the SNpc with human WT α-syn leads to dopaminergic fiber loss in the striatum of each strain.
A. DAB immunostainings for α-syn show stable expression levels in ipsilateral striatum and substantia nigra (delineated by bold dashed lines in LE examples) of all six strains after unilateral injections in the SNpc (as shown by the representative needle). **B**. Sample image representing TH^+ fiber loss in the ipsilateral striatum. Chart shows TH^+ O.D. values representing mean percentage of lesioned striatal dopaminergic fibers compared to respective O.D. values of intact striatum. Data are expressed as mean ± SEM. $^{**}p<0.01$ (Kruskal Wallis with Dunnet *post-hoc* test). Scale bars=500μm. SD: Sprague Dawley; PVG: Piebald Virol Glaxo; BN: Brown Norway; LEW: Lewis; DA: Dark Agouti; LE: Long Evans.

Generating an F2 population of *En1* +/− mice from two different strains gives the basis for linkage analysis

Previous strain difference studies in mice with the *En1* partial KO have determined that SW mice to be more susceptible to this type of KO than Bl6 mice. However no conclusions were made regarding the genetics behind such susceptibility. We aimed at reaching such conclusions by doing a linkage analysis study with these two strains in **paper II**. For linkage analysis to work, specific phenotypes have to be measured and then statistically linked to genetic regions. To generate enough information to run this statistical analysis, a large population is required. So we first generated an F2 population of male mice as a cross between

En1 +/− SW and *En1* +/+ Bl6 in order to have more than 100 mice with mixed genomes and, therefor, mixed phenotypes. We then measured distinct phenotypes common to the *En1* partial KO at 17 weeks of age: dopaminergic cell loss in the SNpc, axonal swellings in the striatum and their size. As predicted we found a large phenotypic spread among the *En1* +/− mice for all three phenotypes, suggesting complex genetic regulation of these traits (Figure 9A). We then compared the averages of the F2 population with averages from WT and +/− parental SW mice, WT parental Bl6, and an N4 generation backcross representing +/− Bl6 (Figure 9B). A correlation analysis was run between cell loss and number of axonal swellings, and no correlation was found (Figure 9C). We therefor decided to use the ratio of axonal swellings and remaining cells, meaning that the more dopaminergic neurons survived in the SNpc by 17 weeks of age, the less axonal swellings per neuron were seen. Thus, at the single time point studied here, axonal swellings correlated with the number of dopaminergic neurons when taking previous loss of dopaminergic neurons into account (Figure 9D).

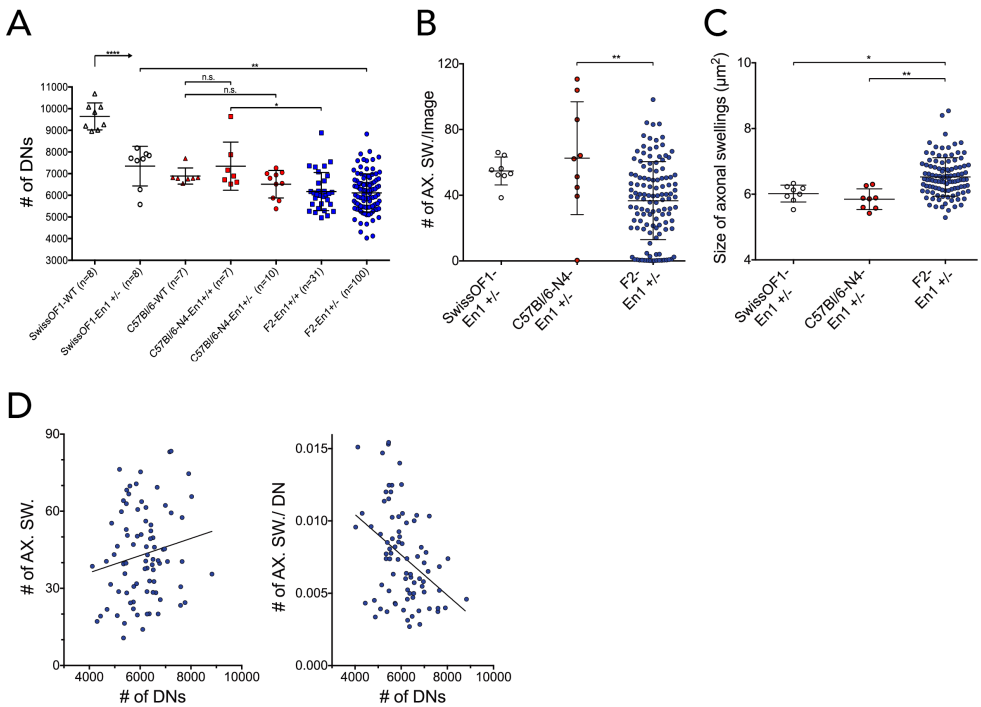

Figure 9. Quantification of dopaminergic cell loss in SNpc, # of axonal swellings, and size of axonal swellings of the different mouse strains and intercrosses.
A. Stereological quantification of dopaminergic neurons (DNs) in SNpc at 17 weeks of age. The mean number of DNs in SW-WT was significantly higher than in all other groups; ****p<0.0001, **p<0.01, *p<0.05. **B**. Number of axonal swellings in parental SW-En1+/-, Bl6-N4-En1+/-, F2-En1+/-. **C**. Size of axonal swellings in parental SW-En1+/-, Bl6-N4-En1+/-, F2-En1+/-. **p<0.01, *p<0.05. **D**. There is no correlation between number of dopamiergic neurons and number of axonal swelling, but there is a correlation between number of remaining dopaminergic neurons and number of swellings per neuron (r = -0.37, p<0.001). Individual data points are shown with mean ± SD.

Several QTLs determine susceptibility to the partial KO of *En1*

All mice were genotyped for *En1* and their DNA was sent for whole-genome SNP genotyping. To identify loci linked to dopaminergic neuron susceptibility to degeneration in the absence of one En1 allele, the number of DNs in SNpc, the load of axonal swellings, and size of axonal sweelings of F2-En1+/− mice at 17 weeks were used in genome-wide linkage analysis employing R-QTL. Out of 377 genotyped SNPs, in the Illumina Mouse LD Linkage Panel, 114 were informative (Figure 10A).

