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PROGRESSIVE MODELS OF PARKINSON'S DISEASE: BEHAVIOURAL AND NEUROCHEMICAL ANALYSES

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Progressive Models of Parkinson's Disease: Behavioural and Neurochemical Analyses
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To my family with love

“Just because you can explain it doesn't mean it's not still a miracle.”
— Sir Terry Pratchett

ABSTRACT

Parkinson's disease (PD) is a common neurodegenerative disorder diagnosed based on the presence of motor symptoms, including resting tremor, postural impairment, bradykinesia and rigidity. The pathological hallmarks are degeneration of midbrain dopaminergic neurons and the accumulation of inclusions containing the misfolded protein α-synuclein (termed "Lewy bodies") throughout the nervous system. However, when the cardinal motor symptoms of PD emerge already 60-80% of the dopaminergic content has been lost, meaning that patients get diagnosed with PD at a very late disease stage. PD is also associated with many non-motor symptoms, which can appear up to 30 years before the onset of the motor symptoms. In order to fully appreciate the aetiology of PD, including mechanisms underlying the earliest pathological events, valid model systems are needed. Thus, the work of this thesis has focused on the characterization and utilization of progressive models of PD, including the cNurr1^{DATCreER} knock-out (KO) model (studies I and II), the Lmx1a/b^{DATCre} KO model (study III), and a viral vector-induced model overexpressing α-synuclein in the ventral tegmental area (VTA) (study IV), allowing investigation of the early pathological stages of PD.

We here report that the cNurr1^{DATCreER} model recapitulated early pathological features of PD, including a progressively reduced expression of several dopaminergic markers in the striatum and ventral midbrain, reduced striatal levels of dopamine and its metabolites, a dopamine fiber pathology not accompanied by midbrain dopaminergic cell death, and profound motor symptoms. cNurr1^{DATCreER} KO animals also displayed enhanced corticostriatal glutamate release compared with wild-type animals. The PD-related features observed in the cNurr1^{DATCreER} model were likely caused by an altered expression of mitochondrial genes, in line with mitochondrial dysfunction being a pathological mechanism proposed in human PD. Using this model in combination with the well-established 6-OHDA lesioned mouse model and trace amine-associated receptor 1 (TAAR1) KO mice, a potentially therapeutic role of TAAR1 was revealed in experimental Parkinsonism and L-DOPA-induced side effects.

Ablation of developmental transcription factors Lmx1a and Lmx1b in dopaminergic neurons induced an early progressive striatal pathology resembling key aspects of PD. Lmx1a/b ablation also impaired both motor functions and non-motor functions, including olfactory and short-term memory functions, but left anxiety- or depressive-like behaviours intact. Moreover, Lmx1b was required for the normal function of the autophagic-lysosomal pathway, and, importantly, it was also decreased in midbrain dopaminergic neurons of patients with PD. These findings point toward a clinically relevant role of Lmx1b in human PD, suggesting that its pathological mechanism is related to impaired degradation of defective proteins, which has been proposed as a pathological mechanism in PD.

Further focusing on the non-motor symptoms of PD, α-synuclein was overexpressed in the rat VTA using adeno-associated viral vectors. The VTA contains dopaminergic neurons projecting to the prefrontal cortex and to limbic regions. Our findings revealed a role of the dopaminergic projections of the VTA in emotional long-term memory, which was vulnerable to α-synuclein pathology. This effect was likely mediated via its innervations to limbic regions including the amygdala, hippocampus and nucleus accumbens.

In conclusion, we here show how both pathological and behavioural Parkinson-like features related to early PD can be induced by ablation of transcription factors Nurr1 and Lmx1a/b, and by overexpression of α-synuclein in VTA. Our experiments also highlight the usefulness of the cNurr1^{DATCreER} KO model as a bilateral model of early PD, and reveal a role of TAAR1 in experimental PD. Hopefully these data will contribute to a better understanding of the early disease stages of PD and ultimately to the development of better treatment regiments.

LIST OF SCIENTIFIC PAPERS

I. Kadkhodaei B, **Alvarsson A**, Schintu N, Ramsköld D, Volakakis N, Joodmardi E, Yoshitake T, Kehr J, Decressac M, Björklund A, Sandberg R, Svenningsson P, Perlmann T. Transcription factor Nurr1 maintains fiber integrity and nuclear-encoded mitochondrial gene expression in dopamine neurons.
Proc Natl Acad Sci U S A (2013), 110(6): 2360-5

II. **Alvarsson A**, Zhang X, Stan TL, Schintu N, Kadkhodaei B, Millan MJ, Perlmann T, Svenningsson P. Role of trace-amine associated receptor 1 in experimental Parkinsonism, L-DOPA responsivity and glutamatergic neurotransmission.
Submitted manuscript

III. Laguna A, Schintu N, Nobre A, **Alvarsson A**, Volakakis N, Jacobsen JK, Gomez M, Sopova E, Joodmardi E, Yoshitake T, Deng Q, Kehr J, Ericson J, Svenningsson P, Shupliakov O, Perlmann T. Dopaminergic control of autophagic-lysosomal function implicates Lmx1a/b in Parkinson's disease.
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IV. **Alvarsson A**, Caudal D, Björklund A, Svenningsson P. Cognitive deficits induced by AAV6-mediated overexpression of human α-synuclein in dopaminergic neurons from ventral tegmental area.
Submitted manuscript

ADDITIONAL PUBLICATIONS

Related published articles not included in the thesis:

1. Stan TL, **Alvarsson A,** Branzell N, Sousa VC, Svenningsson P. NMDA receptor antagonists ketamine and Ro25-6981 inhibit evoked release of glutamate in vivo in the subiculum.
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2. Eriksson TM, **Alvarsson A,** Stan TL, Zhang X, Hascup KN, Hascup ER, Kehr J, Gerhardt GA, Warner-Schmidt J, Arango-Lievano M, Kaplitt MG, Ogren SO, Greengard P, Svenningsson P. Bidirectional regulation of emotional memory by 5-HT1B receptors involves hippocampal p11.
   Mol Psychiatry (2013), 18(10): 1096-105

CONTENTS

LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
CNS	Central nervous system
DAT	Dopamine transporter
DBS	Deep brain stimulation
eCB	Endocannabinoid
GPe	Globus pallidus pars externa
GPi	Globus pallidus pars interna
KO	Knock-out
mDA	Mesencephalic dopaminergic
MFB	Medial forebrain bundle
PD	Parkinson’s disease
ROS	Reactive oxygen species
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
TAAR1	Trace amine-associated receptor 1
TH	Tyrosine hydroxylase
VTA	Ventral tegmental area
WT	Wild-type

1 INTRODUCTION

Parkinson's disease (PD) is a common, slowly progressive neurodegenerative disorder with a pathogenesis that remains largely unknown. It affects 1-2% of the population over 65 years of age. PD is clinically diagnosed based on motor dysfunctions, and pathologically defined by a prominent loss of dopamine synthesizing neurons in the substantia nigra pars compacta (SNc) projecting to the striatum (Lees et al., 2009). A pathological hallmark of PD is the presence of inclusions partially formed by the protein α-synuclein (Lewy bodies) (Braak and Del Tredici, 2008; Braak et al., 2004; Braak et al., 2003). A progressive accumulation of Lewy bodies throughout the central, peripheral, and enteric nervous systems correlate with the development of clinical signs (Braak and Del Tredici, 2008). Most cases of PD are sporadic without any heritability, however, over ten PD genes, including α-synuclein mutations or duplications, have been identified. Pathological studies in postmortem brain samples from end-stage PD patients are characterized by a profound loss of mesencephalic dopaminergic neurons (mDA), above all in the SNc, but also in the ventral tegmental area (VTA) which projects to many limbic regions. Consistently, the cardinal motor symptoms of PD are often preceded by non-motor symptoms, including depression, anosmia, REM sleep disorders, obstipation and mild cognitive impairments (Schrag, 2004). When the motor symptoms appear, up to 80% of mDA neurons have already degenerated (Hornykiewicz and Kish, 1987; Riederer and Wuketich, 1976; Tissingh et al., 1998; Tissingh et al., 1998), indicating that these symptoms appear very late in the disease process. The golden standard treatment in PD is dopamine replacement by its precursor L-DOPA. However, clinical fluctuations, dyskinesias, hallucinations and exaggerated debilitating reward seeking are complications of long-term treatment with L-DOPA (Dunnett and Björklund, 1999). Furthermore, there is currently no treatment that halts or slows the neurodegenerative processes in PD. An increased understanding of the earliest pathological events in PD is key to develop more efficient treatments, and especially to target the neurodegenerative process. To this end, it is also vital to discover biomarkers to detect PD at an earlier disease stage than what is possible today.

1.1 SYMPTOMATOLOGY OF PARKINSON'S DISEASE

1.1.1 Motor symptoms

The earliest descriptions of symptoms resembling Parkinsonism were described as early as 1000 BC in ancient Indian and Chinese writings (Goetz, 2011). The first medical description of PD, referred to as "paralysis agitans" or "shaking palsy", was published by Dr. James Parkinson in 1817. This description was later refined by Jean-Martin Charcot in 1872 (Goetz, 2011), who also suggested the term "Parkinson's disease" to name the disorder. This renaming was based on Charcot's observation that neither tremor nor weakness affect all patients with PD, as the name "shaking palsy" might suggest (Goetz, 2011). The four cardinal symptoms of PD include bradykinesia (slowness of movement), muscular rigidity, resting tremor, and postural and gait impairments (Kalia and Lang, 2015). The symptomatology of PD is heterogeneous, and two major subtypes have been described based on the motor symptomatology: tremor-dominant PD and non-tremor dominant (akinetic-rigid) PD, which have been proposed to have different aetiologies (Marras and Lang, 2013). Tremor-dominant PD is often associated with a slower disease progression and a less debilitating symptomatology compared with non-tremor dominant PD (Kalia and Lang, 2015). The motor symptoms become more severe as PD progresses. Current PD medications (dopamine agonists or dopamine replacement therapy by L-DOPA), are effective in managing the motor symptoms of PD during the early disease phase. However, the emergence of severe side-effects associated with prolonged L-DOPA treatment, including

dyskinesia, psychosis and clinical fluctuations ("on" and "off" periods, fluctuations between responding well and poorly to L-DOPA) (Hely et al., 2005), complicate the management of PD. Moreover, since the diagnosis of PD today is based solely on the motor symptoms of PD, which appear during the later stages of the disease progression, when 60-80% of the striatal dopaminergic content has already been lost (Hornykiewicz and Kish, 1987; Riederer and Wuketich, 1976; Tissingh et al., 1998a; 1998b), there is a call to identify biomarkers for earlier disease stages. This would allow an earlier diagnosis as well as the use of early therapeutic interventions to slow or reverse the disease progression.

1.1.2 Non-motor symptoms

When James Parkinson first described PD in 1819 he defined it as a fundamental motor disorder, but he also recognized an array of non-motor symptoms, including sleep dysfunctions, autonomous nervous system dysfunctions (dysautonomia) and neuropsychiatric symptoms (Parkinson, 1817). These symptoms are often described as equally or even more debilitating than the motor symptoms by the patients (Chaudhuri et al., 2010; Schrag et al., 2000), and are often not responsive to antiparkinsonian medications. However, up until today these symptoms are poorly recognized in the clinic and/or often remain undeclared by PD patients themselves (Chaudhuri et al., 2010; Shulman et al., 2002). Non-motor symptoms can appear early during the pathology, often before the onset of the cardinal motor symptoms. This is called the premotor, or prodromal, phase of PD. The non-motor symptoms of PD commonly include constipation, loss of olfaction, sleep disturbances, anxiety and depression (Abbott et al., 2003; Iranzo et al., 2006; Shiba et al., 2000; Tolosa et al., 2007), and can be divided into distinct neuroanatomical manifestations (Lee and Koh, 2015), as shown in Table 1.1. Notably, these symptoms are not restricted to the central nervous system. Dysfunctions of the autonomic nervous system can precede the motor symptoms by many years, are very common during the early disease stages and progress in severity throughout the course of the disease (Tolosa et al., 2007).

Table 1.1 Neuroanatomy of the symptomatology in PD

Cortical symptoms	Psychosis Cognitive impairments Impulse control impairments
Basal ganglia symptoms	Apathy Restlessness/akathisia ("inability to sit")
Brainstem symptoms	Depression Anxiety Sleep disorders
Peripheral nervous system symptoms	Constipation Pain Orthostatic hypotension Sensory disturbances

Non-motor symptoms are very common in PD. For instance, impaired sense of olfaction and odour discrimination affects up to 90% of patients with PD (Hawkes et al., 1997). Impaired olfaction, however, is not a symptom restricted to PD, but can also be seen in other neurodegenerative disorders including Alzheimer's disease, rendering olfactory impairments less useful as a potential biomarker of PD. Painful sensations have been reported in 40-85% of PD patients (Cury et al., 2015), and are often reported also as "off"-period symptoms (Witjas et al., 2002). Fatigue or daytime sleepiness has been reported in 40-43% of patients (Hilten et al., 1993; Shulman et al., 2001). Cognitive impairments, anxiety and depression are also frequently reported in PD, and have a large impact on the patients' quality of life, as will be discussed further below.

Moreover, PD patients often experience non-motor fluctuations following prolonged L-DOPA treatment, characterized by symptoms which appear only during "off" periods, while other non-motor symptoms are aggravated during "off" periods (Todorova et al., 2014).

1.1.2.1 Cognitive impairments and dementia in Parkinson's disease

At the end of the nineteenth century mental changes were already being reported in patients with PD, including impaired memory, irascibility (short temper), melancholia and dementia. Cognitive impairments range in prevalence between 24-46% in patients with PD depending on cohort characteristics (Aarsland et al., 2010; Foltynie et al., 2004; Muslimovic et al., 2005). Today there is an increasing awareness that mild cognitive impairments are present also in newly diagnosed patients with PD (Foltynie et al., 2004; Muslimovic et al., 2005). However, mild cognitive impairments are also associated with an older age at disease onset, increased disease duration, more severe motor symptoms and a more advanced disease state in PD (Aarsland et al., 2010). Moreover, PD patients who also suffer from depression more often present with cognitive impairments compared with non-depressed subjects (Aarsland et al., 2010).

The most common cognitive impairments in PD involve executive, psychomotor and memory functions (Elgh et al., 2009; Muslimovic et al., 2005), apathy and decreased levels of motivation (den Brok et al., 2015). Mild cognitive impairments have a significant impact on the patients' quality of life and health outcomes (Lawson et al., 2014a, 2014b), particularly because these symptoms can progress to overt dementia, which is a strong predictor of the need for nursing home placement (Aarsland et al., 2000). A systematical review by Aarsland and colleagues, using strict inclusion and exclusion criterions for PD samples, revealed a 31.3% prevalence of dementia in PD patients (Aarsland et al., 2005). However, after 20 years of disease duration dementia can affect up to 83% of PD patients (Hely et al., 2008). Both cortical Lewy bodies and Alzheimer-like pathological changes have been reported to contribute to the manifestation of dementia in PD, whereas the pathology related to mild cognitive impairments in PD is less known, mainly because patients usually survive until these symptoms develop into dementia (Aarsland et al., 2011a). However, evidence point towards cognitive impairments being related to similar, albeit milder, pathological changes as dementia in PD (Aarsland et al., 2011a). Pathological studies in postmortem brain samples from PD patients with dementia revealed a Lewy body density 5 times higher in cortical areas compared with non-demented patients with PD (Apaydin et al., 2002; Hurtig et al., 2000). Lewy bodies are also found in brainstem nuclei, including the noradrenergic locus coeruleus and the serotonergic raphe nuclei, limbic structures, cholinergic forebrain neurons, and in the cerebral cortex (Emre, 2004). In general, antiparkinsonian medications targeting the dopaminergic system have both beneficial and deleterious effects on cognitive tasks, depending on which brain circuitries are involved in the specific task (Cools et al., 2001).

1.1.2.2 Depression and anxiety in Parkinson's disease

The prevalence of depressive symptoms in PD have been reported to range between 35 and 50% (Aarsland et al., 2011b; Shulman et al., 2001), and have been reported as the main determinant of a poor quality of life in PD (Schrag, 2006). Anxiety has been reported in 28-38% of patients with PD (Menza et al., 1993; Shulman et al., 2001; Stein et al., 1990), and has a very high level of comorbidity with depression (Menza et al., 1993), much higher than in depressed patients without PD (Walsh and Bennett, 2001). PD patients do not often present with classic major depression, but usually show less self-blame, guilt, sense of failure and self-destructive thoughts compared with patients suffering from primary major depression (Schrag, 2006). It is also comparatively uncommon that depressed PD patients commit suicide. Depression can also occur as an "off" symptom, and is then responsive to antiparkinsonian treatments (Menza et al., 1990). Degeneration of the mesolimbic and mesocortical dopaminergic pathways, which project from the VTA to limbic and cortical structures, are thought to play a pivotal role in depressive symptoms in PD (Lieberman, 2006). However, depression in PD is thought to be multifactorial and also related to changes in the noradrenergic and especially serotonergic systems (Chan-Palay, 1993; Kostić et al., 1987; Mayeux et al., 1988, 1984; Menza et al., 1999; Mössner et al., 2001), all potentially contributing to the symptomatology. An important observation is that pathological changes in the raphe nuclei and locus coeruleus, which harbour serotonergic and noradrenergic cell bodies respectively, appear before pathological lesions occur in the dopaminergic cell bodies of SNc (Table 1.2). This is consistent with depressive symptoms sometimes occurring before the onset of motor symptoms in PD.

Although traditional antidepressants can be beneficial in the management of depression in PD, they are associated with a high variability in treatment outcomes. The general understanding of antidepressant treatments has also been hampered by the lack of controlled clinical trials evaluating the efficacy in PD. However, the antidepressant pramipexole, which targets D_3 receptors that are extensively expressed in the limbic system, was recently shown to efficiently improve depressive symptoms in PD (Barone, 2011)**.**

1.2 TREATMENT OF PARKINSON'S DISEASE

The current treatment options for PD focus on targeting dopamine depletion and offer only symptomatic relief, without slowing the neurodegenerative processes. Thus, better treatment options are warranted. An emerging area is the use of non-dopaminergic treatment options in the management of PD. Such treatments include anticholinergics, amantadine, MAO inhibitors and deep brain stimulation. However, a better understanding of the disease processes underlying PD is vital in order to slow, halt, or even reverse the neurodegeneration in PD.