Single QTL analysis

With the single QTL analysis, we found no QTLs linked to dopaminergic cell loss or size of axonal swellings but found one QTL in chromosome 15 related to load of axonal swellings per dopaminergic neuron. Interestingly, mice homozygous for SW alleles at this QTL displayed the lowest load of axonal swellings. Bl6 alleles at this locus are thus linked to the presence of more axonal swellings on remaining dopaminergic neurons (Figure 10B).

Multiple QTL analysis

Single QTL analysis assumes there is only one genetic region responsible for a specific phenotype. It is therefor necessary to run a multiple QTL analysis since several genes at different loci could interact to cause a specific phenotype. With this analysis, we discovered eight loci linked to cell loss in the SNpc with interactions between seven of the loci (Figure 10C); seven loci linked to load of axonal swellings in the striatum, with interactions between five of the loci (Figure 10D); and eight loci linked to size of axonal swellings, with interactions between 6 of the loci (Figure 10E). All QTLs with respective interactions are listed in Table 3.

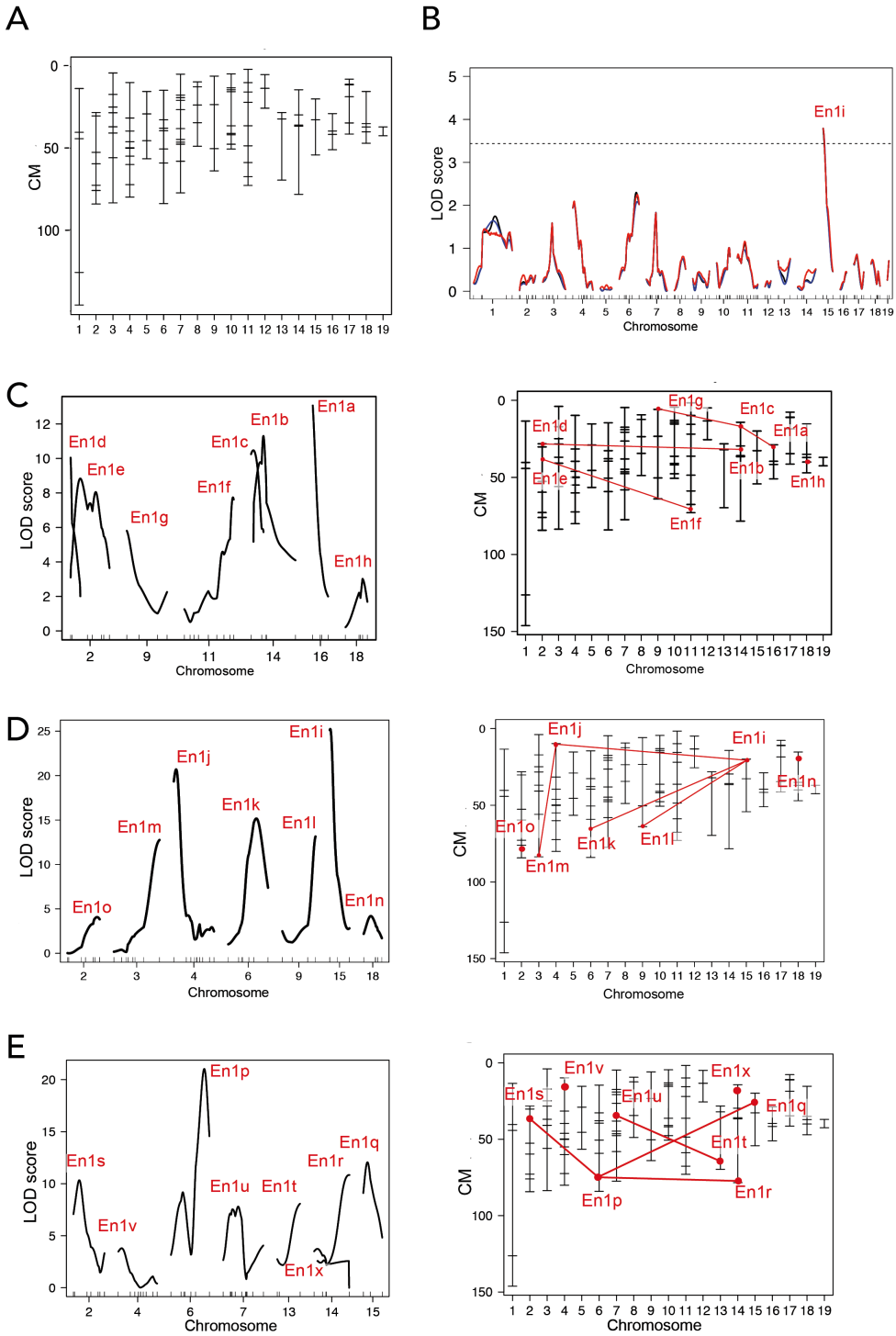
A
B
C
D
E
CM
LOD score
Chromosome
En1i
En1d
En1e
En1g
En1f
En1b
En1c
En1a
En1h
En1j
En1k
En1l
En1m
En1o
En1n
En1p
En1q
En1r
En1s
En1t
En1u
En1v
En1x

Figure 10. QTLs and interactions discovered after single and multiple QTL analysis.
A. Genetic map of F2 population showing physical location of informative SNP markers. **B**. Single QTL scan of load of axonal swellings reveals one QTL at chromosome 15. Solid black - expectation-maximization; red - multiple imputation; blue - Haley-Knott; dotted line: *p*=0.05. **C.** Multiple QTL scan representing number of dopaminergic neurons reveals 8 significant QTLs. Refined chromosomal locations of QTLs with red lines between 7 interacting loci. **D.** Multiple QTL scan representing load of axonal swellings reveals 7 significant QTLs. Refined chromosomal locations with red lines between 5 interacting loci. **E**. Multiple QTL scan representing size of axonal swelling reveals 8 significant QTLs. Refined chromosomal locations with red lines between 6 interacting loci.

Table 3. LOD-score, position and interactions of QTLs in the three respective models.
Full model statistics include *p*-value and LOD scores for the full QTL model with significance LOD threshold in parentheses. *Individual QTL below the respective genome-wide significance LOD threshold (number of DNs in SNpc=7.1, number of axonal swellings per DN=4.8, average size of axonal swellings=8.7). LOD: logarithm of odds, QTL: quantitative trait locus, DN: dopaminergic neuron, SNpc: substantia nigra pars compacta.

Phenotype	QTL	LOD score	Chr	Bayesian credible interval (cM)	Nearest marker	Interactions	Full model statistics	Variance explained
Number of DNs in SNpc	**En1a**	13.1	16	28.9 - 30.4	rs4180773	**En1c**	*p*=2.4E-09 LOD=28.3 (12.2)	74%
	En1b	11.3	14	27.2 - 34.9	rs3695574	**En1d**		
	En1c	10.5	14	14.3 - 22.4	rs3695383	**En1a. En1g**		
	En1d	10.0	2	28.2 - 29.0	rs13476490	**En1b**		
	En1e	8.8	2	36.0 - 67.8	rs3658729	**En1f**		
	En1f	7.7	11	69.5 - 72.9	rs13481230	**En1e**		
	En1g*	5.8	9	5.9 - 12.0	rs13480107	**En1c**		
	En1h*	3.0	18	31.7 - 46.5	rs6320743			
Number of axonal swellings per DN	**En1i**	25.2	15	19.9 - 22.3	rs3674266	**En1k, En1j, En1l**	*p*=1.7E-12 LOD=32.4 (10.1)	80%
	En1j	20.7	4	10.8 - 16.2	rs3653593	**En1i, En1m**		
	En1k	15.1	6	58.1 - 68.4	rs3152403	**En1i**		
	En1l	13.2	9	61.2 - 63.4	rs3694903	**En1i**		
	En1m	12.7	3	76.8 - 83.1	rs3724562	**En1j**		
	En1n*	4.3	18	20.1 - 36.0	rs3669543			
	En1o*	4.1	2	68.3 - 83.8	rs6376291			
Average size of axonal swellings	**En1p**	21.0	6	72.1 - 77.6	rs6387265	**En1q, En1r, En1s**	*p*=7.0E-11 LOD=30.2 (14.1)	74%
	En1q	12.1	15	23.9 - 30.9	rs3699312	**En1p**		
	En1r	10.8	14	69.2 - 78.4	rs3698545	**En1p**		
	En1s	10.3	2	34.7 - 42.7	rs13476507	**En1p**		
	En1t*	8.1	13	61.2 - 69.7	rs6316705	**En1u**		
	En1u*	7.8	7	15.7 - 36.2	rs3696018	**En1t**		
	En1v*	3.8	4	9.8 - 22.8	rs3653593			
	En1x*	3.7	14	14.2 - 29.2	rs3695383			

The DA.VRA1 congenic strain proves useful for studying susceptibility to neurodegeneration

Linkage analysis studies in rats have linked alleles in *Vra1,* in the PVG rats, to neuroprotection after VRA, a model of nerve injury. Later, rats carrying these alleles were backcrossed multiple times to DA to create the DA.VRA1 congenic strain. The congenic strain has been shown to be neuroprotected after VRA (Lidman et al., 2003) and TBI (Al Nimer et al., 2013), suggesting a major role played by *Vra1* in susceptibility to neurodegeneration in these models. We decided to use this congenic strain to test whether *Vra1* would have the same neuroprotective properties in two animal models of PD: the partial lesion of the nigro-striatal pathway with three deposit injections of 6-OHDA (**paper III**), and the rAAV vector-mediated overexpression of human WT α-syn in the SNpc (**paper IV**). In both models we found higher TH^+ fiber O.D. in the striatum of DA.VRA1 rats compared to control DA rats (Figure 11A, B). However, while DA.VRA1 rats have more dopaminergic cell survival in the ipsilateral SNpc after α-syn overexpression compared to DA (Figure 11D), no significant differences were found after injections of 6-OHDA (Figure 11C).

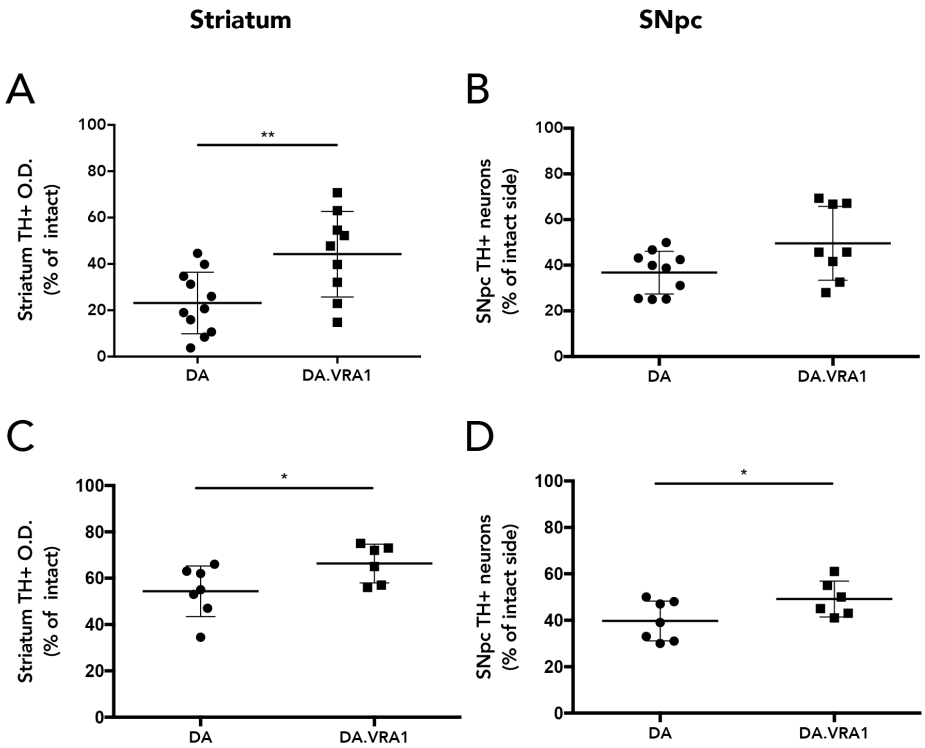

Figure 11. The *Vra1* locus confers protection of the nigrostriatal pathway after striatal 6-OHDA injections and nigral rAAV-mediated overexpression of human WT α-syn.
A. Optical density quantification of TH^+ fibers in the lesioned dorsolateral relative to intact dorsolateral striatum at 8 weeks post 6-OHDA lesion and **C.** α-syn overexpression. DA.VRA1 rats display a higher density of TH^+ fibers in the lesioned striatum compared to DA in both models. **B**. Stereological quantification of TH^+ neurons in the SNpc at 8 weeks post 6-OHDA lesion and **D**. α-syn overexpression. While a trend towards more TH^+ cells is noticeable in **B**, there is no significant difference between the two strains. DA.VRA1 rats display a higher density of TH^+ cell count compared to DA in **D**. Individual data points and mean ± SD are shown. **p*<0.05 ***p*<0.01.