1.2.1.1 L-DOPA

Dopamine replacement therapy by L-DOPA (L-3,4-dihydroxyphenylalanine, also called levodopa) is currently the golden standard and the most efficient treatment for PD, despite its disabling side effects. L-DOPA is a precursor of dopamine which, as opposed to dopamine itself, can cross the blood-brain barrier. Once L-DOPA has entered the brain it will be converted to dopamine by the surviving dopaminergic neurons. In order to prevent L-DOPA from being converted to dopamine in the peripheral nervous system, L-DOPA must be co-administered with a peripheral L-DOPA decarboxylase inhibitor to prevent peripheral side effects.

In patients suffering from PD, the therapeutic effect of L-DOPA is gradually lost, whereas L-DOPA induced dyskinesias, or abnormal involuntary movements, emerge in response to previously therapeutic doses of L-DOPA. No antiparkinsonian medications slow the progression of PD, and as dopaminergic neurons continue to degenerate, there are progressively fewer nigrostriatal dopaminergic terminals that can convert L-DOPA into dopamine, store dopamine in synaptic vesicles and subsequently release dopamine. Eventually L-DOPA is decarboxylased into dopamine and released by serotonergic neurons, which lack inhibitory D_2 autoreceptors and thus are unable to properly buffer dopamine levels (Carta et al., 2007; Rylander et al., 2010). This results in large fluctuations in extracellular dopamine in response to L-DOPA administration, and subsequently to functional pre- and postsynaptic changes throughout the basal ganglia. The molecular mechanisms behind the emergence of L-DOPA induced dyskinesias are still poorly understood. However, L-DOPA induced dyskinesias can be reduced by the weak NMDA receptor antagonist amantadine, suggesting that the glutamatergic system is involved (Jenner, 2008). Changes in corticostriatal connectivity, which are related to glutamatergic neurotransmission and altered density of glutamatergic AMPA and NMDA receptors, have been implicated in the induction of dyskinesias (Jenner, 2008). This has been further explored in study II of this thesis.

1.2.1.2 Dopamine agonists

Dopamine agonists have fewer side effects and are often used during early disease stages of PD to delay the use of L-DOPA and the subsequent onset of L-DOPA induced side effects (Kuzel, 1999; Lange, 1998; Montastruc et al., 1999; Pahwa et al., 2004). Although dopamine agonists are associated with fewer side effects compared with L-DOPA, they are not as efficient as L-DOPA as the disease progresses (Lange, 1998; Montastruc et al., 1999; Pahwa et al., 2004). One commonly used dopamine agonist is ropinirole, which acts on D_2-like receptors (D_2, D_3 and D_4 receptors) with the highest affinity for D_3 receptors (Kulisevsky and Pagonabarraga, 2010; Pahwa et al., 2004). Ropinirole has been used in study II to investigate the modulatory role of dopamine receptors on corticostriatal glutamate release in experimental Parkinsonism.

Apomorphine is a non-selective dopamine agonist that activates both D_1-like and D_2-like dopamine receptors. It has a rapid onset of effect, making it effective in patients experiencing both predictable and unpredictable “off” periods, and can also be useful when the patient is anticipating an “off” period (Trenkwalder et al., 2015). Subcutaneous apomorphine infusions can be useful in the treatment of PD for patients who experience troublesome “off” periods with oral medications and who would otherwise need very frequent apomorphine injections (Trenkwalder et al., 2015). Apart from being useful in the management of motor fluctuations, there is also evidence that apomorphine can have effects on non-motor symptoms associated with “off” periods (Trenkwalder et al., 2015). Apomorphine is also commonly used to investigate the efficacy of 6-OHDA lesions, which are used to induce experimental Parkinsonism in rodents. When apomorphine is injected systemically into a hemiparkinsonian rodent with a unilateral 6-OHDA lesion, a characteristic rotational behavior is induced, its intensity reflecting the level of 6-OHDA induced neurodegeneration. This supersensitive response induced by apomorphine treatment is associated with the stimulation of postsynaptic dopamine receptors in the denervated hemisphere, which become supersensitized following dopamine denervation, causing the animal to rotate contralaterally in response to apomorphine (Creese and Snyder, 1979; Creese et al., 1977).

1.2.1.3 Deep brain stimulation

PD is associated with increased activity of the SNc and the GPi. Deep brain stimulation (DBS) via electrodes targeting the subthalamic nucleus (STN) and the GPi is sometimes used to treat advanced treatment-resistant PD and L-DOPA induced dyskinesias, by simulating the effects of lesions to these regions reversibly, without destroying the brain tissue (Fasano et al., 2012). Although DBS has well-documented therapeutic effects, including almost immediate therapeutic effects on tremor, rigidity and bradykinesia (Volkmann et al., 2002), the mechanisms of action remain elusive (Kringelbach et al., 2007).

1.2.1.4 Immunotherapy

Abundant evidence has indicated a role of α-synuclein in the neuronal death in PD, and even that α-synuclein may propagate the neurodegenerative process in a prion-like manner (Visanji et al., 2013). In light of this, targeting extracellular α-synuclein via immunization-based therapies has emerged as a potential consideration (Kalia et al., 2015). The results of the first clinical trial of a vaccine composed of immunogenic peptides mimicking the C-terminus of α-synuclein have not yet been published (Kalia et al., 2015).

1.2.1.5 Stem cell therapy

The implantation of fetal ventral mesencephalic cells into the brain of patients with PD has proven to be beneficial in some patients, but the usefulness of such therapies is limited by ethical concerns and the limited availability of donor tissue. Although *in vivo* animal experiments have shown that fetal grafts can survive and integrate into the host striatum, release dopamine and restore motor functions (Brundin et al., 1986; Nikkhah et al., 1995), human clinical trials have remained inconclusive, with some patients even developing severe dystonia and dyskinesia (Luo et al., 2009). Embryonic stem cells have also been proposed in the therapy of PD, and can circumvent the logistic complications associated with fetal grafts since they can be grown *in vitro*. However, undifferentiated embryonic stem cells are prone to form tumours *in vivo* (Luo et al., 2009). The risk of tumourigenesis is also a concern when using stem cells that have been differentiated *in vitro*. The use of pluripotent adult stem cells holds more promise for future therapeutic management, since it avoids the ethical problems associated with the use of embryonic stem cells. These cells can instead be harvested from the central nervous system (CNS) and blood marrow of a postmortem donor, or even potentially from the patient itself, and can differentiate into a variety of cells in response to growth factors (Luo et al., 2009). The clinical usefulness of such stem cell therapies remains to be further explored (Trounson et al., 2011).

1.2.1.6 Gene therapy

Gene therapy, performed via the injection of viral vectors that replace or correct a defective gene, can potentially be used to treat many disorders by altering the disease environment. In the case of PD, this would be done by the induction of genes or transgenes that favour neuronal survival in the basal ganglia. Another strategy is to increase the efficacy and reduce the side effects of current pharmacological and surgical treatments. One obstacle in the treatment of neurological disorders is the blood-brain barrier, which hinders most viral vectors to enter the CNS. Adeno-associated virus (AAV) subtype 9 has the ability to cross the blood-brain barrier (Duque et al., 2009), making it useful for targeting pathological processes of the brain. Many trials have suggested safety and efficacy of gene therapy in PD (Bartus et al., 2013), but the majority of phase II experiments have failed to show any beneficial effects beyond the placebo effect (Bartus et al., 2013). Whereas studies using

neurotrophic factor GDNF to protect dopaminergic neurons were associated with unwanted side effects (Nutt et al., 2003), the failure of gene therapy involving the growth factor neurturin was suggested to be associated with the need of earlier delivery, optimally before any extensive neurodegenerative damage has occurred (O'Connor and Boulis, 2015). This highlights the need for the discovery of biomarkers that would enable an earlier diagnosis and thus earlier therapeutic management of PD.

1.3 PATHOGENESIS OF PARKINSON'S DISEASE

1.3.1 Lewy bodies and α-synuclein

PD is characterized by an accumulation of misfolded protein aggregates, containing ubiquitin and the insoluble misfolded protein α-synuclein, in vulnerable neurons of the enteric, peripheral and central nervous system (Golbe, 1999). These inclusions were first described by Fritz Heinrich Lewy in 1912, and were termed "Lewy bodies" by Konstantin Nikolaevich Tretiakoff in 1919 (Goedert et al., 2012). Lewy bodies and Lewy neurites (when inclusions appear in neuronal processes) do not appear during healthy ageing (Thal et al., 2004). In postmortem tissue from patients with PD or prodromal PD, Lewy bodies are found throughout the CNS as shown in Table 1.2. Lewy neurites are most numerous in the substantia nigra and in the CA2 and CA3 regions of the hippocampus in postmortem tissue (Love, 2005). Although Lewy bodies are the most studied pathological feature of PD, aggregated α-synuclein is also found in particulate form in cell bodies (Kuusisto et al., 2003). α-synuclein is enriched at presynaptic terminals, where it, under healthy conditions, promotes the assembly of the SNARE machinery required for vesicle fusion, and also appears to be protecting nerve terminals against injury (Chandra et al., 2005).

It is not known why α-synuclein misfolds and aggregates in PD, but several mechanisms, including dysfunctional protein folding and degradation have been proposed. The α-

Table 1.2 Symptomatology related to pathological stage of PD

Symptom	Related brain structures	Braak stage
Olfactory impairments	Olfactory bulb, Anterior olfactory nucleus	1 1
Constipation	Dorsal nucleus of the vagus	1
Depression, anxiety	Locus coeruleus Raphe nuclei Hippocampus	2 2 4
Sleep disorders	Locus coeruleus Pedunculopontine nucleus Basal forebrain	2 3 3
Motor functions	Substantia nigra	3
Apathy, impaired motivation	Ventral tegmental area Amygdala	3 3
Cognitive dysfunctions, dementia	Hippocampus Amygdala Nucleus basalis Frontal cortex	4 4 4 5

Adapted from (Braak et al., 2004; Braak et al., 2003; Dickson et al., 2009; Tolosa et al., 2007).

synuclein protein is normally unfolded, but depending on its environment it can adopt partially folded structures as well as monomeric and oligomeric alpha helix and beta sheet conformations, or different types of aggregates including amyloid-like fibrils (Uversky, 2003). Oxidation of dopamine has been shown to affect α-synuclein aggregation *in vitro* in a pH-dependent manner (Pham et al., 2009), which may explain the pronounced vulnerability of dopaminergic neurons in PD.

The role of α-synuclein in the pathological processes of PD has not yet been established. It has been suggested that α-synuclein inclusions can interfere with axonal transport (Saha et al., 2004) and interrupt neurotransmitter production by reducing the functional levels of the rate-limiting enzymes TH and ChAT (Beach et al., 2008; Dugger and Dickson, 2010). However, it is also known that nerve cells harbouring Lewy bodies or Lewy neurites can survive for decades, which has raised questions on the toxic role of α-synuclein inclusions in the pathology of PD. It has even been suggested that α-synuclein inclusions may have a neuroprotective role (Lee et al., 2006), although this view is generally considered controversial. α-synuclein overexpression experiments have demonstrated that dopaminergic neurons are more vulnerable to α-synuclein overexpression compared with other neurons, possibly due to the higher iron content and dopamine's ability to generate reactive oxygen species (ROS) which leads to the induction of oxidative stress, a process which, in PD, may be further aggravated by mitochondrial dysfunction (Xu et al., 2002). However, non-dopaminergic neurons are also known both to present with Lewy bodies and to degenerate in PD (Braak et al., 2003). The reduced vulnerability of dopaminergic neurons of the VTA in PD compared with dopaminergic neurons of the SNc is not known, but has been attributed to a comparatively higher expression of dopamine transporters and lower expression of VMAT2 in SNc neurons, which would render these particular neurons more susceptible to dopamine-mediated toxicity (Gainetdinov et al., 1998)

1.3.2 Pathological staging

The pathological process of PD progresses slowly, and there is extensive evidence that the neuronal damage appears before the onset of clinical symptoms and progresses for many years before fully manifested (Del Tredici et al., 2002). The topographic spread of the neuronal pathology in PD is characteristic and predictive, and, upon autopsy, PD can be classified as one of six neuropathological stages based on α-synuclein deposition, called Braak stages (Braak et al., 2003) (Table 1.2). During the first stage, lesions are found in the dorsal nucleus of the vagus nerve (the tenth cranial nerve projecting from medulla oblongata to the heart, lungs and gastrointestinal system) in the brain stem, and in the olfactory bulb. During stage 2, the Lewy pathology spreads through the medulla oblongata and pontine tegmentum. At stage 3, Lewy bodies spread to the substantia nigra and the amygdala, which correlates with the onset of the first motor symptoms, commonly rigidity, resting tremor or gait disturbances. At stage 4, the Lewy pathology has spread to the temporal cortex. During subsequent disease stages, 5 and 6, lesions reach the neocortex, which is paralleled by cognitive dysfunctions (Braak et al., 2004; Braak et al., 2003; Goedert et al., 2012).

1.3.2.1 The basal ganglia

The basal ganglia are a selection of nuclei that play a key role in the control of body movements. The first coherent model of the basal ganglia circuitry was developed starting in the middle of the 80s (Albin et al., 1989; DeLong, 1990; Penney and Young, 1986), and described how the basal ganglia integrated information from different brain regions and generated feedback signals to the cerebral cortex. Today it is known that the basal ganglia have many functions beyond merely being an integrational relay structure. The term "basal

ganglia" refers to all gray matter structures located at the base of the cerebral hemispheres, but it is commonly used only to describe the interconnected nuclei substantia nigra pars reticulata (SNr) and SNc, striatum (caudate and putamen), globus pallidus pars externa (GPe) and GPi, and the STN (Obeso et al., 2008). A simplified schematic drawing of the basal ganglia and their projection areas is presented in Figure 1.3. The striatum is the primary input nucleus of the basal ganglia. In humans and primates, the dorsal striatum consists of the caudate nucleus and putamen, separated by the internal capsule, whereas the ventral striatum consists of nucleus accumbens, the olfactory tubercle and islands of Calleja. In rodents the caudate nucleus and putamen are not visually separated, but the neuronal subpopulations are still organized into different functional compartments (Zeiss, 2005). The striatum consists of medium spiny neurons (MSN), representing nearly 95% of all striatal neurons (Kreitzer and Malenka, 2008), which either express stimulatory dopamine D_1 receptors or inhibitory dopamine D_2 receptors. A small proportion of MSN express both receptor types (Bertran-Gonzalez et al., 2010). MSN provide inhibitory control by releasing γ-aminobutyric acid (GABA) onto the regions that they innervate. The striatum receives dopaminergic innervation from the SNc, and glutamatergic innervation from striatum and thalamus, which project onto the MSN. Dopamine release will activate dopaminergic receptors expressed on MSN, resulting in activation of the "direct" motor pathway (MSN expressing D_1 receptors) projecting to GPi/SNr, or blockade of the "indirect" motor pathway (MSN expressing D_2 receptors) projecting to GPe, which in turn inhibits the STN. The STN in turn sends glutamatergic projections to GPi/SNr, the output region of the basal ganglia, which in turn send inhibitory GABAergic projections to thalamus. Activation of D_1 receptors of the direct pathway leads to a net reduction of inhibition of the thalamus and initiation of movement. Activation of inhibitory D_2 receptors of the indirect pathway leads to inhibition of thalamus and inhibition of movements. Under healthy conditions there is a very delicate balance between the activation of the direct and indirect basal ganglia pathways. PD is pathologically

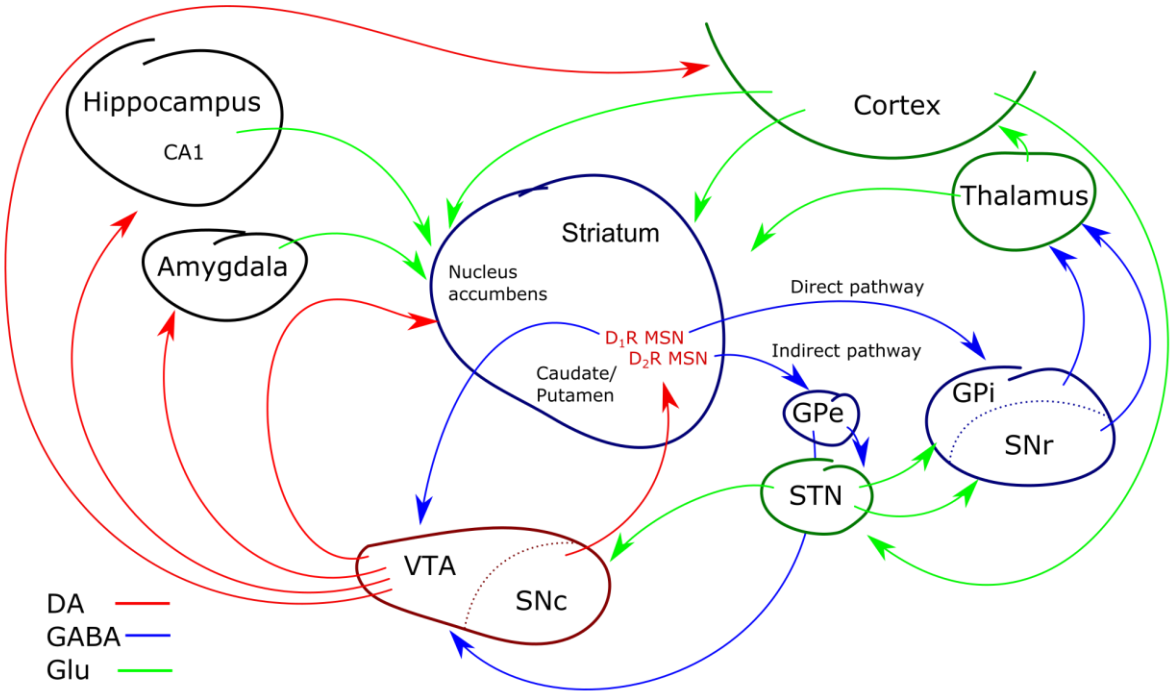


Figure 1.3 A simplified schematic drawing of the basal ganglia, including the nigrostriatal, corticostriatal, mesolimbic, mesocortical and mesoamygdaloid pathways. DA: dopamine; Glu: glutamate. Adapted from (Russo and Nestler, 2013).

characterized by a loss of dopaminergic neurons originating in the SNc projecting to the striatum, leading to an imbalance between the direct and indirect pathways and thus between the initiation and inhibition of movement, ultimately resulting in the cardinal motor symptoms of PD (Obeso et al., 2008; Wichmann and DeLong, 1998). PD is also characterized by a disinhibition of the glutamatergic STN, which is thought to contribute to the progressive neurodegeneration due to the excitotoxic properties of glutamate. The striatum also contains interneurons, the most prominent being the tonically active cholinergic interneurons which inhibit cortical glutamatergic output, and GABAergic fast-spiking interneurons which mediate intrastriatal feed-forward inhibition (Obeso and Lanciego, 2011). The basal ganglia also receive input from sub-cortical nuclei, including serotonergic innervation from the raphe nuclei, and noradrenergic innervation from locus coeruleus (McHaffie et al., 2005).

Today it is established that the basal ganglia not only mediate the control of movement, but also motor learning, working memory and executive functions. It is also believed that the VTA plays a role in the non-motor symptoms of PD. Dopaminergic neurons of the VTA degenerate to between 36-55% in patients with PD (Uhl et al., 1985), meaning that they are relatively spared compared with dopaminergic neurons of the SNc. The VTA is a heterogeneous structure consisting of subpopulations of dopaminergic neurons with discrete projections and different electrophysiological properties (Roeper, 2013). For instance, the anterior and posterior VTA play different functional roles with regards to behavioural functions (Sanchez-Catalan et al., 2014). Apart from the well-studied dopaminergic neurons, the VTA also harbours GABAergic and glutamatergic neurons, as well as neurons that co-release dopamine and glutamate, which create different functional outputs (Walsh and Han, 2014). Recently a cluster of mainly GABAergic cells were identified, termed the tVTA, which form synapses with cells of the VTA and SNc and thus possibly can control the activity of dopaminergic neurons (Sanchez-Catalan et al., 2014). The projection from the VTA to the nucleus accumbens, a part of ventral striatum, compromises a well-characterized reward circuitry of the brain, and is critical for the recognition of rewards and for the initiation of their consumption, thus playing a major role in addictive disorders (Koob and Le Moal, 2008). This circuitry is also involved in the recognition of aversive stimuli. The VTA also sends dopaminergic projections to basolateral amygdala, the CA1 region of the hippocampus and the prefrontal cortex (Russo and Nestler, 2013). These interconnected regions play key roles in reward, learning and memory functions, and receive serotonergic input from midbrain raphe nuclei and noradrenergic input from locus coeruleus (Russo and Nestler, 2013). These regions are also proposed to play roles in mood disorders (Drevets et al., 1992; Fu et al., 2004; Mayberg et al., 2000; McEwen, 1999; Sheline et al., 2001; Siegle et al., 2007).

1.3.3 Non-dopaminergic neurotransmitter systems in PD

PD does not exclusively affect the dopaminergic system, but is today considered a multisystem disorder. There is growing evidence that a non-dopaminergic pathological process underlies the non-motor symptoms of PD, and that this process may precede the degeneration of the dopaminergic system, as demonstrated in Table 1.2. The dopaminergic neurons projecting from the SNc to the striatum receive modulatory input from several neurotransmitter systems including the glutamatergic, cholinergic, serotonergic and noradrenergic systems, both at the cell bodies and at the terminals where these receptor types are expressed. Moreover, the GABAergic MSNs of the striatum express acetylcholine, glutamate, serotonin, adenosine, opiate and cannabinoid receptors on both cell bodies and terminals (Jenner, 2015). Nicotinic cholinergic receptors of the α4β2 and α6β2 subtypes are expressed on striatal cholinergic interneurons where they play a key role in regulating striatal activity, possibly playing a role in the paradoxical protective effect of tobacco

smoking in PD (discussed further in 1.3.5.2). Corticostriatal glutamate release is essential for voluntary movement, and may also exert neurotoxic effects when dysregulated, making it a promising target for therapeutic interventions in PD. Another glutamatergic region is the STN, which sends glutamatergic innervations to several regions of the basal ganglia, and in turn is innervated by GABAergic neurons. The work of this thesis has focused on the role of the glutamatergic system and trace-amine associated receptor 1 in PD, which are further elaborated upon below.

1.3.3.1 The glutamatergic system in Parkinson's disease

Glutamatergic neurotransmission plays a crucial role in cognitive processes such as learning and memory, and alterations in glutamatergic neurotransmission has been implicated in non-motor symptoms associated with PD, particularly cognitive dysfunctions (Gravius et al., 2010; Hsieh et al., 2012). Consistently, the antiglutamatergic drug memantine, an N-methyl D-aspartate (NMDA) receptor antagonist, is used in the clinic to alleviate cognitive impairments and dementia in PD (Aarsland et al., 2009; Litvinenko et al., 2010).

The general role of glutamate in the basal ganglia is to alter the excitability of neurons; to hyperpolarize or depolarize neurons (Conn et al., 2005). The striatum is the principal input structure of the basal ganglia, and receives excitatory glutamatergic input from neocortex and thalamus (McGeer et al., 1977; Smith and Villalba, 2008), as shown schematically in Figure 1.3. The striatum also receives extensive dopaminergic innervation from SNc, making it a key region for integration of dopaminergic and glutamatergic input (Bouyer et al., 1984). Excitotoxic damage caused by excess glutamate has been proposed as a mechanism contributing to neurodegeneration (Rodriguez et al., 1998). The STN is glutamatergic and projects to the substantia nigra, including the SNc, where it is in a position to exert nigral excitotoxic cell damage (Rodriguez et al., 1998). Moreover, degeneration of nigrostriatal dopaminergic neurons leads to excess spontaneous glutamatergic corticostriatal activity, which has been demonstrated in the unilaterally 6-hydroxydopamine (6-OHDA) lesioned rat model using electrophysiology (e.g. (Gubellini et al., 2002)). Although the modulation of corticostriatal glutamatergic transmission by D_2-like receptors has been demonstrated previously by electrophysiology in slice preparations (e.g. (Cepeda et al., 1993), the

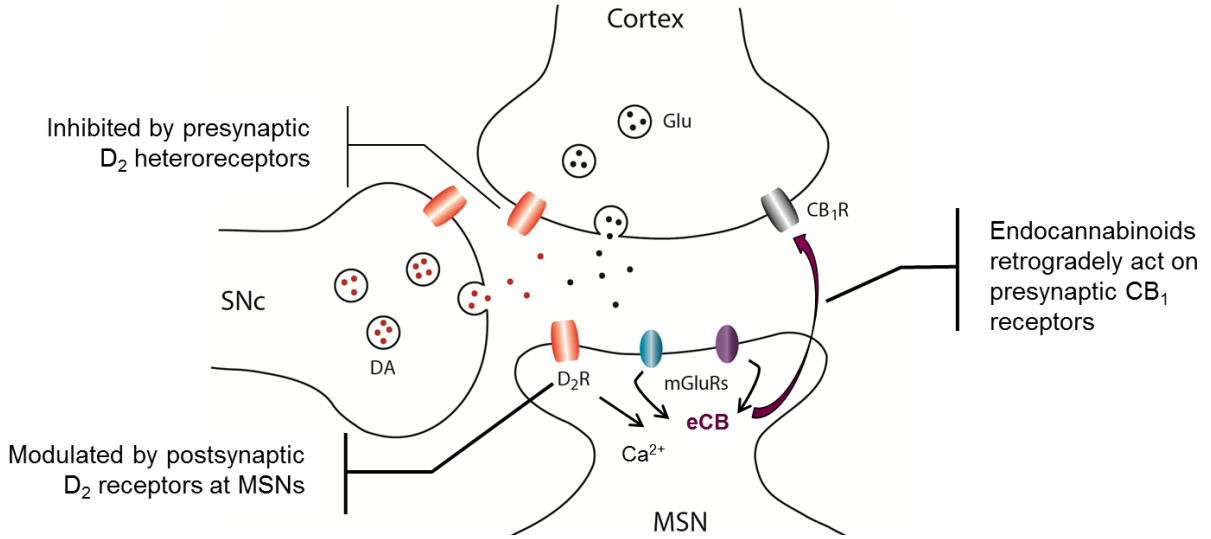


Figure 1.4 The modulation of corticostriatal glutamate release. A simplified schematic drawing of a corticostriatal synapse, indicating the potential roles of dopamine D_2 receptors and retrograde endocannabinoid release in the inhibition of glutamate release. CB_1R: cannabinoid receptor type 1; DA: dopamine; eCB: endocannabinoids; Glu: glutamate; mGluR: metabotropic glutamate receptor.

underlying mechanism remains controversial. One interpretation is that activation of presynaptic D_2 heteroreceptors at corticostriatal terminals suppresses synaptic transmission (Wang and Pickel, 2002). On the other hand, there is support for the role of the endocannabinoid (eCB) system in modulating glutamate release from cortical afferents projecting to striatum, possibly via D_2 receptor induction (Mathur and Lovinger, 2012) (Figure 1.4). This mechanism has been explored further in study II.

1.3.3.2 Trace amines and trace amine-associated receptor 1

Endogenous trace amines, which are structurally related to monoamines and exist in low (0.1-10 nM) concentrations throughout the mammalian brain, have in recent years emerged as potential modulators of dopaminergic functions (Branchek and Blackburn, 2003). Altered trace amine concentrations have been associated with neuropathological disorders related to the monoaminergic systems, including schizophrenia, depression and PD (Branchek and Blackburn, 2003). However, the functional role of trace amines remained elusive until 2001, when a family of receptors activated by trace amines, termed "trace amine-associated receptors", were discovered (Bunzow et al., 2001; Borowsky et al., 2001).

Trace amine-associated receptor 1 (TAAR1) has been identified as a modulator of many functions related to the monoaminergic systems, and increasing evidence suggest that TAAR1 can play a role in disorders related to monoaminergic systems, including PD. TAAR1 is expressed in monoaminergic systems of the brain, including the VTA and substantia nigra (Bunzow et al., 2001; Di Cara et al., 2011; Miller et al., 2005; Xie et al., 2007). Previous *in vitro* experiments by Xie and Miller (Xie and Miller, 2009, 2008, 2007), using cell cultures and striatal synaptosomes, reported that TAAR1 agonism inhibited dopamine reuptake by the dopamine transporter (DAT), caused internalization of DAT, and reversed DAT function resulting in increased dopamine efflux, subsequently causing an increase in extracellular dopamine levels. There is convincing data that TAAR1 and D_2 receptors are highly co-expressed in dopaminergic neurons of the substantia nigra (Borowsky et al., 2001; Di Cara et al., 2011), where they may interact by forming heterodimers (Espinoza et al., 2011). This indicates that TAAR1 can modulate dopaminergic functions by interacting with several components of dopaminergic signaling.

Agonists at TAAR1 have been proposed as potential means to treat neuropsychiatric disorders characterized by enhanced dopaminergic neurotransmission (Revel et al., 2013, 2011). TAAR1 is activated by a wide range of endogenous ligands, including trace amines, biogenic amines including dopamine and dopamine analogs, 3-methylated metabolites of catecholamines, and by synthetic substances including amphetamine derivatives and ergolines (Bunzow et al., 2001). None of these compounds are selective to TAAR1, which hampered the design and interpretation of mechanistic studies before the development of the first specific TAAR1 antagonist EPPTB (Bradaia et al., 2009) and agonist RO5166017 (Revel et al., 2011). Previous experiments in wild-type (WT) animals showed no behavioural effects following oral administration of RO5166017 except for a minor decrease in locomotor activity (Revel et al., 2011). A high dose of RO5166017 also elicited a mild anxiolytic effect in the stress-induced hyperthermia paradigm, and prevented cocaine-induced hyperactivity and stereotypies (Revel et al., 2011). None of these effects were present in TAAR1 knock-out (KO) mice, suggesting mediation via TAAR1. RO5166017 administered intraperitoneally (i.p.) reduced locomotor activity in mice lacking DAT, a rodent model characterized by spontaneous hyperactivity, but failed to do so in mice that lacked both DAT and TAAR1 (Revel et al., 2011), suggesting that this effect was mediated by TAAR1 independently of DAT. RO5166017 also blocked hyperlocomotion induced by an NMDA-receptor antagonist, an effect abolished in TAAR1 KO animals, suggesting that TAAR1 activation may mediate an antipsychotic effect (Revel et al., 2011). Also, the full TAAR1 agonist RO5256390 and the

partial agonist RO5263397 were found to mediate antipsychotic-like, pro-cognitive and anti-depressant effects in rodents and primates (Revel et al, 2013), further supporting TAAR1 as a potential target for treatment of negative and cognitive symptoms of schizophrenia.

Another factor that has hampered studies of the functions of TAAR1 is that the TAAR1 antagonist EPPTB does not readily cross the blood-brain barrier. Although TAAR1 KO animals have been developed, it is possible that a congenital deletion of TAAR1 leads to developmental adaptations, which would otherwise be circumvented by the use of a selective antagonist. Previous experiments in mice, where the TAAR1 gene was targeted, showed that TAAR1 KO mice did not display any genotype-related differences regarding their general state of health, motor functions, overall activity or anxiety-like behaviour compared to control animals, apart from a deficit in the prepulse inhibition test (Lindemann et al., 2008; Wolinsky et al., 2007), suggesting a schizophrenia-like phenotype. Moreover, TAAR1 KO animals expressed a 262% higher level of high-affinity D_2 receptors in the striatum compared with WT mice (Wolinsky et al., 2007), as well as a higher sensitivity towards amphetamine. The latter was manifested as enhanced amphetamine-induced extracellular dopamine and noradrenaline concentrations in the striatum as measured by microdialysis, and higher locomotor activity in the open field test compared with control animals (Achat-Mendes et al., 2012; Lindemann et al., 2008; Wolinsky et al., 2007). Slice experiments in the VTA of TAAR1 KO mice revealed enhanced spontaneous firing of dopaminergic neurons in KO compared with WT animals, suggesting that TAAR1 mediates a dampening effect on the activity of dopaminergic neurons upon activation (Lindemann et al., 2008), which is supported by slice experiments using specific ligands (Bradaia et al., 2009; Revel et al., 2011). However, the enhanced dopamine neuron firing rate observed in TAAR1 KO animals did not enhance basal levels of extracellular dopamine as detectable by microdialysis (Lindemann et al., 2008). It is highly likely that the enhanced potency of antipsychotic drugs in TAAR1 KO animals is related to the elevated proportion of high-affinity D_2 receptors in this model (Wolinsky et al., 2007), which in turn may be a developmental adaptation induced by a congenital absence of TAAR1. Paradoxically, mice overexpressing TAAR1 did not display any basal behavioural phenotype, but displayed elevated concentrations of dopamine and noradrenaline in nucleus accumbens, and an increased firing rate of dopaminergic neurons in the VTA (Revel et al., 2012). These mice were also less sensitive to amphetamine.

Changes in dopamine release and dopamine receptor binding is implicated in the behavioural sensitization of psychostimulants (Steketee and Kalivas, 2011). Amphetamine, metamphetamine and 3,4-methylenedioxymethamphetamine (MDMA) are agonists of TAAR1, suggesting that TAAR1 may mediate the reinforcing effects of these compounds (Achat-Mendes et al., 2012; Bunzow et al., 2001; Di Cara et al., 2011). Indeed, previous work demonstrated that TAAR1 KO mice exhibit more psychomotor effects compared with WT mice in response to amphetamine and metamphetamine respectively, and increased striatal release of biogenic amines compared with WT mice in response to amphetamine (Achat-Mendes et al., 2012; Lindemann et al., 2008; Wolinsky et al., 2007). *In vitro* experiments revealed that metamphetamine enhances dopamine efflux from cells via a TAAR1-dependent mechanism (Xie and Miller, 2009). Moreover, a partial and a full TAAR1 agonist each suppressed cocaine-seeking after withdrawal from chronic cocaine self-administration, suggesting TAAR1 as a potential target for the treatment of cocaine addiction (Pei et al., 2014).

These data from previous studies highlight a multifaceted role of TAAR1 in a range of functions related to the dopaminergic system. In study II, we have on further delineating the role of TAAR1 in experimental PD.

1.3.4 Pathogenesis of Parkinson's disease

Several mechanisms contributing to cell death have been proposed in PD. However, the reason for the vulnerability of some cell types and resistance of other cell types in PD is not adequately understood. The mechanisms most relevant to this thesis are described below.

1.3.4.1 Mitochondrial dysfunction and oxidative stress

Defective mitochondrial function, accompanied by increased oxidative stress, has been shown to play an important role in the pathogenesis of PD (Schapira, 2008). The normal function of mitochondria involves the production of energy in the form of ATP via the mitochondrial respiratory chain, as well as the regulation of cell death, calcium metabolism and the production of ROS. The mitochondrial respiratory chain, also called the electron transport chain, consists of enzymatic complexes I-IV, which are embedded in the inner mitochondrial membrane. Complexes I-IV catalyse redox reactions where electrons are transferred from a donor, produced by the Krebs cycle in the matrix of the mitochondrion, to an acceptor molecule (Mitchell, 2011). These redox reactions create an electrochemical gradient through the inner mitochondrial membrane which drives the synthesis of ATP (Mitchell, 2011). Mitochondria are also a main source of ROS and reactive nitrogen species (RNS), produced by their multiple electron carriers, but they also normally harbour extensive antioxidant defence mechanisms to prevent harmful oxidative stress (Lin and Beal, 2006). Mitochondria damage, induced by ROS for instance, may result in an imbalance between ROS generation and removal, and subsequently to enhanced ROS levels (Andreyev et al., 2005). This in turn would increase the amount of cell damage and misfolded proteins, thus increasing the demand for protein removal by the ubiquitin-proteasome system. Complex I deficiency has been described in postmortem brain samples from patients suffering from PD (Schapira et al., 1989). It should be noted that many of the genes associated with PD have been shown to affect mitochondrial functions.