The *Vra1* locus mediates higher expression of *Gsta4* to protect dopaminergic neurons in two PD models

Ström et al. found *Gsta4* as candidate gene for *Vra1* in VRA and TBI (Strom et al., 2012). We therefor examined gene expression levels of *Gsta4* in both our rat PD models; the 6-OHDA injection model since the toxin generates high levels of oxidative stress in dopaminergic neurons (Tieu, 2011), and the α-syn overexpression model since evidence suggests that high levels of α-syn lead to mitochondrial impairment, which in turn leads to lipid peroxidation (Esteves et al.,

2009; Rocha et al., 2017). Higher expression levels of *Gsta4* were found in both the striatum and midbrain of 6-OHDA-injected DA.VRA1 rats at 2 days post injection compared to DA (Figure 12A). The same was true after α-syn overexpression in the SNpc at 3weeks (Figure 12B).

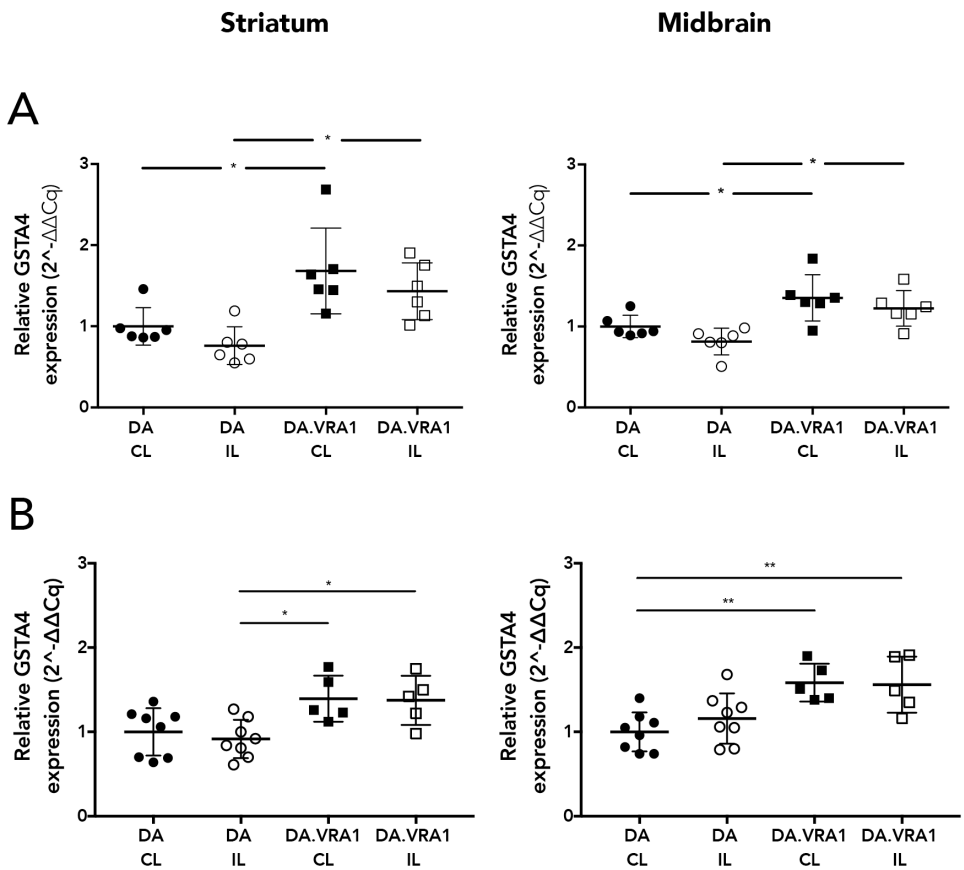

Figure 12. *Gsta4* gene expression in the striatum and midbrain after striatal 6-OHDA lesion and α-syn overexpression.
A. At 2 days post 6-OHDA lesion, *Gsta4* expression is significantly higher in DA.VRA1 compared to DA for both the ipsilateral and the contralateral sides of the striatum and midbrain. **B**. Similar results are seen at 3 weeks post α-syn overexpression. A one-way ANOVA was used to calculate gene expression differences between groups and Bonferroni's multiple comparisons test was run as a *post-hoc*. Gene expression levels are related to the mean value for DA at the IL side. Individual data points and mean ± SD are shown; *p<0.05 **p<0.01.

GSTA4 co-localizes with astrocytes as degeneration progresses

Next we examined the cellular localization of GSTA4 in the rat brain by double immunofluorescent labeling with a marker for astrocytes (GFAP), a marker for microglia (IBA1), and a marker for neurons (NeuN), and found GSTA4 expressed in the cell bodies of astrocytes in both the 6-OHDA model and the rAAV-α-syn model after 8 weeks of the respective injections (Figure 13A-F).