The brain is the most metabolically active organ of the body, and thus its internal iron concentration is very high (Hare et al., 2015). Superoxide and H_2O_2 are intermediates of oxygen reduction and potent oxidants in the presence of iron. When reacting with ferrous iron, Fe^{2+}, they will form hydroxyl radicals, which can cause cytotoxicity by lipid peroxidation. The production of hydroxyl radicals is normally handled by specific antioxidant defense mechanisms. Neurons of the SNc contain neuromelanin, a byproduct of dopamine synthesis which facilitates iron accumulation (Sulzer et al., 2000). Although neuromelanin has been proposed to increase cellular vulnerability of PD, it should be noted that the more resistant VTA neurons contain similar amounts of neuromelanin as neurons of the SNc (Dias et al., 2013). Iron-mediated oxidative stress is prevented by sequestration of free iron by the cytosolic protein ferritin, which can store up to 4500 excess iron atoms that are released when required to prevent free iron from reaching high concentrations (Rouault, 2013). Ferritin also catalyzes the oxidation of ferrous iron (Fe^{2+}) to the more stable form ferric iron (Fe^{3+}) upon storage. Ageing is associated with enhanced accumulation of iron, which happens specifically in naturally iron-rich regions, including the basal ganglia and cortex, probably by mechanisms including increased ferritin levels and increased activity of haem oxygenase-1, which releases free iron by degrading haem. Several studies have reported that mechanisms facilitating iron transport from the SNc are deficient in PD (Jin et al., 2011; Lei et al., 2012). Consistently, iron accumulation is higher in the neurons of the SNc compared with other cell types. Early-life iron exposure has been proposed to be a contributing factor to PD in later life, although the impact of dietary iron on the risk of developing PD remains controversial (Hare et al., 2015).

Mitochondria are believed to contribute to ageing via the accumulation of mitochondrial DNA mutations (Corral-Debrinski et al., 1992) and the production of ROS. Ageing is a prominent risk factor not only for PD, but also for other neurodegenerative disorders including Alzheimer's disease and ALS. Indeed, oxidative damaged has been implicated in age-induced cognitive decline in humans (Lu et al., 2004). Moreover, enhancement of the natural antioxidant defence of mitochondria has been reported to promote longevity in *drosophila* (Ruan et al., 2002; Sun et al., 2002) and mice (Schriner, 2005).

Since mitochondrial dysfunction has been strongly implicated in the pathogenesis of PD, drugs that improve mitochondrial functions may be neuroprotective in PD. However, clinical studies using vitamin E with coenzyme Q10, an electron carrier for mitochondrial complexes I and II of the electron transport chain and a scavenger of free radicals, and creatinine, were terminated prematurely due to lack of effect in early PD (Kalia et al., 2015). One complication of using ROS scavengers in the treatment of neurodegenerative disorders is that they impair autophagy, which in itself may be useful in the treatment of neurodegenerative disorders (Metcalf et al., 2012), as discussed below.

1.3.4.2 Dysfunctional protein folding and degradation

Many proteins must fold into a specific three-dimensional structure in order to gain functional activity, and misfolding may generate protein aggregation or the generation of toxic species (Hartl et al., 2011). α-synuclein aggregation has been associated with dysfunctional proteostasis, i.e. of the cellular machineries that normally handle protein folding and the degradation of dysfunctional proteins. This includes the chaperone systems that regulate protein folding and the refolding of misfolded proteins (Hartl et al., 2011), the ubiquitin-proteasomal pathway as well as the autophagy-lysosomal pathway, which are responsible for the elimination of toxic proteins (Tyedmers et al., 2010).

Protein degradation via the ubiquitin-proteasomal pathway is the most common mechanism to degrade proteins in the mammalian cytosol and nucleus (Ciechanover et al., 1980; Hershko et al., 1983, 1982). This mechanism requires two steps. During the first step, termed “conjugation”, multiple ubiquitin molecules are covalently attached to the protein destined for degradation. During the second step, termed “degradation”, the ubiquitin-tagged protein is degraded by the 26S proteasome. Ubiquitin is often a constituent of Lewy bodies, which has led to the common hypothesis that dysfunctions of the ubiquitin-proteasomal pathway may contribute to PD. It should be noted that ubiquitin is also associated with other functions besides proteasomal targeting, including the autophagic-lysosomal system (Matsuda and Tanaka, 2010).

The autophagic-lysosomal pathway plays an important function in neurons by removing aggregation-prone proteins. Autophagy is the catabolic mechanism by which cells degrade dysfunctional and redundant cellular components via lysosomes. This process allows recycling of larger cellular components, removal of toxic aggregates, and promotes cellular survival by maintaining energy levels during starving (Metcalf et al., 2012). There are three described forms of autophagy: macroautophagy; microautophagy and chaperone-mediated autophagy. Macroautophagy is the main autophagy pathway and used to remove damaged organelles and redundant proteins. Cytoplasmic components targeted for degradation are isolated within double-membraned vesicles called autophagosomes, which then mature by fusing with endosomes and lysosomes to induce degradation via acidic lysosomal hydrolases and recycling of the contents of the autolysosome (Eskelinen, 2008). During microautophagy, cytoplasmic contents are taken up directly by the lysosome via inward folding of the lysosomal membrane (Mijaljica et al., 2011). Chaperone-mediated autophagy is a complex mechanism that specifically targets cytosolic proteins marked with a

recognition site for an hsc70-containing complex. This pathway allows proteins to bind a chaperone, after which they are translocated via the lysosome membrane for degradation. Autophagy normally plays a part in programmed cell death, in the degradation of infectious particles, and in cellular repair mechanisms. Failure of autophagy to degrade damaged proteins and organelles has been implicated in the accumulation of cell damage, aging and neurodegenerative disorders, including both PD and Alzheimer's disease (Cuervo et al., 2005; Menzies et al., 2015; Rajawat et al., 2009).

There are indications of relationships between abnormal protein accumulation and degradation, oxidative stress, and mitochondrial dysfunction. For instance, α-synuclein overexpression in mice treated with the neurotoxin MPTP has been shown to impair mitochondrial function, enhance oxidative stress and aggravate MPTP-induced nigral neurodegeneration, indicating an interaction between α-synuclein and MPTP *in vivo* (Song et al., 2004). It has been proposed that α-synuclein may interact with mitochondria by interacting with their outer membranes, by being transported into mitochondria via protein transportation mechanisms, or by impairing the transcription of nuclear encoded genes required for mitochondrial function (Beal, 2004). Moreover, rotenone, a complex I inhibitor used to induce experimental Parkinsonism in rodents, increases the generation of α-synuclein aggregates, an effect which was abolished following the recovery of mitochondrial metabolism (Lee et al., 2002). The clearance of damaged mitochondria via autophagy is partly mediated by PARKIN and PINK1, two genes implicated in PD (Durcan and Fon, 2015).

1.3.4.3 Neuroinflammation

Chronic inflammation is a major characteristic of PD, and patients display increased levels of pro-inflammatory molecules including TNF-α, iNOS and IL-1β both in cerebrospinal fluid and postmortem brain (Dobbs et al., 1999; Mogi et al., 1994; Reale et al., 2009). Today, there is increasing evidence that the immune system, especially glial cells, plays a role in PD also during early disease stages, indicating that this inflammatory response may not be triggered by the neurodegenerative process (Herrero et al., 2015). It is known that microglial cells, which play an important neuroprotective role by maintaining the neuronal environment and scavenging the CNS for pathogens, are activated during the neurodegenerative process in PD (Sanchez-Guajardo et al., 2015). Microglial changes, termed "microgliosis", have commonly been described in patients with PD and in animal models, and appear in regions characterized by α-synuclein pathology. In animal models of PD recapitulating α-synuclein pathology, microgliosis appeared before neuronal death (Sanchez-Guajardo et al., 2015).

Genome-wide analysis studies have revealed a number of susceptibility loci related both to innate and adaptive immune functions associated with an increased risk of developing PD (Herrero et al., 2015). Moreover, several genes associated with familial and sporadic PD, including LRRK2, SNCA, PARK2, PARK7 and PLA2G6 are expressed in microglia and astrocytes, which suggests a role of these genes in neuroinflammation (Russo et al., 2014). Interestingly, prolonged use of non-steroidal anti-inflammatory drugs, and of ibuprofen in particular, may reduce the risk of developing PD (Rees et al., 2011). Ageing is also known to affect the immune system *per se*, and advancing age has been specifically associated with chronic mild inflammation of the SNc, which may render these dopaminergic neurons more vulnerable to neurodegeneration in PD (Kanaan et al., 2010).

1.3.4.4 Excitotoxic cell damage

The excitatory neurotransmitter glutamate plays an important role in the normal basal ganglia circuitry, and neurodegeneration of dopaminergic nigral neurons results in altered

dynamics throughout the basal ganglia, as explained further in 1.3.3.1. This imbalance may accelerate neuronal degeneration due to excitotoxic processes (Blandini et al., 2000). This also means that glutamate release may provide a target for neuroprotective treatments. However, due to rapid release and reuptake mechanisms, glutamatergic neurotransmission has been technically difficult to measure *in vivo* using conventional methods. Thus, previous *in vivo* microdialysis studies of striatal tonic glutamate concentrations following neurotoxin-induced dopamine denervation have been inconsistent; reporting enhanced (Meshul et al., 1999), unaltered (Corsi et al., 2003; Robelet et al., 2004), or decreased (Reid et al., 1990) glutamate concentrations following lesioning. These inconsistent results may be related to methodological differences, including sampling rate and size of the probe, species used, anesthetic agent used, and time span elapsed between lesion and recording, the latter which may affect the level of compensatory regeneration of multiple neurotransmitter systems. Therefore, in study II we used an enzyme-based microelectrode array that allowed detection of low glutamate levels on a subsecond time scale, in order to monitor the fast temporal fluctuations of glutamatergic neurotransmission in anaesthetized PD models (Hascup et al., 2008).

1.3.5 Aetiology of Parkinson's disease

The cause of sporadic PD is unknown, but is generally believed to be caused by a combination of factors, including environmental exposures and gene-environment interactions.

1.3.5.1 Genetic factors

Genetic factors have been linked to around 10% of all PD cases, which are commonly cases of early-onset PD. Mutations in up to 18 chromosomal regions, termed PARK1-18, have been associated with PD (Klein and Westenberger, 2012). Six of these genes are known to cause monogenic, familial PD: SNCA (PARK1 and 4), LRRK2 (PARK8), Parkin (PARK2), DJ-1(PARK7), PINK1 (PARK6) and ATP13A2 (PARK9) (Klein and Westenberger, 2012). SNCA encodes α-synuclein, a constituent of Lewy bodies both in familial and sporadic PD (Dehay et al., 2015). Three missense mutations (A53T, A30P and E46K), duplications and triplications of the SNCA gene are known to cause PD (Bekris et al., 2010). LRRK2 is a tyrosine kinase-like protein, which has been suggested to be involved in neuroinflammation (Russo et al., 2014). It is present in the cytoplasm and the mitochondrial outer membrane (Ito et al., 2007). PARK2 encodes the protein Parkin, which is localized in the cytosol and Golgi apparatus (Shimura et al., 1999). Parkin can also localize to mitochondria, where it has been proposed to modulate the transcription and replication of mitochondrial DNA in proliferating cells (Kuroda et al., 2006), to protect mitochondrial DNA from oxidative damage (Rothfuss et al., 2009), to modulate the activity of mitochondrial complex 1 and the production rate of ROS (Kuroda et al., 2006), and to play a role in targeting proteins for proteasomal degradation (Lonskaya et al., 2013). PARK7 encodes the protein DJ-1, a cytoplasmic protein that can translocate to the mitochondria (Zhang et al., 2005). It has been proposed to harbour antioxidant properties (Abou-Sleiman et al., 2003; Canet-Avilés et al., 2004), to scavenge ROS via auto-oxidation (Taira et al., 2004), and to prevent aggregation of α-synuclein (Batelli et al., 2008). PINK1 encodes a mitochondrial protein of unknown function expressed both in the brain and in systemic organs, which is proposed to protect from stress-induced mitochondrial dysfunction (Silvestri et al., 2005). ATP13A2 is a lysosomal membrane protein with an ATPase domain, and its function is currently unknown (Ramirez et al., 2006). Common variation in 3 genes, LRRK2, SNCA and microtubule-associated protein tau (MAPT), and loss-of-function mutations in the glucocerebrosidase gene (GBA) have also been shown to be susceptibility factors for PD (Bonifati, 2014).

Table 1.3. Genes associated with monogenic PD

Gene (also known as)	Official full name	Function of protein in PD
SNCA (PD1; NACP; PARK1; PARK4)	Synuclein, alpha (non A4 component of amyloid precursor)	Constituent of Lewy bodies
LRRK2 (PARK8; RIPK7; ROCO2; AURA17; DARDARIN)	Leucine-rich repeat kinase 2	Associated with mitochondria Neuroinflammation?
PARK2 (PRKN; PDJ; AR-JP; LPRS2)	Parkin RBR E3 ubiquitin protein ligase	Modulates mitochondrial functions Proteasomal degradation
PARK7 (DJ-1; HEL-S-67p)	Parkinson protein 7	Antioxidant ROS scavenger
PINK1 (BRPK; PARK6)	PTEN induced putative kinase 1	Unknown Associated with mitochondria
ATP13A2 (CLN12; KRPPD; PARK9; HSA9947)	ATPase type 13A2	Unknown

1.3.5.2 Environmental factors

Several lifestyle factors have been associated with a decreased risk for developing PD, including smoking and coffee consumption (Derkinderen et al., 2014). A meta-analysis demonstrated strikingly that PD was 60% lower among cigarette smokers compared with never smokers, and 30% lower in coffee drinkers compared with non-coffee drinkers.

The mechanism underlying the protective effect of smoking is not known, but one proposed mechanism is that tobacco smoke can inhibit the uptake of neurotoxins by inhibiting MAO-B and/or by binding to nicotinic receptors, thus competitively inhibiting potential neurotoxins (Morens et al., 1995). Nicotinic cholinergic receptors of the α4β2 and α6β2 subtypes are expressed on striatal cholinergic interneurons, where they play a key role in regulating striatal activity and dopamine release (Quik et al., 2009). Nicotinic acetylcholine receptors have been shown to exert neuroprotective and antidyskinetic effects in experimental animal models of PD, and, in particular, α7 nicotinic acetylcholine receptors have been proposed as therapeutic targets in the management of PD (Quik et al., 2015). However, a study in 8 patients with early-onset Parkinsonism on the effects on nicotine chewing gum showed improved symptoms after taking nicotine chewing gum, but only in patients who were smokers (Mitsuoka et al., 2002). A short-term trial on the effects of nicotine chewing gum in 46 patients with PD showed no effects on PD symptoms (Clemens et al., 1995)

As for coffee, this protective effect may be mediated via caffeine, since caffeine from non-coffee sources, but not decaffeinated coffee, has also been shown to be inversely associated with the risk of developing PD (Ascherio et al., 2001). Caffeine has been shown to down-regulate neuroinflammatory responses and nitric oxide production (Yadav et al., 2012), thus potentially preventing cell death. It has also been shown that in the human

brain, at doses normally consumed, caffeine increases the levels of dopamine D_2 and D_3 receptors in the striatum, which may account for the stimulating effect of caffeine (Volkow et al., 2015). Caffeine is an adenosine receptor antagonist which binds to all types of adenosine receptors (Ribeiro and Sebastio, 2010). Adenosine receptors of the A_2A subtype have been shown to reduce the postsynaptic effects of dopamine depletion, resulting in alleviation of the motor symptoms of PD (Schwarzschild et al., 2006). In the striatum, A_2A receptors colocalize with D_2 receptors and form A_2A-D_2 receptor heteromers, whereby they elicit opposite actions on neuronal adenylate cyclase and cAMP production (Ferré et al., 2007). Thus, A_2A receptors inhibit D_2 receptor signalling upon activation, whereas A_2A blockade enhances D_2 receptor signalling (Pollack and Fink, 1995). A_2A receptor blockade has been shown in both preclinical and clinical studies to enhance the effect of L-DOPA treatment and to alleviate L-DOPA-induced dyskinesias (Dungo and Deeks, 2013). The selective A_2A receptor antagonist istradefylline was recently approved for adjunctive treatment of PD in Japan (Dungo and Deeks, 2013), specifically used to reduce "off" time during L-DOPA treatment (LeWitt et al., 2008). In view of the recent hypothesis that PD starts in the gut (discussed in section 1.3.6), it is also interesting to note that both coffee consumption (Jaquet et al., 2009; Nakayama and Oishi, 2013) and smoking cessation (Biedermann et al., 2013) have been shown to affect the human intestinal microbiota.

Occupational risk factors, including welding and exposure to heavy metals including aluminium, amalgam, copper, iron, lead, manganese or zinc have been proposed to increase the risk of PD, but no conclusive evidence has been found (Lai et al., 2002). Increased iron levels have been found in the substantia nigra of PD patients, where it may contribute to increased formation of free radicals. As mentioned in 1.3.4.1, the effects of dietary iron on the risk of developing PD remains inconclusive. Another occupational risk factor is exposure to pesticides, which will be discussed in part 1.4 since several successful animal models are based on this association.

Type 2 diabetes mellitus, a metabolic disorder associated with several psychiatric and neurodegenerative disorders (Kalaria, 2009; Llorente and Urrutia, 2006), has also been shown to be associated with an increased risk of developing PD, independently of coffee consumption, smoking or body weight (Hu et al., 2007). Diabetes may also be a risk factor for drug-induced Parkinsonism (Ma et al., 2009). Moreover, PD patients suffering from diabetes mellitus suffer from more severe cognitive impairments compared with non-diabetic PD patients (Bohnen et al., 2014). Chronic inflammation, impaired mitochondrial function and oxidative stress have also been proposed in the pathogenesis of type 2 diabetes, and a shared pathophysiology with PD has been proposed since the 1960s, but remains controversial (Lima et al., 2014). The role of insulin in the CNS is not yet well understood. However, insulin receptors are expressed throughout the CNS, including the VTA, SNr, hippocampus, amygdala and olfactory regions (Unger et al., 1991a, 1991b), where they appear to modulate a vast array of functions ranging from feeding behaviour and energy storage to cognitive functions (Bloemer et al., 2014; Filippi et al., 2013; Sandoval et al., 2009). Patients with PD have reduced levels of insulin receptors in the basal ganglia, which correlates with the loss of tyrosine hydroxylase (TH) mRNA (Moroo et al., 1994). Moreover, dopamine depletion has been shown to influence insulin levels and increase insulin resistance in experimental models of PD (Morris et al., 2011, 2008). Dopaminergic drugs, including L-DOPA and dopamine agonists, also influence insulin production, insulin resistance and glycaemic control (Sirtori et al., 1972). Interestingly, recent preclinical and clinical studies have shown that drugs developed for the treatment of diabetes, including analogues of incretin peptides and a GLP-1 receptor agonist, may have neuroprotective effects both in PD and in Alzheimer's disease (Hölscher, 2014; 2012).

1.3.6 Parkinson's disease and the gut

Gastrointestinal dysfunction has, during the past 30 years, been recognized as a frequent and debilitating non-motor symptom of PD (Pfeiffer, 2011; 2003). Notably, gastrointestinal complications may arise 12-20 years before the onset of motor symptoms in patients subsequently diagnosed with PD, suggesting that these symptoms may appear before any nigral pathology (Abbott et al., 2001; Savica et al., 2009). Consistently, PD is associated with Lewy body accumulation in the enteric nervous system (Kupsky et al., 1987; Wakabayashi et al., 1989). Moreover, loss of dopaminergic neurons in the enteric nervous system has been reported in PD patients with severe constipation (Singaram et al., 1995). Gastrointestinal dysfunction has also been recapitulated by experimental models of PD, including 6-OHDA-, MPTP- and rotenone-treated rodents (Anderson et al., 2007; Greene et al., 2009; Natale et al., 2010; Tian et al., 2008). In recent years, a role of colonic inflammation has been proposed in PD (Clairembault et al., 2014; Devos et al., 2013). Indeed, based on the postmortem topographic distribution of Lewy bodies in PD patients, Braak and colleagues proposed that PD may originate outside the CNS, possibly via the exposure to an unidentified environmental pathogen entering postganglionic neurons via the intestinal lining of the gastrointestinal tract, subsequently spreading via the vagal nerve to the CNS (Braak et al., 2006; Braak et al., 2003). This hypothesis is supported by a study reporting that full truncal vagotomy is associated with a decreased risk of developing PD later in life, both compared with the general population and with patients that underwent super-selective vagotomy (Svensson et al., 2015). A recent study also reported that α-synuclein from the brain lysate of a PD patient injected into the wall of the gastrointestinal tract of Sprague Dawley rats was taken up and transported retrogradely via the vagal nerve to the brain (Holmqvist et al., 2014). These observations shed new light on the hypothetic aetiologies of PD.

1.3.7 Gender differences in Parkinson's disease

PD has been estimated to affect up to 3% of the population in the industrialized world, affecting 1% of people over the age of 60 (Nussbaum and Ellis, 2003). Several reports found a higher prevalence of PD in men compared with women (de Lau and Breteler, 2006), which may be linked to the potential neuroprotective properties of oestrogen demonstrated both *in vitro* and *in vivo* (Saunders-Pullman, 2003). Premenopausal women have been reported to display a worsening of symptoms and a need for more L-DOPA during menstruation, when oestrogen levels are reduced (Quinn and Marsden, 1986; Tolson et al., 2002). Moreover, oestrogen replacement in postmenopausal women has been shown to improve cognitive functions (Jacobs et al., 1998; Shaywitz et al., 1999; Sherwin, 1988; Verghese et al., 2000), the latter which may delay the development of cognitive impairments and dementia in PD (Marder et al., 1998). The clinical features of PD also appear to differ between female and male patients. Women have been reported to develop more dyskinesias at equivalent doses of L-DOPA, to require lower doses of L-DOPA compared with men (even when doses are adjusted for body weight), and to have a milder motoric symptomatology after 5 years of disease progression compared with male patients (Lyons et al., 1998; Penney et al., 1996). Gender differences in the manifestation of non-motor functions have also been reported in drug-naïve PD patients, with male patients performing significantly worse on odour identification tests and cognitive tests compared with female patients, and female patients presenting with higher anxiety rates and poorer visuospatial abilities compared with male patients (Liu et al., 2015). Interestingly, genetic factors may also have a different impact in women and men. A recent study of the link between the genes LMX1A and LMX1B, which were the focus of study III, and PD, revealed that four single nucleotide polymorphisms (SNPs) in LMX1A and two SNPs in LMX1B were associated with PD in women but not in men, whereas, conversely, two SNPs

in LMX1A and two SNPs in LMXB were associated with PD in men but not in women (Bergman et al., 2009). This indicates that PD-associated genes may interact with oestrogen or other sex-specific transcription factors, thus affecting the subsequent risk of developing PD (Bergman et al., 2009). Apart from hormonal differences between women and men, it is possible that occupational and life-style factors, which, for instance, may affect the exposure to environmental toxins, also account for gender differences in PD. Interestingly, gender differences may also differ with ethnicity since the higher incidence rates of PD in men are reported exclusively in studies conducted in Western populations, but not in Asian populations (Alves et al., 2008).

1.4 EXPERIMENTAL MODELS OF PARKINSON'S DISEASE

Valid animal models that mimic the progressive disease state of PD are essential tools to better understand the early pathogenesis of PD. The two toxin-based animal models predominantly used in preclinical PD research, the 6-OHDA-induced model and the MPTP-induced model, induce an acute ablation of the dopaminergic system and thus do not successfully model the progressive nature of PD. Very few studies have been performed to address the early pathophysiology of PD, partially due to the absence of valid progressive animal models. In the studies presented in this thesis, we have evaluated novel models of progressive PD and used them to learn more about the early pathological changes in PD-like states. In addition, we have used 6-OHDA lesioned mice as a well characterized comparison to the novel models investigated. We also used 6-OHDA lesioned mice to study L-DOPA-induced side effects, since this model is particularly useful to this end. Below is a brief overview of current experimental models of PD.

1.4.1 Toxin-induced models of Parkinson's disease

Neurotoxin-induced models represent the oldest experimental PD models. The common feature of all neurotoxin-induced models is their ability to induce oxidative stress, thus damaging dopaminergic neurons in a way that mimics the pathology of human PD.

1.4.1.1 The 6-hydroxydopamine lesioned model

The first toxin-induced animal model of PD to be generated was the 6-OHDA model (Ungerstedt et al., 1974). In this well-established model, dopaminergic neurons projecting from the SNc to the striatum are unilaterally ablated by the neurotoxin 6-OHDA. 6-OHDA is a hydroxylated analogue of dopamine with a high affinity for DAT, which does not cross the blood-brain barrier and thus must be locally injected into the brain. Upon DAT-mediated transportation of 6-OHDA into the neuron, 6-OHDA accumulates in mitochondria where it inhibits complex I (Blandini and Armentero, 2012). 6-OHDA can also auto-oxidate, resulting in the production of hydrogen peroxide (H_2O_2) (Blandini and Armentero, 2012). 6-OHDA is commonly injected directly into the SNc or into the medial forebrain bundle (MFB), the latter harbouring projections of the A9 dopaminergic cell group, which originates in the SNc and terminates in the striatum. Dopaminergic neurons start to degenerate 12 hours after the 6-OHDA injection, and after 2-3 days there is a marked loss of dopaminergic terminals in the striatum accompanied by dopamine depletion, subsequently leading to a 90-100% loss of dopaminergic neurons (Blandini and Armentero, 2012). It is common to perform SNc/MFB 6-OHDA lesions unilaterally, leaving one hemisphere intact, which increases the viability of the animals and provides a useful model system to study and quantify L-DOPA-induced dyskinesias and stereotypies (Lundblad et al., 2004; Ungerstedt and Arbuthnott, 1970). Unilaterally lesioned animals display a characteristic rotational behaviour when challenged with drugs that stimulate striatal dopamine receptors directly or indirectly, such as apomorphine, L-DOPA and amphetamine

(Ungerstedt and Arbuthnott, 1970; Ungerstedt, 1971; Von Voigtlander and Moore, 1973). In another 6-OHDA-induced model the injections are made into the striatum, often bilaterally, resulting in a comparatively milder and progressive loss of dopaminergic neurons over 4-6 weeks post-injection (Sauer and Oertel, 1994).

1.4.1.2 The MPTP model

It is known that humans exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) present with a syndrome that involves many key features of PD, including neurological symptoms and a selective neurodegeneration of dopaminergic neurons. The selective nigrostriatal toxicity of MPTP was first described in 1982, following the occurrence of chronic Parkinsonism in four young drug users from Northern California after injecting a preparation of synthetic heroin contaminated with MPTP (Langston et al., 1983). MPTP is transformed into its active metabolite 1-methyl-4-phenylpyridinium ion (MPP+) by MAO-B upon crossing the blood-brain barrier, and is then transported into dopaminergic neurons by DAT where it blocks mitochondrial complex I activity (Blandini and Armentero, 2012). Systemic injections of MPTP induce L-DOPA responsive Parkinsonism that recapitulate the cardinal symptoms of PD, and has thus proved useful for modelling PD in mice and primates. Rats, however, are resistant to systemic MPTP injections via a mechanism currently not understood. The MPTP-model has highlighted both the involvement of mitochondria in PD-associated neurodegeneration, and the role of pesticides and environmental toxins in the aetiology of PD (see below), since there are structural similarities between MPTP, MPP+ and pesticides, including paraquat. One limitation of the MPTP-induced model is that the nigral lesions are not accompanied by the formation of Lewy bodies (Forno et al., 1996), one key pathological hallmark of PD in humans.

1.4.1.3 Rotenone and paraquat

Chronic exposure to pesticides is a known risk factor of PD, which has led to numerous studies on agricultural pesticides and neurodegeneration, and to the discoveries of additional toxin-induced animal models of PD. Chronic systemic injections of the pesticide rotenone induces Parkinsonism in rats by entering dopaminergic neurons in a DAT-independent manner and inhibiting mitochondrial complex I (Betarbet et al., 2000). Inhibition of complex I leads to the formation of ROS (Seaton et al., 1997), and subsequently to selective nigrostriatal dopaminergic degeneration with a relative sparing of VTA neurons, and, notably, cytoplasmic inclusions reminiscent of Lewy bodies (Betarbet et al., 2000). Weaknesses of the rotenone model include a high level of variability in the individual response to rotenone injections making the lesions hard to standardize, and a high risk of mortality due to heart, liver, kidney and gastrointestinal toxicity (Blandini and Armentero, 2012). Moreover, due to its high specificity to dopaminergic neurons, rotenone treatment fails to recapitulate pathological changes outside the dopaminergic system associated with PD (Blandini and Armentero, 2012).

The herbicide paraquat, which is structurally similar to MPP+, is also used as a systemic model of PD. Unlike MPTP and rotenone, paraquat does not pass the blood-brain barrier. Instead, paraquat may enter the brain via amino acid transporters (Blandini and Armentero, 2012), and dopaminergic neurons via DAT (Rappold et al., 2011). Inside the neuron paraquat can undergo redox cycling which yields the free radical superoxide, subsequently inducing oxidative stress-mediated neurotoxicity (Rappold et al., 2011). As opposed to MPP+ and rotenone, paraquat has a low affinity to complex I, thus complex I does not appear to be part of its neurotoxic mechanism (Blandini and Armentero, 2012).

1.4.2 Genetic models of Parkinson's disease

About 95% of PD cases are sporadic, or without any genetic linkage (Dauer and Przedborski, 2003). Nevertheless, the recent discovery of different genetic mutations, including SNCA (the gene encoding α-synuclein), LRRK2, PARK2, PARK7 and PINK1, has led to the development of genetic models of PD (Dawson et al., 2010). There is also support for a role of dysfunctional transcription factors in PD. Several developmental transcription factors, including Nurr1 and Lmx1b, remain expressed in mature dopaminergic neurons, where they play a role in the neuronal maintenance. Nucleotide polymorphisms in genes encoding these transcription factors have been associated with PD in humans (Bergman et al., 2010, 2009; Grimes et al., 2006; Haubenberger et al., 2011; Tang et al., 2012; Xu et al., 2002; Zheng et al., 2003), making them promising experimental targets and useful for generating models of construct validity.

1.4.2.1 The cNurr1^{DATCreER} mouse model

Nurr1, also known as NR4A2, is a developmental transcription factor predominantly expressed in the brain (Law et al., 1992; Zetterström et al., 1996), which continues to be expressed in mature dopaminergic neurons of the midbrain. Nurr1 is a nuclear receptor required for the differentiation and maintenance of functional mDA neurons of the ventral midbrain (Castillo et al., 1998; Kadkhodaei et al., 2009; Perlmann and Wallén-Mackenzie, 2004; Saucedo-Cardenas et al., 1998; Wang et al., 2003; Zetterström et al., 1997). The expression of Nurr1, however, is gradually decreased upon ageing (Chu et al., 2002). Analyses of postmortem brain tissue revealed down-regulation of Nurr1 in neurons containing α-synuclein inclusions in PD patients compared with healthy age-matched controls (Chu et al., 2006). Nurr1 expression levels were also decreased in peripheral blood lymphocytes in patients with PD compared with age-matched controls (Liu et al., 2012). The Nurr1^{DATCreER} mouse model, where Nurr1 is ablated in young adult animals, is characterized by a slowly developing mDA neuron dysfunction culminating in loss of cell integrity and cellular abnormalities that resemble key aspects of the disease progression of PD (Kadkhodaei et al., 2013, 2009). This includes reduced expression of dopaminergic markers including TH, DAT and VMAT throughout the nigrostriatal and mesocorticolimbic pathways (Kadkhodaei et al., 2013). Nurr1 is also involved in the expression of receptor tyrosine kinase RET (Decressac et al., 2012), the receptor for glial cell line-neurotrophic factor (GDNF) which has neuroprotective effects in neurotoxin-induced models of PD (Aron and Klein, 2011; Björklund et al., 2000; Kirik et al., 2004; Kordower, 2000; Ramaswamy et al., 2009). As revealed in study I of this thesis, Nurr1 is also involved in the expression of nuclear-encoded mitochondrial genes (Kadkhodaei et al., 2013), which is consistent with mitochondrial dysfunction being proposed as a leading cause of dopaminergic degeneration in PD (Dawson and Dawson, 2003). Interestingly, the novel Nurr1 agonist SA00025 was recently shown to have both neuroprotective and anti-inflammatory effects in a neuroinflammation-exacerbated 6-OHDA lesioned rat model of PD (Smith et al., 2015).

1.4.2.2 The Lmx1a/b mouse model

In addition to the cNurr1^{DATCreER} mouse model, the less well characterized Lmx1a/b KO mouse model, which harbours a germline deletion of the developmental transcription factors Lmx1a and b, has been characterized in study III. Lmx1a and Lmx1b are two highly related LIM homeodomain transcription factors essential for the development of mDA neurons (Deng et al., 2011; Yan et al., 2011). Under normal development, Lmx1a cooperates with Lmx1b to regulate proliferation, specification and differentiation of neuronal stem cells into mDA neurons (Yan et al., 2011), and they continue to be expressed also in postmitotic differentiating mDA neurons. Lmx1a is vital for the differentiation of mDA neurons,

whereas Lmx1b also appears to be required for the survival of these neurons, and is co-expressed with TH and paired-like homeodomain transcription factor 3 (Pitx3), a marker for mDA neurons (Alavian et al., 2014). Combined null mutations in Lmx1a and Lmx1b have previously been shown to result in disrupted Wnt1 signaling, decreased proliferation of progenitor cells and decreased neurogenesis, ultimately resulting in abolished generation of mDA neurons (Deng et al., 2011; Yan et al., 2011). However, the exact function of Lmx1a and Lmx1b after specifying dividing neural progenitors has remained unknown. Interestingly, genetic variants in the LMX1A and LMX1B genes have been linked to an increased risk of developing PD in humans (Bergman et al., 2009). In study III we explored the role of Lmx1a/b in adult neurons with a focus on PD pathology and symptomatology, revealing that Lmx1a/b ablation induced a phenotype strikingly similar to early PD.

1.4.2.3 α-synuclein overexpressing models

α-synuclein is a major constituent of Lewy bodies, as discussed in detail in 1.3.1. α-synuclein can induce PD when mutated, or when the WT form is increased by gene duplication or triplication (Douglas et al., 2007). One problem with transgenic overexpression of α-synuclein is that α-synuclein pathology is very widespread in PD. A successful PD model must mimic this broad pathological distribution; thus, the phenotype of these models depends to a great extent on the promoter used to drive transgene expression (Chesselet, 2008). Notably, transgenic α-synuclein overexpressing mouse models do not exhibit any progressive loss of dopaminergic neurons, but present with several functional abnormalities of the nigrostriatal system (Dawson et al., 2010; Giráldez-Pérez et al., 2014). Aggregated α-synuclein appears to induce toxic effects by impairing mitochondrial functions and by impairing proteasomal and lysosomal systems. The most promising transgenic α-synuclein overexpressing mouse model, which overexpresses human A53T mutated α-synuclein under the mouse prion promoter, exhibits age-dependent neurodegeneration (Chesselet, 2008) and mitochondrial dysfunctions, including damaged mitochondrial DNA and mitochondrial degeneration, highlighting a relationship between α-synuclein and mitochondrial functions (Martin et al., 2006).

An alternative to the transgenic approach is to use stereotaxic injections of viral vectors to overexpress α-synuclein in specific sets of neurons to induce a PD-like pathology. α -synuclein overexpression in neurons of the substantia nigra induced by this approach result in axonal pathologies and progressive cell loss (Kirik et al., 2002; Klein et al., 2002; Lo Bianco et al., 2004). In rats, intranigral α-synuclein overexpression induced by AAV vectors led to the development of motor impairments and a prominent loss of dopaminergic neurons in the SNc, but not in the VTA, although signs of α-synuclein toxicity was found also in the latter area (Ulusoy et al., 2010). This bears resemblance to the VTA being less susceptible to neurodegeneration in patients with PD. In study IV we used AAV vectors to overexpress α-synuclein specifically in the VTA to study the impact on PD-related symptoms.

2 AIMS

Valid animal models are key to understand the pathophysiological processes in neurological and psychiatric disorders. The overall aim of this thesis was to characterize and validate genetically induced progressive models of Parkinson's disease to improve understanding of how pathophysiological mechanisms contribute to the symptomatology and pathology of Parkinson's disease. We were specifically interested in the role of developmental transcription factors in behavioural functions and neurotransmitter release. We also focused on the evaluation of a potential therapeutic role of TAAR1 in the treatment of Parkinson's disease, using both neurotoxin-induced and a newly characterized genetically induced model. The specific aims of this thesis included:

1. Characterization of the cNurr1^{DATCreER} mouse line to determine whether this model recapitulated features of Parkinson's disease.
2. Investigation of the role of TAAR1 in L-DOPA responsivity and glutamate release using models of Parkinson's disease, including 6-OHDA lesioned mice and cNurr1^{DATCreER} KO mice.
3. Characterization of the Lmx1a/b^{DATCre} mouse line to determine whether this model recapitulated features of Parkinson's disease.
4. Investigation of the role of α-synuclein overexpression in the VTA in the symptomatology of Parkinson's disease.