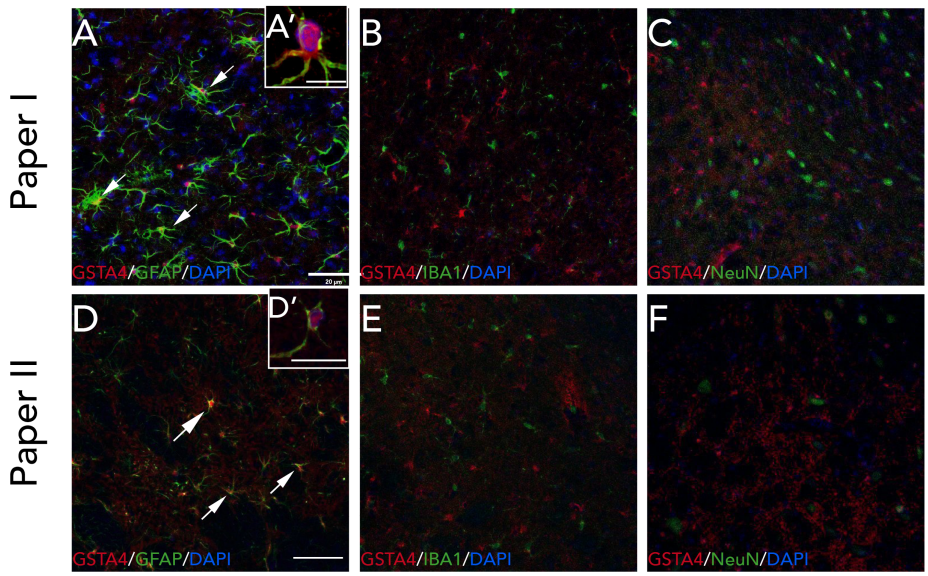

Figure 13. Expression of GSTA4 in midbrain astrocytes after striatal 6-OHDA lesion and α-syn overexpression.
A-C.Immunofluorescent staining of GSTA4 combined with cell-specific markers for (**A**) astrocytes; GFAP, (**B**) microglia; IBA1 and (**C**) neurons; NeuN in the SNpc 8 weeks after 6-OHDA lesion. **D-F**. The same is shown but in SNpc 8 weeks after α-syn overexpression. GSTA4 staining co-localized with GFAP (**A,D**) but not IBA1 (**B,E**) or NeuN (**C**,**F**), suggesting astrocytic expression. (**A-C**; **D-F**) Scale bar Pictures taken at 20x=20µm. **A'**. Zoomed in 60x image showing co-localization of GSTA4 with GFAP, mainly expressed in the soma. Scale bar=50µm. (**D'**) Pictures taken at 60x, scale bar=100µm. All markers were combined with nuclear marker DAPI (blue).

Discussion

The studies included in this thesis aim at advancing our search for candidate genes responsible for susceptibility to dopaminergic neurodegeneration in different animal models of PD. Identifying rat strain differences within a specific PD model, finding QTLs that regulate specific PD-related phenotypes in mice, and studying the effects of a rat QTL on susceptibility to dopaminergic neurodegeneration are only stepping stones in the pursuit to determine genetic risk factors for idiopathic PD. Here, this thesis will discuss the impact of the results and possible future applications.

The importance of uncovering genetic risk factors for PD

PD affects around 2 people for every 10,000 people at age of onset (about 60 years old) worldwide and the incidence increases with age. Therefore, age is the largest risk factor for the disease, with the number of PD cases on the rise due to longer life expectancy (Dauer & Przedborski, 2003), and a cure has yet to be found. Therapies for managing symptoms of PD exist, but none are able to stop or slow down disease progression. This is because current medication is focused on compensating for missing dopamine in the striatum, but there is no medication able to prevent the pathological hallmarks of the disease. Finding new therapeutic strategies is difficult, especially since there is still very little consensus disease etiology.

While we are familiar with some of the pathological mechanisms related to PD, like dopaminergic cell death in the SNpc, accumulation of toxic α-syn oligomers, inflammation, and oxidative stress mechanisms leading cellular dysfunction, the majority of genetic risk factors behind these mechanisms are still undiscovered. Most cases of PD are complex, where environmental factors as well as genetic ones determine susceptibility for acquiring the disease. Genes are likely to interact with each other and with environmental factors to cause idiopathic PD (Horowitz & Greenamyre, 2010; Singh et al., 2014); therefor, learning more about disease etiology is extremely challenging.

This thesis addresses this challenge by deploying three tools that are effective in determining candidate genes in animal models that can later be used in association studies in humans: exploring strain differences after α-syn overexpression in rats (**paper I**), performing linkage analysis in *En1* +/− mice (**paper II**), and using a congenic rat strain to analyze the protective effects of the Vra1 locus after 6-OHDA lesions (**paper III**) and after α-syn overexpression (**paper IV**).

Genetic variation in rats causes different responses to α-syn overexpression

We, as human individuals, are genetically heterogeneous, and this causes us to respond differently to specific stimuli, whether they are environmental or intrinsic. Studying strain differences in a controlled environment is helpful in simulating this type of genetic heterogeneity: if two or more animal strains show differences in susceptibility to a specific neurodegenerative factor, it means that their genetic make-up helps determine those differences. Several genetic approaches are then available to help search for candidate genes responsible for those differences.

Animal strain differences in certain PD models have already been utilized in PD research. One of the most prevalent models for such studies has been the MPTP mouse model thanks to its effectiveness in targeting the nigrostriatal pathway and being chemically similar to PD-related environmental compounds, such as paraquat and rotenone (Meredith & Rademacher, 2011). It's been shown that Bl6 mice are more susceptible to MPTP in terms of dopaminergic depletion and certain behavioral deficits compared to CF1 white, mice (Riachi & Harik, 1988) CBA/Ca, and Swiss-Webster (Sundstrom et al., 1987). Still no major study has been performed in a rat α-syn overexpression model when examining strain-dependent genetic susceptibility to neurodegeneration. However, on the heels of studies showing strain differences in susceptibility to nerve injury (Lundberg et al., 2001) and the genetics behind such susceptibility (Piehl et al., 1999; Swanberg et al., 2009), we have recently addressed the effects of α-syn overexpression in two studies using DA rats and a DA-background congenic strain of rats. In one study, we observed differential microglial activation between DA and DA.VRA1 rats after α-syn overexpression due to allelic variation of Mhc2ta. The congenic strain carries PVG alleles in the *Vra4* locus and therefore displays lower Mhc2ta expression levels compared to DA rats, which leads to higher susceptibility to α-syn overexpression (Jimenez-Ferrer et al., 2017). This lead us to hypothesize that different rat strains could potentially have neuroprotective traits, which could later be isolated through linkage analysis and from then mapped in order to find candidate genes.

In **Paper I**, we tested the susceptibility of six different strains to a specific neurodegenerative insult: unilateral overexpression of α-syn in the SNpc. We measured phenotypes similar to some of the ones seen in PD patients: motor deficits and midbrain dopaminergic cell and fiber loss. We found strain-dependent variation within those phenotypes. Regarding behavior, striatum-dependent motor deficits related to adjusting steps in the stepping test developed linearly as a function of lesion severity in all animals. However, we observed that, when looking at each strain independently, the deficit occurred more dramatically in SD rats, than in others with a similar percentage of lesion. This points to possible genetic factors that increase susceptibility to dopaminergic depletion in the striatum by limiting compensatory mechanisms. (Blesa et al., 2017). These include the reduction of dopamine transporter (DAT) to enhance pre-synaptic uptake of dopamine (Lee et al., 2000), or changes to other neurotransmitter systems like serotonin, which has been shown to compensate for dopamine loss (Wile et al., 2017).

Much of what we know about the rAAV-mediated overexpression of α-syn comes from work done in SD rats (Decressac et al., 2012b; Gombash et al., 2013; Maingay et al., 2005). Some of those studies indicate that striatal fiber dysfunction and relative dopamine loss induced by the injection of human WT α-syn in the SNpc may come ahead of dopaminergic cell loss (Kirik et al., 2002). Lundblad et al. suggest that this might be because of striatal synaptic impairment seen already at 3 weeks post injection (Lundblad et al., 2012). This could have large impacts on motor behavior. Interestingly, while our SD rats display similar levels of behavior deficits (approximately 40% contralateral paw deficit in both tests) and dopaminergic fiber loss (approximately 50% TH^+ fiber loss), PVG rats show quite a large disparity between these measurements (20-30% behavioral deficits; more than 70% TH^+ fiber loss). Since previous studies have shown that a minimum of 50-60% dopaminergic nigral loss is necessary to observe substantial behavior deficits (Kirik et al., 2002), doing a stereological count of SNpc neurons within each strain would help unravel this mystery. Nevertheless, these results suggests that PVG rats are less susceptible to dopaminergic synaptic dysfunction, and, in fact, it may also be that the compensatory mechanisms mentioned above are more robust in PVG rats. Previously, PVG rats have been shown to be less susceptible to VRA, a nerve injury model (Lundberg et al., 2001), compared to other inbred rat strains, including, LEW, DA, and BN, which resulted in new knowledge about strain-dependent genetic regulation of glial reactivity and neuron death after mechanical nerve injuries.

The work in **paper I** gave us more knowledge about strain-dependent genetic regulation dopaminergic neurodenegeration. From here, a linkage analysis approach could be used to find QTLs linked to such regulation, which could subsequently lead to the discovery of new candidate genes.

Several QTLs determine susceptibility to neurodegeneration in *En1* +/− mice

Linkage analysis is better known as an approach for mapping monogenic traits. In fact, mutations in the *SNCA* and *LRRK2* genes were originally linked to familial PD (Polymeropoulos et al., 1997; Zimprich et al., 2004). This is because genetic linkage is based on the co-segregation of genetic markers; therefor, if a genetic region contains risk alleles, affected family members will carry it more often. Overall this means that linkage often relies on linkage disequilibrium (the nonrandom association of alleles at different loci). With this concept in mind, inbred animal strains can be generated in order to fix certain alleles and study them. Thus, through linkage analysis, differences found among inbred animal strains can be linked and mapped to QTLs.

We used linkage analysis in **paper II** to map loci regulating PD-like neuropathology in a spontaneous PD mouse model: the partial KO of *En1*. Two different mouse strains, SW and Bl6, were shown to respond differently to this partial KO: SW mice display PD-like pathology with preferential loss of dopaminergic neurons in the SNpc, while Bl6 do not show such pathology (Sgado et al., 2006). We were able to identify a total of 22 QTLs, 6 of which either overlap or are in close proximity to one another, that explain the vast majority of the variation in dopaminergic neuron neurodegeneration and axonal pathology in an F2 population intercross between those two strains. We chose to map three distinct features of PD-like pathology: loss of dopaminergic neurons in the SNpc, load of axonal swellings in the striatum, and size of these swellings. While the single QTL analysis revealed only one QTL linked to load of axonal swellings (*En1i*), the multiple QTL analyses revealed several loci with high LOD scores and interactions for all three phenotypes measured. This isn't surprising since a large number of QTLs and interactions between loci are typical for complex traits. Knowing that the etiology of 90% of PD cases is complex with multiple interacting genetic and environmental risk factors (Horowitz & Greenamyre, 2010), the presented QTLs linked to PD-like pathology in the En1 mouse model are particularly relevant to idiopathic PD.

In regards to PD pathophysiology, dopaminergic neurodegeneration is a classic hallmark. The En1 mouse model is therefor extremely relevant for PD studies, especially considering that *En1* is part of a complex network of transcription factors that orchestrate the development and survival of dopaminergic neurons (Veenvliet et al., 2013). With this in mind, it is not surprising that the number of dopaminergic neurons in the genetically heterogeneous F2 mice show large variation with the partial KO of *En1*.

As alluded to already in rat studies (Kirik et al., 2002), recent research in mice also suggests that dopaminergic cell loss in the SNpc comes with axonal changes in these neurons that precede such cell loss. This has been called Wallerian-like axonal degeneration (Mack et al., 2001), where degeneration starts at the nerve terminals and protracts back to the cell bodies. Spheroidal dystrophic axonal swellings with accumulated beta- and gamma-synuclein have been found in the hippocampus of PD patients (Galvin et al., 1999). These post-mortem studies support the notion that axonal failure is a significant prodromal hallmark of PD, and these are also reflected in animal PD models (Chu et al., 2012). Mice with the partial KO of *En1* with SW background display abnormal TH-stained axonal swellings as early as 8 days after birth (Nordstroma et al., 2015). These swellings have been shown to increase in number and in size over time. Not much is know about what causes these swellings or what they are composed of, but a recent study suggests that an unordinary accumulation of mitochondria and electron-dense vacuoles are within the swollen structures (Nordstrom et al., 2015). Bl6 mice presenting the partial KO of do not present such axonal deformities (Sgado et al., 2006; Veenvliet et al., 2013). In the present study, we did not observe significant nigral cell loss in 17-week old Bl6-N4-*En1*+/- mice; however, the the abundance of striatal axonal swellings seems to be similar to that seen in SW *En1*+/- mice of the same age. This could be interpreted as a sign of delayed nigrostriatal degeneration in *En1* heterozygous Bl6 mice – a feature that doesn't seem to pertain to SW mice.