3 MATERIALS AND METHODS

EXPERIMENTAL MODELS

cNurr1^{DATCreER} KO mice (I, II)
TAAR1 KO mice (II)
Lmx1a/b^{DATCre} KO mice (III)
6-OHDA lesioned mice (II)
α-synuclein overexpressing Sprague-Dawley rats (IV)

BEHAVIOURAL METHODS
Motor functions

Open field test (I, III)
Ledged beam test (I, II, III)
Pole test (I, II, III)
Cylinder test (II)
Rotation test (II)

Learning and memory functions

Passive avoidance (III, IV)
Novel object recognition (III)
T-maze (III)

Anxiety-like behaviour

Elevated plus maze (III)

Depressive-like behavior

Porsolt swim test (III)
Sucrose preference test (III)

PHARMACOLOGICAL TREATMENTS

Acute and chronic systemic administration (I, II)
Tamoxifen treatment (I, II)
Local brain administration (II)

STEREOTACTIC TECHNIQUES

In vivo amperometry (II)
6-OHDA lesioning (II)
Adeno-associated viral vectors (IV)
Methylene Blue intracranial infusion (II)

HISTOLOGY

Autoradiography (II)
Nuclear Fast Red counterstaining (II)

BIOCHEMICAL TECHNIQUES

Immunoblotting (II)

3.1 PHARMACOLOGICAL COMPOUNDS USED IN THE PRESENT WORK

NAME	FUNCTION
L-DOPA	Dopamine precursor
Ropinirole	Selective dopamine D_2-like receptor agonist ($D_3 > D_2 > D_4$)
Apomorphine	Non-selective dopamine agonist
RO5166017	Selective TAAR1 agonist
EPPTB	Selective TAAR1 antagonist
AM251	Inverse CB_1 cannabinoid receptor agonist
Tamoxifen	Estrogen receptor antagonist

3.2 GENERAL BEHAVIOURAL PROTOCOLS USED IN THE PRESENT WORK

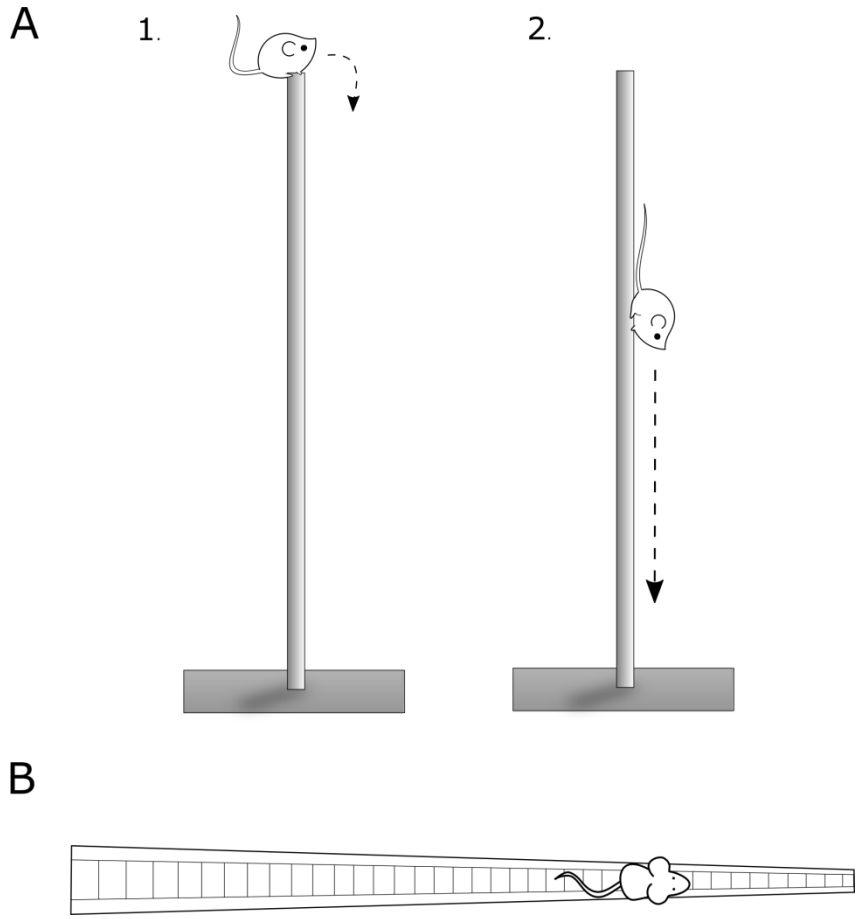


Figure 3.2.1 Tests of motor functions in the pole test and beam traversal tests. (A) In the pole test, mice are placed head-up on top of a vertical pole (diameter: 8 mm, height: 55 cm) and trained for 1 day to turn and descend the pole back into their home cage. On the day of the test, animals perform 3 trials and the time to orient downward, called t-turn (1), and the total time to turn and descend the pole, called t-total (2), are measured at a maximum duration of 120 seconds. (B) In the beam traversal test, mice are trained for two consecutive days to traverse a beam leading to their home cage. On the test day, a grid (1 cm^2) of corresponding width is placed 1 cm above the beam and the mice are videotaped while traversing it. The time to walk across the beam, the number of steps and errors (defined as a limb slipping through the grid) are calculated.

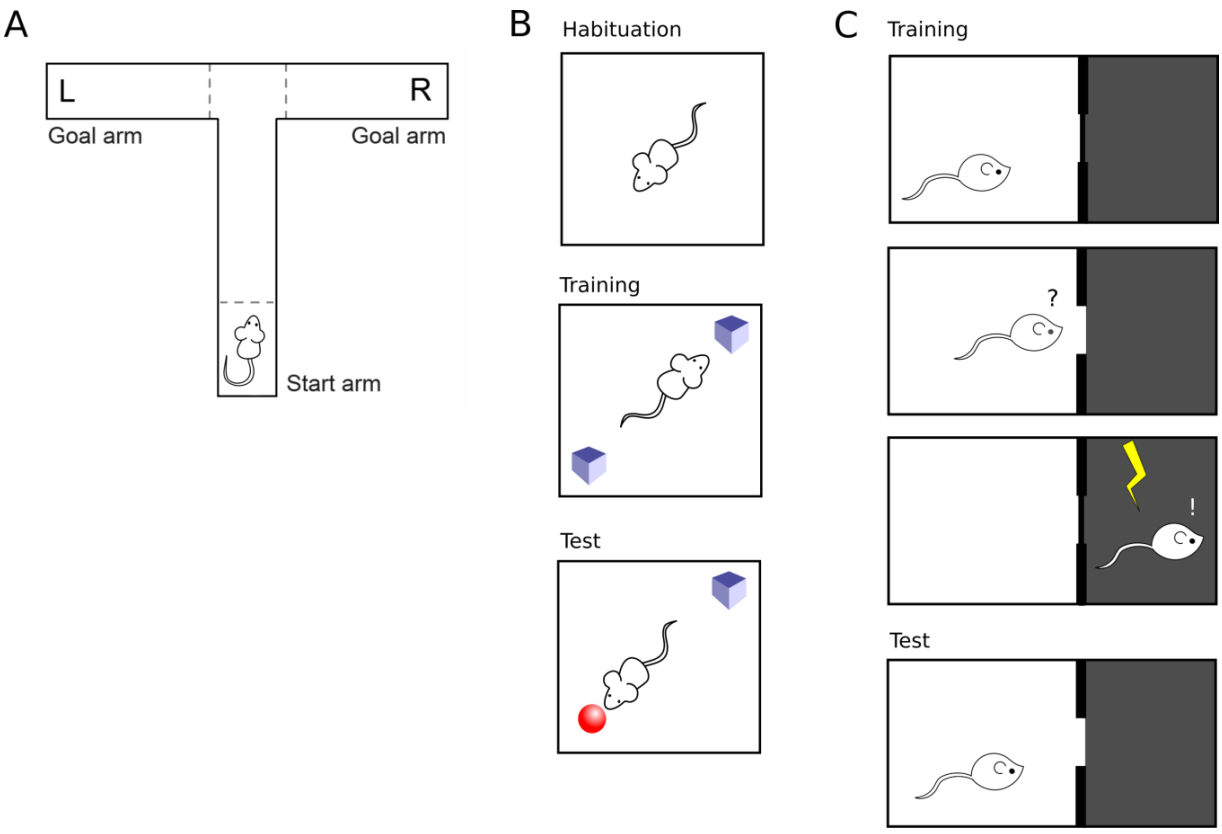


Figure 3.2.2 Tests of cognitive skills. (A) The T-maze is a test of working memory. A spontaneous alternation paradigm was used in this thesis, based on the natural tendency of rodents to alternate between opposite goal arms during natural exploration. This paradigm has the advantage that it does not require any training or food rewards. Each test starts with a forced trial followed by 14 choice trials. After each completed arm choice the mouse is placed back into the start arm and confined for 5 seconds before each new consecutive trial. One point is assigned for each choice arm alternation. (B) The novel object recognition test is a test of object memory, and is useful for evaluating short-term memory in mice. A habituation session is performed in an empty open field arena to let the animals habituate to the test environment. 24 h after the habituation phase, the mice are allowed to explore the open field arena for 5 minutes in the presence of two identical objects. The memory test (5-10 minutes) is performed 1-4 hours after the training session, during which one of the previous familiar objects is replaced with a novel object. Object exploration time (typically defined as nose < 1 cm from object) can be measured manually or automatically using a tracking software connected to a ceiling mounted camera. A relative discrimination index is calculated as $(Time_{Novel}-Time_{Familiar})/(Time_{Novel}+Time_{Familiar})$ based on the exploratory time of each object, and a significant preference for the novel object indicates intact object memory. (C) The passive avoidance test is used to assess emotional contextual memory. The apparatus consists of two compartments connected via an automatic door: one brightly illuminated with white walls, and one with black walls covered by a ceiling. During the test session the mouse is placed in the bright compartment and the step-through latency into the dark compartment is measured. Once the mouse enters the dark compartment with all four paws the automatic door is closed and a current is delivered through the grid floor (0.3 mA) for 2 seconds. 30 seconds after step-through the mouse is removed and placed into its home cage. The test session (9 minutes, no current) is typically performed 24 hours after the training session. The mouse is then placed back into the bright compartment and the step-through latency to enter into the dark compartment is measured. A significantly prolonged step-through latency during the test session indicates intact emotional contextual memory.

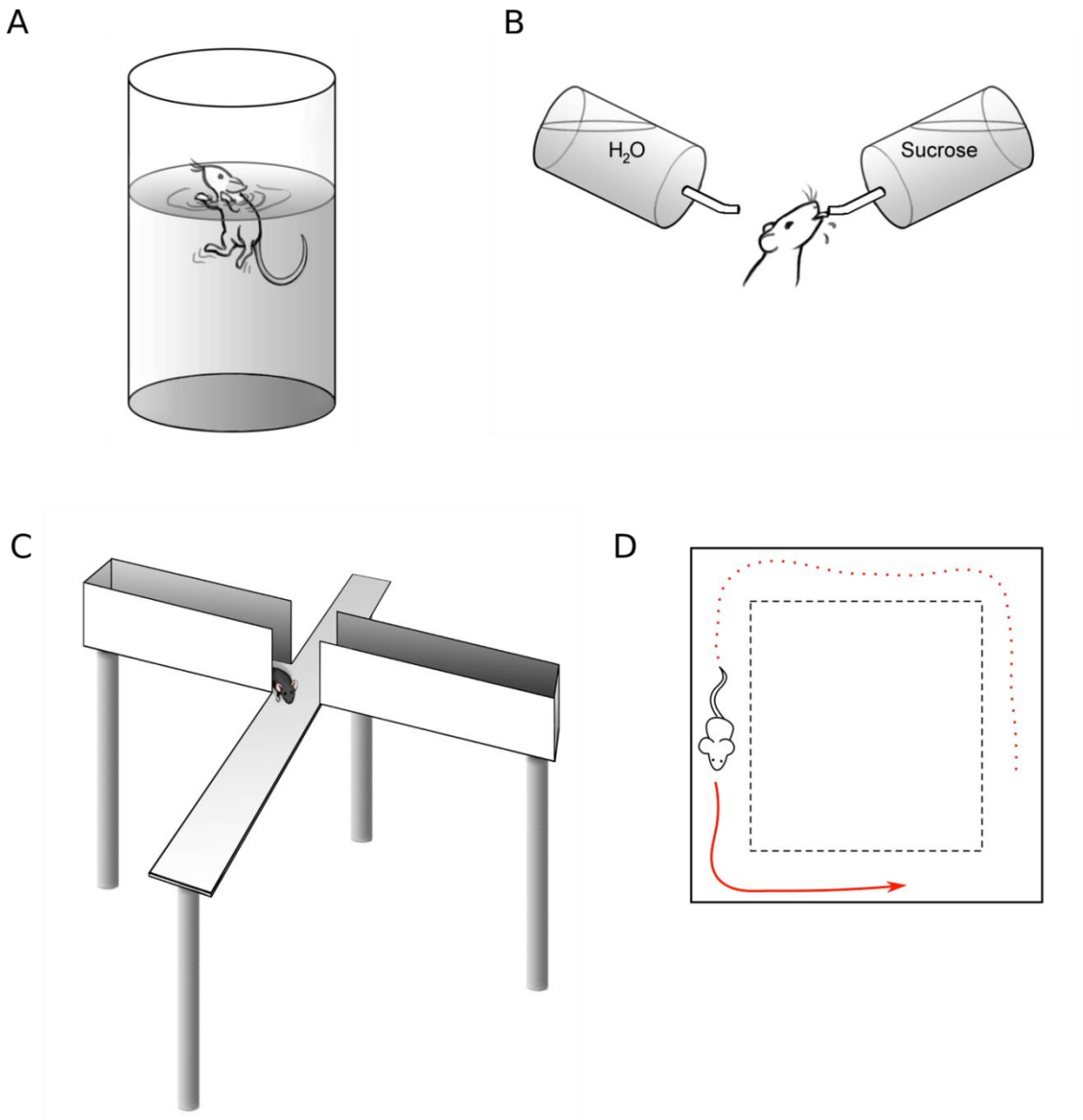


Figure 3.2.3 Tests of anxiety- and depressive-like behaviour. (A) The Porsolt swim test is based on behavioral despair, measured as inactive floating after initial swimming or climbing. This test is sensitive to acute antidepressant treatment, which increases the time spent swimming and climbing. Each test session lasts for 5-6 minutes (water temperature: 23-25°C) and is typically videotaped for subsequent analysis. (B) The sucrose preference or sucrose consumption test is a measure of anhedonia, based on the preference of a sucrose solution over water. Two bottles, one containing the sucrose solution and one containing tap water, are presented for 3 consecutive days, positions interchanged each day. Sucrose preference is calculated as a percentage of the total volume consumed. (C) The elevated plus maze is used to assess anxiety-like behavior. The apparatus consists of four arms elevated one meter above the ground, two with high walls and two without walls. Rodents, being prey animals, naturally prefer the closed arms and avoid the open arms. During testing, each animal is individually placed in the center of the apparatus and allowed to explore it for 5 minutes. The number of entries into the open and closed arms and the total time spent in each pair of arms are measured and compared. (D) Thigmotaxis behavior in an open field arena, defined as the animal staying in close proximity of the walls, can be used as a measure of anxiety since rodents tend to avoid bright, open areas depending on the level of anxiety.

3.3 *IN VIVO* AMPEROMETRY

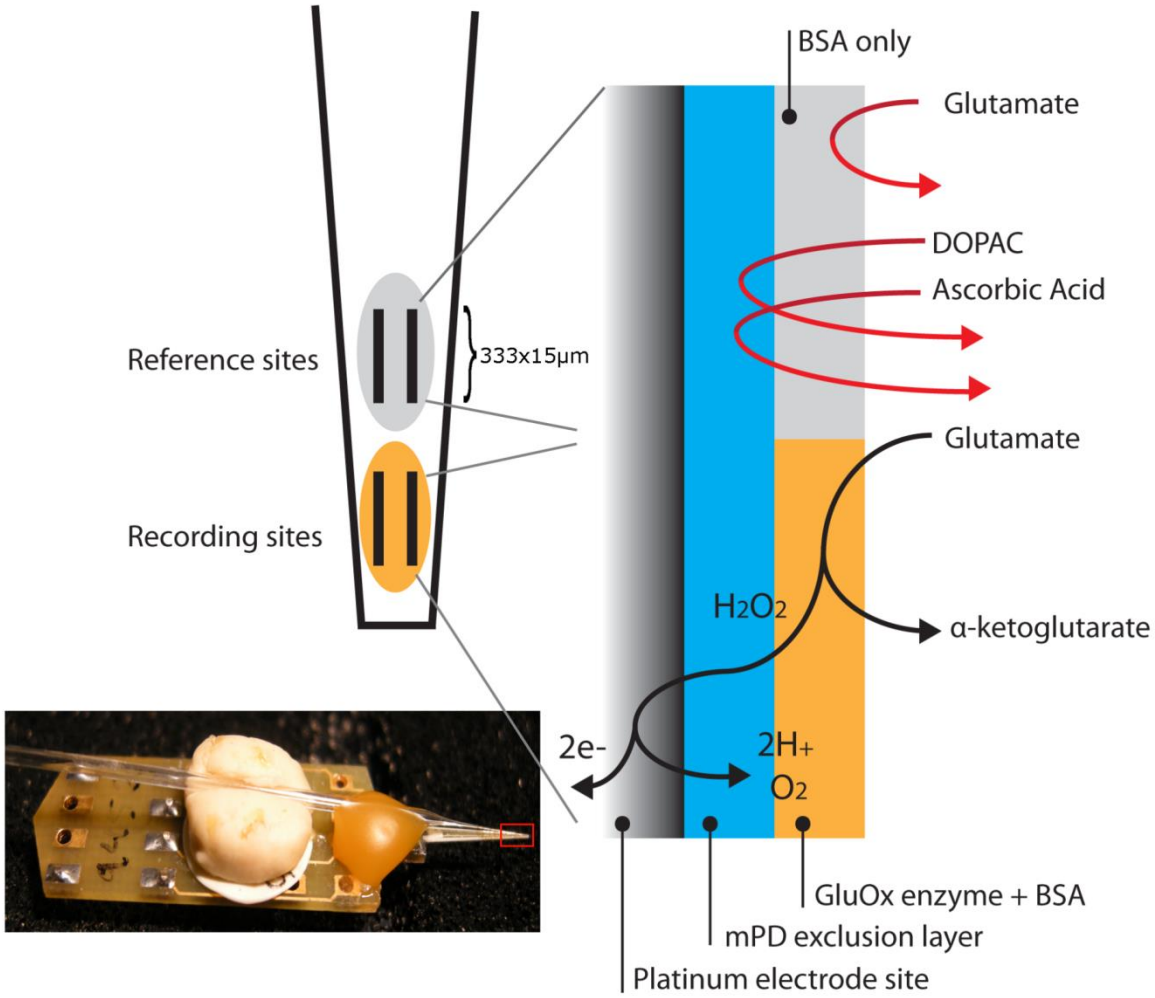


Figure 3.3.1 Schematic drawing of a microelectrode array (MEA) of the S2 model used for *in vivo* glutamate recordings. Each MEA has four platinum electrode sites (15 x 333 µm) organized as pairs. Prior to experiments two platinum sites are coated with glutamate oxidase (GluOx), which cause enzymatic breakdown of glutamate into α-ketoglutarate and peroxide (H_2O_2), whereas the second pair of sites are coated with an inactive protein matrix (BSA) and used for self-referencing. All four sites are coated with a protective layer of metaPhenylene-diamine dihydrochloride (mPD) to block interferents such as dopamine, DOPAC and ascorbic acid. Peroxide is oxidized at the platinum site, resulting in the release of two electrons that create a current measured by a Fast Analytical Sensing Technology (FAST 16-mkII) system. Responses recorded at the reference sites are subtracted from those generated at the recording sites during analysis, with the resulting signal representing glutamate measurements. A micropipette is aligned between the four sites and used for local delivery of 70 mM KCl and the compound of interest to stimulate depolarization and evoke glutamate release.