Although further fine-mapping is required to find candidate genes within the QTLs found here, candidates in, or in close proximity to these loci can already be discussed thanks to them being previously tied to PD pathogenesis. *Lmx1b,* for example, lies close to one of our QTLs (*En1d*) linked to dopaminergic cell death in this study, and it has recently implicated PD-like pathology (Laguna et al., 2015). *Foxa2* is another candidate; mice on the SW background with its partial deletion show abnormalities in motor behavior and an associated progressive loss of dopamine (Kittappa et al., 2007). This gene is found on chromosome 2, close to QTL *En1o*, which is linked to load of axonal swellings per dopaminergic neuron. Interestingly, *En2,* which has been shown to compensate for loss of *En1* (Sgado et al., 2006)*,* was not found in any of the QTLs, suggesting that there is no *cis*-effect of *En2* protecting DNs in Bl6. There might, however, be *trans* effects, meaning that another QTL may act indirectly on *En2* and induces its expression.

Overall this work is an important step towards candidate gene discovery. QTLs themselves represent a genetic region that may contain hundreds of genes, so more work is necessary to extrapolate a more refined group of genes or even one single gene within that QTL that can be considered candidates, and this can be done by congenic mapping. Congenic mapping consists in generating congenic strains by transferring a QTL of interest from a donor strain to a recipient strain, therefor

isolating that QTL for further analysis. This has been done, for example, after the discovery of the *Vra1* QTL in PVG rats, which were found to be less susceptible to neurodegeneration after VRA and TBI (Lundberg et al., 2001).

The *Vra1* QTL protects rats from PD-like pathology

Susceptibility to neurodegeneration has been studied in rats after VRA (Swanberg et al., 2009). The QTL *Vra1* on rat chromosome 8 was linked to neuroprotection after VRA (Lidman et al., 2003) and TBI (Al Nimer et al., 2013). After congenic mapping, *Gsta4* was found to be the main candidate gene within *Vra1* for conferring neuroprotection through a *cis*-effect on gene expression, likely caused by polymorphisms within or near *Gsta4* affecting gene transcription (Al Nimer et al., 2013; Strom et al., 2012; Swanberg et al., 2009). Since *Gsta4* regulates lipid peroxidation, and since there is evidence that lipid peroxidation, and oxidative stress in general, are major factors in PD (Blesa et al., 2015; Castellani et al., 2002; Zarkovic, 2003), we hypothesized that *Vra1* would have a neuroprotective effect in rat models of PD. To test this hypothesis, we compared the response of DA rats vs DA.VRA1 congenic rats to the 6-OHDA-induced unilateral partial striatal lesion (**paper III**) and to the unilateral rAAV-mediated overexpression of α-syn in the SNpc (**paper IV**). Both these models were chosen because together they produce a wide range of phenotypes that resemble PD.

Dopaminergic neurodegeneration driven by the 6-OHDA lesion

The loss of dopaminergic neurons and fibers in the nigrostriatal pathway is one of the phenotypic hallmarks of PD. The 6-OHDA rat model has been extensively exploited to mimic this aspect of the disease. Moreover, the progressive degeneration of the nigrostriatal pathway driven by the partial striatal lesion is preferable for studying mild to moderate parkinsonian phenotypes, which mimic early stage parkinsonism, and the effect of neuroprotective agents or alleles (Deumens et al., 2002). We therefor used this model in **paper III** to test the neuroprotective effects of *Vra1*.

Our results show that PVG alleles in the locus protect DA.VRA1 congenic rats from loss of dopaminergic fibers in the striatum when compared to the control DA strain. In fact, DA.VRA1 rats displayed an almost 2-fold higher striatal dopaminergic fiber density compared to DA at 8 weeks after the lesion. A similar trend of neuroprotection in the DA.VRA1 was found for number of TH^+ cells counted in the SNpc, with a higher proportion of surviving neurons in the congenic strain, but this difference was not statistically significant. The less

obvious difference in the SNpc could be due to GSTA4 affecting an ongoing degenerative process that starts at dopaminergic projections in the striatum, and has a delayed, retrograde effect on cell somas in the SNpc. Studying strain differences in nigral dopaminergic cell counts at additional, and later, time point would be required to confirm this.

Toxic α-syn accumulation driven by the rAAV-α-syn viral vector

The striatal 6-OHDA model is effective at reproducing progressive neurodegeneration of nigrostriatal pathway similar to that seen in PD patients. Yet, this model does not cover other pathogenic mechanisms of PD, such as the production of toxic α-syn species or impaired protein degradation (Venda et al., 2010). The functional link between α-syn and PD is very robust: Lewy bodies, which are made up of mainly α-syn aggregates are present in both familial and idiopathic PD, and the *SNCA* gene has been linked to familial PD and associated to the risk of developing idiopathic PD (Lill, 2016). The rat rAAV-α-syn model induces unilateral overexpression of α-syn, progressive dopaminergic neurodegeneration and motor impairment (Kirik et al., 2002). The rAAV vector used in the current study incorporates the WPRE element, which amplifies the expression of the transgene (Decressac et al., 2012b). The rAAV-α-syn model induces more progressive behavioral impairments compared to the striatal 6-OHDA model, probably due to the buildup of toxic α-syn oligomers leading to deficits in synaptic function (Decressac et al., 2012a; Venda et al., 2010).

In **paper IV**, we observed that striatal dopaminergic fiber density in the lesioned side was significantly higher in the congenic rats compared to DA at 8 weeks, and similar evidence of *Vra1*-mediated neuroprotection was observed for midbrain dopaminergic cell bodies by stereological cell counts of TH^+ and vesicular monoamine transporter 2 $(VMAT2)^+$ neurons. VMAT2 is typically packages dopamine when dopamine is released at the synapse, and can thus be used as a marker for dopaminergic neurons (Lohr & Miller, 2014). We used this marker to be sure that the cell loss determined by TH^+ staining wasn't simply an indication of TH downregulation instead of actual cell loss. These results are in line with the observations made in **paper III**, and are promising for future research on PD therapeutic molecules. Due to the genetic heterogeneity of PD, a drug of choice should be able to induce therapeutic effects efficiently in different people. Therefor, since this thesis shows the promising neuroprotective effects of *Gsta4* in two very different PD models, it can be analyzed in samples of patient and control cohorts to determine whether it associates to PD.