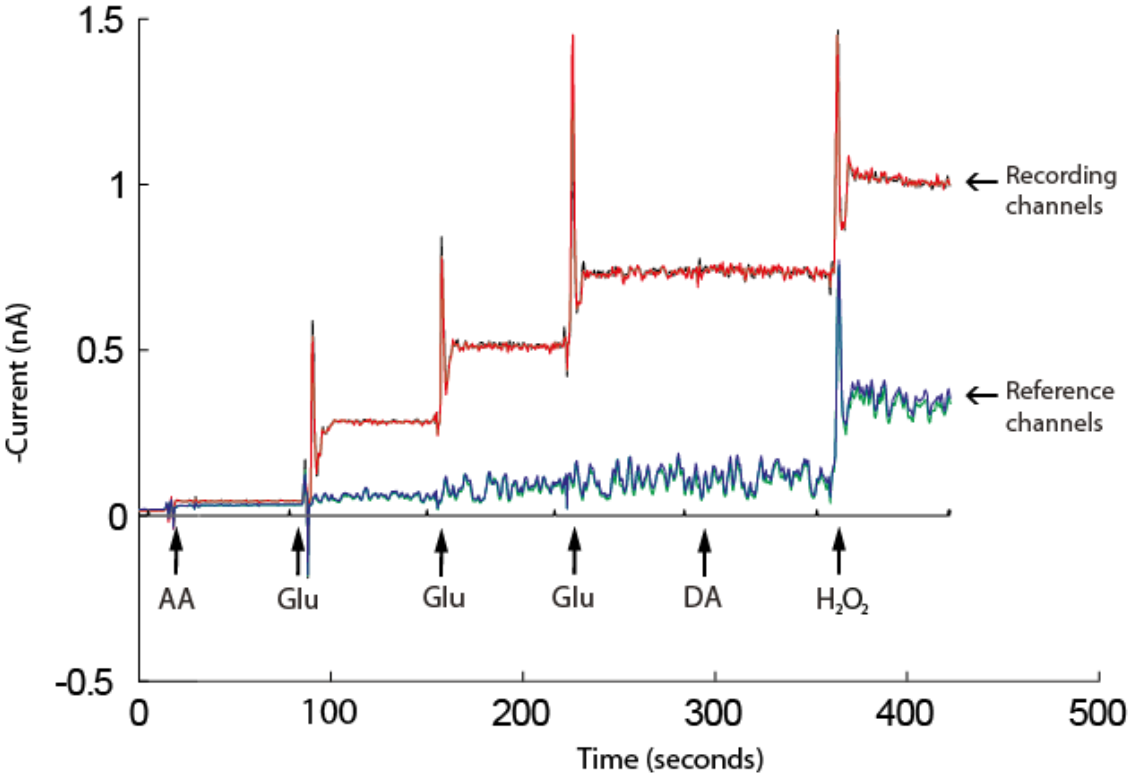


Figure 3.3.2 ***In vitro*** **calibration of MEA.** Before each experiment, electrodes are calibrated *in vitro* to determine the selectivity and sensitivity for glutamate against ascorbic acid. Electrodes are suspended in 0.05 M phosphate-buffered saline (pH 7.4, 37°C), and analytes of interest are added to the buffer to determine the response, detected as a change in current. The electrode sites coated with GluOx should respond to glutamate and the reporter molecule H_2O_2, but not to the interferents ascorbic acid (AA) and dopamine (DA), which are repelled by the mPD coating. The electrode sites with no GluOx coating should respond only to molecules not repelled by mPD and are used for self-referencing. Only electrodes that fulfill certain calibration criterions based on their selectivity and sensitivity for L-glutamate are selected for *in vivo* experiments.

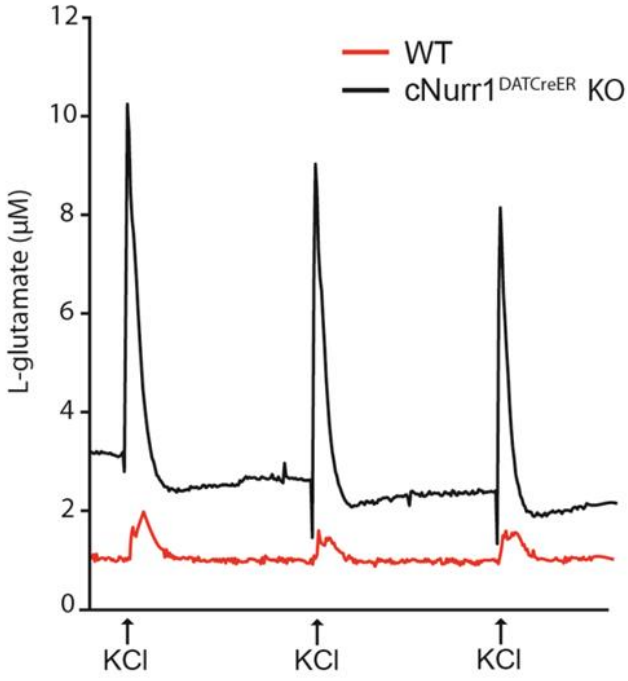


Figure 3.3.3 ***In vivo*** **recording.** The arrows indicate KCl ejections, which are typically made for 1 second at 1-minute intervals during experiments.
Compounds of interest can be mixed with KCl for local delivery. KCL ejections are followed by increased concentration of glutamate at the recording channels. During analysis, the signal recorded at the reference channels is subtracted from that of the recording channels to remove background noise. The graph shows a striatal recording from a cNurr1^{DATCreER} KO mouse superimposed on that of a WT mouse. Data from these experiments are presented in study II.

4 RESULTS AND DISCUSSION

The constituent studies focus on the characterization of models of experimental Parkinsonism, and on the utilization of these models to identify disease mechanisms related to Parkinson's disease. Below is a brief summary of the thesis work, including some relevant unpublished findings. More comprehensive descriptions are found in the full papers.

4.1 AIM I: CHARACTERIZATION OF THE TRANSGENIC cNURR1^{DATCreER} MOUSE MODEL

The transcription factor Nurr1 has been associated with PD in humans and is essential for the development of ventral midbrain dopamine neurons. Paper I aimed at investigating the role of Nurr1 in adult dopaminergic neurons, focusing on factors relevant for PD. Conditional Nurr1 gene targeted mice, in which Nurr1 was ablated selectively in mature dopaminergic neurons by tamoxifen treatment (cNurr1^{DTACreER} KO mice), were used. Immunostaining was performed to detect Nurr1, dopaminergic markers and cell integrity in developing and mature neurons of the ventral midbrain and striatum. High-pressure liquid chromatography was performed in dissected brain tissue of the striatum and ventral midbrain to detect dopamine and its metabolites. Laser-microdissected dopaminergic neurons were used for RNA extraction and next-generation mRNA sequencing to identify Nurr1-regulated genes. Behavioural analyses focusing on motor functions were performed to assess the behavioural phenotype. These experiments revealed that Nurr1 ablation resulted in a progressive pathology associated with reduced striatal dopamine, impaired motor behaviours (Figures 4.1A and B), and dystrophic axons and dendrites, and that Nurr1 regulates a battery of genes expressed in dopaminergic neurons, including nuclear-encoded mitochondrial genes. These experiments indicate that Nurr1 has a key function in sustaining the high respiratory levels in dopaminergic neurons, and that Nurr1 ablation in mice recapitulates early features of PD disease. Based on the findings in Paper I, the cNurr1^{DTACreER} KO mouse model was found to hold promise as a valid model of progressive PD, and subsequent experiments are currently performed to further characterize this model with a focus on non-motor functions in PD.

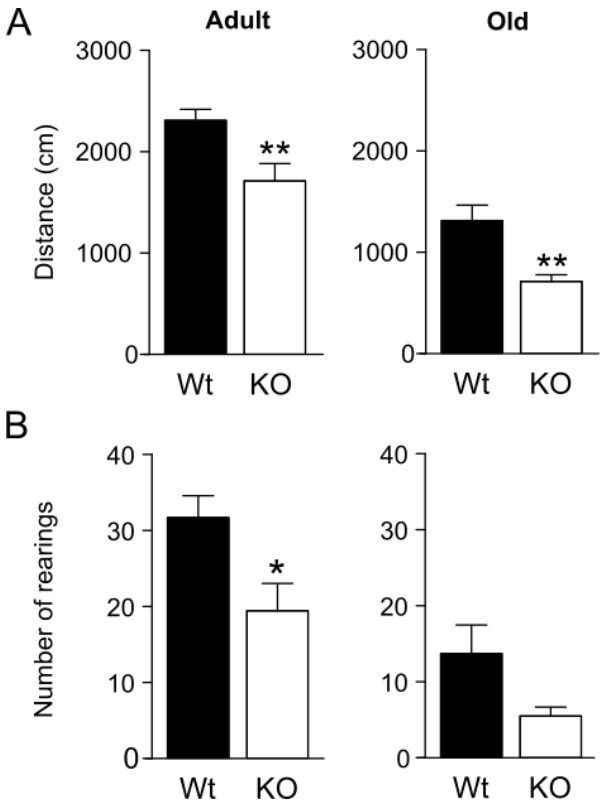


Fig 4.1. Open-field behavior in adult and old cNurr1^{DATCreER} KO and WT mice. (A) Both adult and old cNurr1^{DATCreER} KO mice moved a significantly shorter distance in an open field arena compared with age-matched WT mice. (B) Adult, but not old, cNurr1^{DATCreER} KO mice performed fewer rearings compared with age-matched WT mice.

4.2 AIM II: THE ROLE OF TAAR1 IN L-DOPA RESPONSIVITY AND GLUTAMATE RELEASE IN MODELS OF PARKINSON'S DISEASE

As discussed in detail in 1.3.3.2, TAAR1 is a recently discovered receptor that modulates dopaminergic transmission. Paper II aimed at investigating the influence of TAAR1 on factors related to PD, including L-DOPA-induced sensitization and corticostriatal glutamate release, using pharmacological interventions, Parkinsonian mouse models and TAAR1 KO mice. Mice with unilateral medial forebrain bundle 6-OHDA lesions were used to model L-DOPA sensitization following subchronic L-DOPA treatment. Mice with intrastriatal 6-OHDA lesions and the previously characterized cNurr1^{DATCreER} KO mouse model were used to model progressive dopaminergic neurodegeneration. Presynaptic glutamate release was measured by *in vivo* amperometry (described in 3.3), whereas postsynaptic glutamate transmission was detected by immunoblotting for phosphorylation of AMPA-receptor subunits. These experiments revealed that TAAR1 KO mice had higher levels of the dopaminergic markers TH and DAT than WT mice, and were less vulnerable to intrastriatal 6-OHDA injections. TAAR1 KO mice sustaining medial forebrain bundle 6-OHDA lesions were more markedly supersensitive to subchronic L-DOPA treatment both in terms of rotations, abnormal involuntary movements and AMPA receptor phosphorylation compared with WT mice. Conversely, pretreatment with the TAAR1 agonist RO5166017 reduced supersensitization to L-DOPA in 6-OHDA-treated WT mice, in parallel with a reduction in L-DOPA-induced AMPA receptor phosphorylation. Corticostriatal glutamate release was enhanced both in the lesioned hemisphere of 6-OHDA-treated mice (Figures 4.2.1 and 4.2.2) and in cNurr1^{DATCreER} KO mice compared with respective controls (Figure 4.2.2), whereas nigral glutamate release remained unaltered in the Parkinsonian state of both models (Figure 4.2.3). These findings highlight similarities between these two experimental models with respect to both striatal and nigral glutamate release. In cNurr1^{DATCreER} KO mice, the enhanced corticostriatal glutamatergic transmission was reversed by the TAAR1 agonist and by the D_2-like receptor agonist ropinirole via a CB_1 receptor dependent mechanism (Figure 4.2.4). These data reveal that TAAR1 modulates behavioral responses to L-DOPA, and regulates pre- and postsynaptic glutamate neurotransmission in the striatum (Figure 4.2.5).

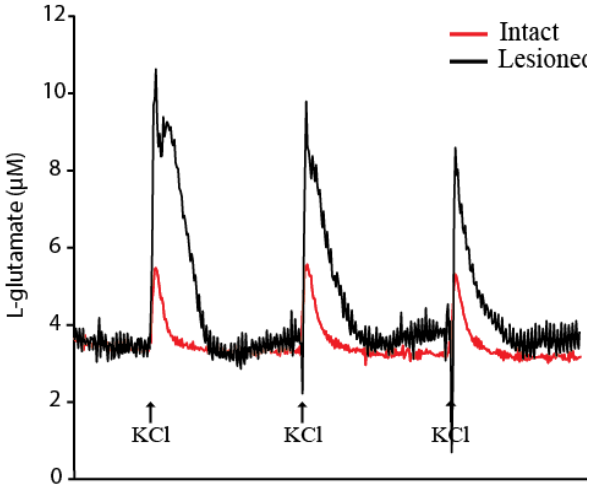
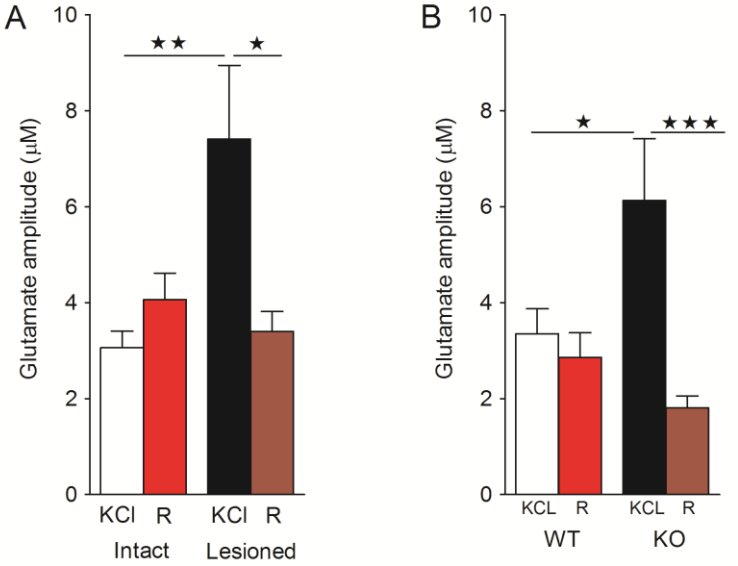


Figure 4.2.1 *In vivo* amperometry in striatum of 6-OHDA lesioned mice. Two superimposed *in vivo* amperometry L-glutamate recordings from one intact and one 6-OHDA lesioned hemisphere, showing enhanced glutamate amplitudes in the lesioned striatum (black trace).

Figure 4.2.2 Striatal glutamate release in two models of PD. Striatal glutamate release was enhanced in the Parkinsonian state, i.e. in the lesioned hemisphere of 6-OHDA lesioned mice (A), and in cNurr1^{DATCreER} KO mice (B), highlighting similarities between these two models. Statistics: Two-way ANOVA followed by Fisher's LSD *post hoc* test, **p*<0.05; **p<0.01; ****p*<0.001



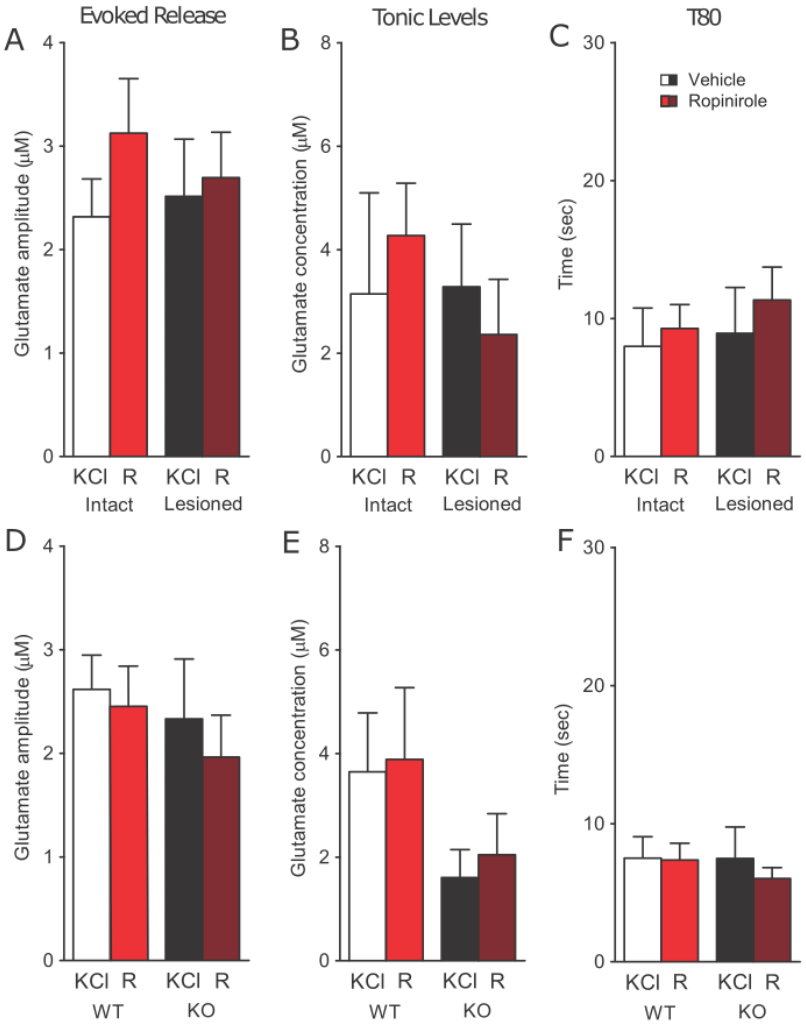


Figure 4.2.3 Glutamatergic neurotransmission in SNc in two models of PD. No changes in glutamate amplitudes (A), tonic glutamate levels (B) or the glutamate reuptake rate (T80) (C) were found in SNc of 6-OHDA lesioned mice. Similarly, no changes in glutamate amplitudes (D), tonic glutamate levels (E) or the glutamate reuptake rate (F) were found in SNc of Nurr1^{DATCreER} KO mice compared with WT mice. This indicates that excitotoxic glutamate release in SNc plays little role in these two models.