Astrocytic *Gsta4* mRNA expression levels drive neuroprotection

To further investigate the neuroprotective effects seen on striatal dopaminergic fibers in the DA.VRA1 congenic strain, we analyzed mRNA expression of *Gsta4*, the candidate gene from the *Vra1* QTL (Al Nimer et al., 2013; Strom et al., 2012). *Gsta4* plays an important role in GSH metabolism, which is heavily involved in clearing of oxidative stress and lipid peroxidation products (Schulz et al., 2000). HNE has been shown to be elevated in PD patients (Selley, 1998). Incidentally, the degenerative process driven by 6-OHDA is thought to be due to accumulation of ROS within dopaminergic neurons (Glinka et al., 1997). In **paper III,** we decided to examine *Gsta4* expression at 2 and 7 days after 6-OHDA injections. This choice was made since Ström et al. found the highest levels of *Gsta4* expression between 1 and 5 days after nerve injury in DA and PVG.1AV1 rats (Strom et al., 2012) and since previous studies show high levels of striatal free radical species from 25 min to 7 days after 6-OHDA lesion (Henze et al., 2005). Our data indicate that *Gsta4* expression is higher in both the injured and intact striatum and midbrain of DA.VRA1 compared to DA rats at 2 days but not 7 days, suggesting that the gene exerts its neuroprotective effects within the first days after 6-OHDA lesion.

Evidence suggests that α-syn overexpression increases oxidative stress levels, which is a key feature of human PD. Both *in vivo* and *in vitro* models have shown that accumulation of α-syn can lead to mitochondrial dysfunction through the inhibition of Complex I, which in turn leads to the production of ROS (Moon & Paek, 2015). Furthermore, a study by Shearn et al. on chronic alcohol consumption in a GSTA4 null mouse, has shown that GSTA4 works as a mitochondrial detoxifier (Shearn et al., 2016). This strongly suggests that α-syn toxicity is partly mediated by oxidative stress mechanisms, mainly acting through the mitochondria in dopaminergic cells and involving GSH metabolism.

In **paper IV**, we examined mRNA expression levels of *Gsta4* at 3 weeks post-injection since the first signs if neurodegeneration, synaptic impairment, and behavioral deficits are visible starting at this time point (Decressac et al., 2012b; Kirik et al., 2002). We found that *Gsta4* is more highly expressed in DA.VRA1 rats compared to DA. This is observed on both the ipsilateral and the contralateral side of the striatum and midbrain, meaning that α-syn itself does not cause the higher expression levels, but is rather a strain-dependent factor. This is similar to what we see in **paper III** after 6-OHDA injections, and in both models we can infer that a modest increase in *Gsta4* gene expression in the DA.VRA1 congenic strain can partially protect midbrain dopaminergic cell projections and somas from degeneration.

GSTA4 is expressed in astrocytes

Not much is know about the cellular localization of GSTA4 and the cell types expressing it in the brain. We decided to investigate this in **paper III**, and found expression of in astrocytes of both the striatum and of the SNpc, not in microglia or neurons at 8 weeks post lesion. The same histological expression pattern was obtained after α-syn overexpression at the same time point in **paper IV**. It's been shown that astrocytes play a pivotal role in clearing oxidative stress products (Wilson, 1997). Moreover, astrocytes have been shown exhibiting high GSH activity. This is important because GSH uses GSTA4 to degrade HNE (Heales et al., 2004; Sagara et al., 1993). One mechanism by which astrocytes might be neuroprotective is through their release of GSH to neurons, which has been observed after nitric oxide exposure in-vitro (Heales et al., 2004). Moreover, it's been shown that α-syn can accumulate in astrocytes of the forebrain of PD patients, and it's been suggested that α-syn spreads from neurons to astrocytes (Croisier & Graeber, 2006). A recent study by Lindström et al. points out the important role of astrocytes in α-synucleinopathies. They show that in a co-culture system astrocytes engulf large amounts of α-syn oligomers but are subsequently not able to degrade them completely, which leads to the formation of inclusions. This is most likely brought on by a dysfunctional lysosomal system. Astrocytes also showed signs of mitochondrial damage caused by the accumulation of these α-syn oligomers (Lindstrom et al., 2017).

The fact that we see a similar neuroprotective phenotype of DA.VRA1 rats in the α-syn overexpression model as in the striatal 6-OHDA model strongly suggests that the *Vra1* locus encoding *Gsta4* regulates key processes in PD-like dopaminergic neurodegeneration. Overall this data suggests that *Gsta4* could be an important target for future translation studies, and should be considered as a candidate gene for genetic association to PD. Therefor, GSTA4 could become therapeutic target for PD treatment.

Concluding Remarks

The genetics of common neurodegenerative diseases like PD is crucial to fully understand its etiology and in order to find new treatments that slow down the progression of the disease or even cure it. This task is easier for familial cases of PD where an inherited gene mutation is enough to cause PD, however, for idiopathic cases, the task is much more challenging. The combination of factors is at play in most cases of PD making it much more difficult to find a direct cause, therefor researching genetic factors conferring susceptibility to acquiring PD is essential. This thesis is aimed at doing just that by utilizing different PD animal models and experimental tools necessary for discovering candidate genes within these models that can be studied in human samples at a later time point.

We report that six rat strains are differentially susceptible to unilateral overexpression of α-syn in the SNpc, indicating that genetic differences among the strains are crucial for determining that susceptibility.

We used linkage analysis to uncover several mouse loci that are responsible for either reducing susceptibility in Bl6 mice or augmenting susceptibility in SW mice to a partial *En1* KO.

Finally, we found the DA.VRA1 congenic rat strain to be less susceptible to dopaminergic neurodegeneration in two separate PD models: the 6-OHDA striatal lesion and the rAAV-mediated overexpression of α-syn. Since here the locus had already been identified, we focused on a candidate gene within this locus to test its role in neuroprotection within both models. Our results suggest that *Gsta4* plays an important role in this neuroprotection, and we hypothesize that it is mainly exerted through astrocytes.

This thesis contributes to the discovery of several candidate genes involved in susceptibility to neurodegeneration in the different animal models. It also paves the way for future studies within these models to find even more candidates, and more importantly to studies in human samples by testing these candidate genes in PD patient and control cohorts.

Overall, this research brings us one step closer to determining genetic risk factors for idiopathic PD.

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