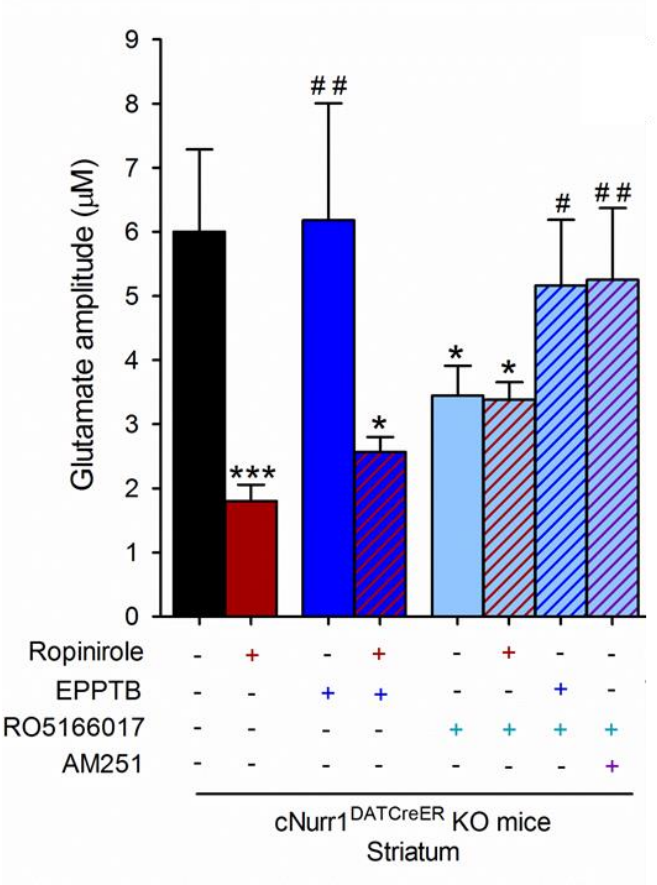


Figure 4.2.4 Pharmacological modulation of striatal glutamate release. The enhanced glutamate release in cNurr1^{DATCreER} KO mice was inhibited by local administration of D_2 receptor agonist ropinirole (100 µM). Blockade of CB_1 receptors by AM251 did not affect striatal glutamate release, but attenuated ropinirole's effect. This supports previous data showing that D_2 receptors attenuate dysfunctional glutamate release via a mechanism involving CB_1 receptors, possibly via retrograde endocannabinoid signaling. The TAAR1 antagonist EPPTB (10 nM) did not affect corticostriatal glutamate release or the effect of ropinirole. The TAAR1 agonist RO5166017 (500 nM) inhibited enhanced glutamate release. Blockade of CB_1 receptors by AM251 attenuated the effect of RO5166017. No effects were seen in WT animals. This suggests that local TAAR1 agonism, via CB_1 receptors, mediates a rapid attenuating effect on the hyperglutamatergic state in experimental PD. Statistics: One-way ANOVA followed by Fisher's LSD *post hoc* test, *p<0.05; **p<0.01; ***p<0.001 *vs.* KO veh; $^{\#}p$<0.05; $^{\#\#}p$<0.01 *vs.* KO ropinirole.

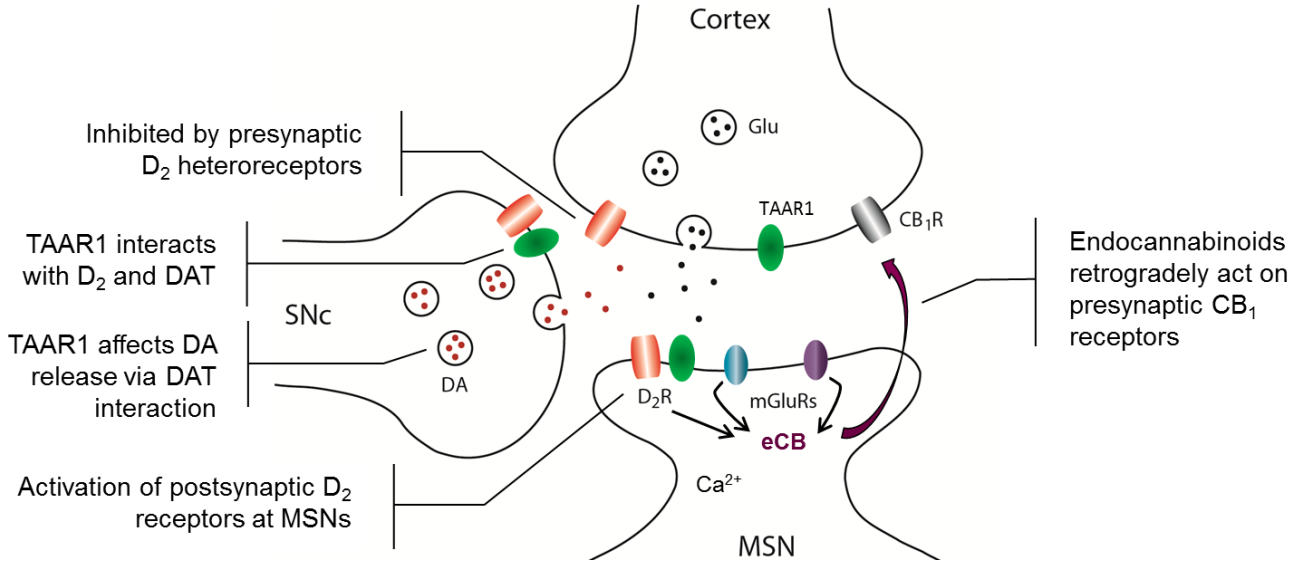


Figure 4.2.5 Potential modulation of corticostriatal glutamate release by TAAR1. A simplified schematic drawing of a corticostriatal synapse and the possible expression of TAAR1 relevant for interactions with the dopaminergic system and the modulation of glutamate release. TAAR1 (green) may be located in a position to modulate corticostriatal glutamate release by interacting with DAT at dopaminergic terminals of the SNc, by interacting with D_2 receptors (red) at dopaminergic or glutamatergic terminals, or at MSN of the indirect pathway. TAAR1 may affect glutamate release indirectly by enhancing dopamine release from nigrostriatal terminals, thus enhancing activation of postsynaptic D_2 receptors similarly to ropinirole. This effect would be abolished by blockade of CB_1 receptors. CB_1R: cannabinoid receptor type 1; DA: dopamine; eCB: endocannabinoids, Glu: glutamate; mGluR: metabotropic glutamate receptor.

4.3 AIM III: BEHAVIOURAL CHARACTERIZATION OF THE cLMX1A/B^{DATCre} TRANSGENIC MOUSE MODEL

Paper III focused on the roles of the developmental transcription factors Lmx1a and Lmx1b in the maintenance of neuronal properties of relevance for neuropathological conditions, particularly PD. Lmx1a and Lmx1b are two highly related LIM homeobox transcription factors required for the early specification of neuronal stem cells into ventral mDA neurons. The function of Lmx1a and Lmx1b in maturing and adult mDA neurons was investigated by analysing the consequences of mDA neuron-specific ablation in conditional knockout mice using histology, in situ hybridization, stereological cell counting, behavioural tests, electrophysiology, high-performance liquid chromatography, electron microscopy, and immunoblotting. The cLmx1a/b^{DATCre} mouse model used in these experiments harboured floxed LMX1A and LMX1B and a cre recombinase expressed under DAT, which starts to be expressed at embryonic day 13.5. Immunostaining of Lmx1b was also performed in human postmortem tissue from healthy subjects and patients with PD. This study revealed that conditional ablation of Lmx1a/b after mDA neuron specification resulted in abnormalities that resemble early features of PD. Expression of Lmx1a was rapidly decreased at postnatal stages, whereas Lmx1b remained continuously expressed throughout life. Electron micrographs revealed an abnormal morphology of dopaminergic nerve terminals following Lmx1a/b ablation, characterized by giant nerve terminals and an accumulation of autophagosomes and endosome-like structures in the axoplasm. Lmx1b expression was shown to be decreased in neuromelanin-positive neurons in postmortem brain tissue from PD patients. A battery of behavioural tests was performed in adult mice (6-9 months of age) and old mice (18-21 months of age). A summary of the behavioural phenotype is shown in Figure 4.3. The behavioural test battery revealed that both adult and old animals were impaired in the wooden block test (Figure 4.3A), a test of social olfactory functions. Both adult and old animals also required longer time to complete the pole test compared with WT animals (Figures 3.2.1A and 4.3D), whereas old animals required a longer time to complete the beam traversal test (Figure 3.2.1B), indicating a presence of progressive motor impairments. Adult animals were also impaired in the novel object recognition test (Figures 3.2.2B and 4.3B), indicating a mild cognitive phenotype emerging early in the pathology, likely related to pathological changes in the prefrontal cortex and striatum as detected in adult animals. No alterations in exploratory behaviour were revealed in the novel object recognition or open field tests (Figure 4.3C and E). Both working memory and long-term memory functions, assessed using the T-maze (Figure 3.2.2A) and passive avoidance tests (Figure 3.2.2C), remained intact in Lmx1a/b^{DATCre} KO mice at both age intervals. No depressive- or anxiety-like behaviours were found in Lmx1a/b^{DATCre} KO mice using the Porsolt swim test (Figure 3.2.3A), sucrose preference test (3.2.3B), elevated plus maze (3.2.3C) or by assessing thigmotaxis in the open-field test (Figures 3.2.3D and 4.3F). This suggests that genetic ablation of Lmx1a/b does not induce any emotional behaviour, and although a significant loss of TH was found in the VTA in old animals, this lack of emotional phenotype suggests that there were no major alterations in the serotonergic and noradrenergic neurotransmitter systems. These findings indicate that Lmx1a/b-ablation in dopaminergic neurons lead to impaired motor functions, impaired short-term memory and impaired olfaction in adult animals. Lmx1a/b was also required for the normal execution of the autophagic-lysosomal pathway, for the integrity of dopaminergic nerve terminals and for long-term mDA neuronal survival. In conclusion, the results of this study reveal that Lmx1b is required for the function of mature midbrain mDA neurons, and that this model recapitulates several features of PD in humans. Lmx1b may thus be associated with the pathogenesis of PD, likely via its role in the autophagic-lysosomal pathway.

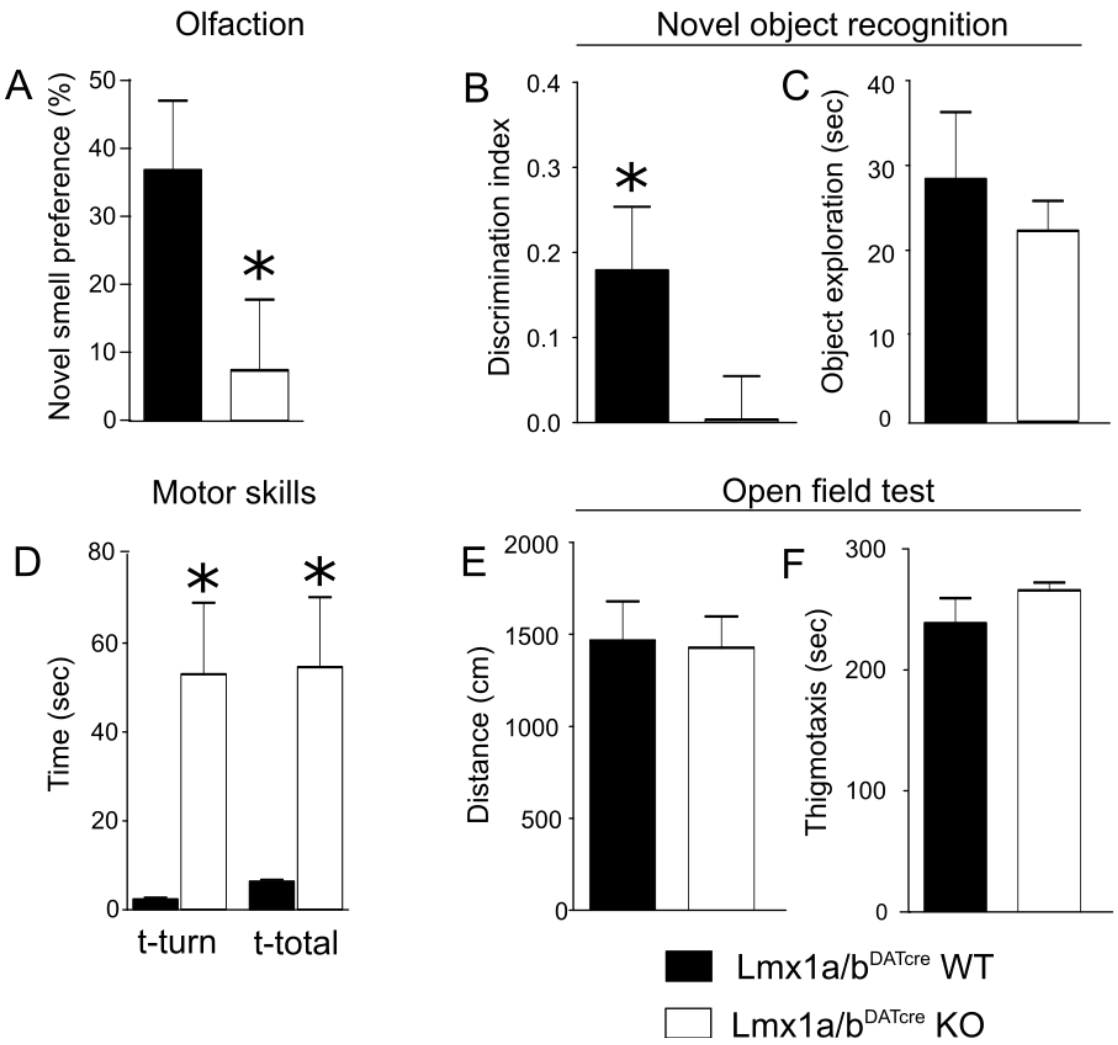


Figure 4.3 Summary of the behavioural phenotype of adult WT and Lmx1a/b^{DATCre} KO animals, revealing olfactory, cognitive and motor impairments, but intact exploratory behaviour. (A) The wooden block test revealed a significantly decreased preference for the novel smell of an unknown animal compared to the animal's own smell in Lmx1a/b^{DATCre} KO animals. (B) Lmx1a/b^{DATCre} KO animals did not prefer a novel object before a familiar one, seen as a discrimination index close to 0, whereas WT animals prefered the novel object. (C) There was no difference between the genotypes in the total time spent exploring the object pair during the training phase, indicating that the poor performance of Lmx1a/b^{DATCre} KO animals was not related to altered exploratory behaviour. (D) Lmx1a/b^{DATCre} KO animals required a longer time to turn and climb down during the pole test compared with WT animals, indicating impairments of posture and fine motor skills. (E) The total distance travelled and (F) the time spent in the thigmotaxis zone did not differ between the genotypes in the open field test, indicating intact exploratory behaviour and lack of anxiety-like behaviour in Lmx1a/b^{DATCre} KO animals. The open field test is an important control measure, since most behavioural tests are affected by the basal level of exploratory behaviour.

4.4 AIM IV: THE ROLE OF α-SYNUCLEIN OVEREXPRESSION IN THE VTA IN THE SYMPTOMATOLOGY OF PARKINSON'S DISEASE

The accumulation of Lewy bodies, misfolded protein aggregates containing the misfolded protein α-synuclein, is a major pathological hallmark of PD. However, neurodegeneration in PD is not restricted to the substantia nigra, but also affects the VTA, where α-synuclein aggregates are also found in early stages of the disease. The VTA mainly projects to the frontal cortex, the ventral striatum, amygdala and the hippocampus, and degeneration of these projections are thought to play a role in the cognitive and emotional symptoms of PD. Lewy body accumulation in the VTA correlates with the onset of cognitive impairments, but the impact of the VTA in the symptomatology of PD remains elusive. In study IV, we overexpressed α-synuclein specifically in the VTA using AAV vector constructs in order to evaluate the pathological impact on motor- and non-motor symptoms across time. Immunohistochemical stainings were performed to assess neuropathological features. The ledged beam test, described in Figure 3.2.1A, was used to monitor motor functions, and the step-through passive avoidance test was used to evaluate emotional contextual memory functions. Step-through passive avoidance is a paradigm based on Pavlovian fear conditioning, and is described in Figure 3.2.2C. Being prey animals and nocturnal, rodents naturally prefer dark areas over brightly illuminated ones. By coupling a preferred dark compartment (the conditioned stimulus) with an aversive stimulus (the unconditioned stimulus), in this case a weak electric current, the animal can be taught to prefer the light compartment, i.e. to display passive avoidance of the dark compartment. Fear learning is dependent on the amygdala (Phillips et al., 2010) and prefrontal cortex (Abercrombie et al., 1989; Gonzalez et al., 2014), whereas the hippocampus is involved in complex memory functions, including the mental representation of complex environments (Baarendse et al., 2008; Kim and Fanselow, 1992; Morris et al., 1982; Selden et al., 1991), but not in basic associative functions (Schmaltz and Theios, 1972; Solomon, 1977; Winocur et al., 1987).

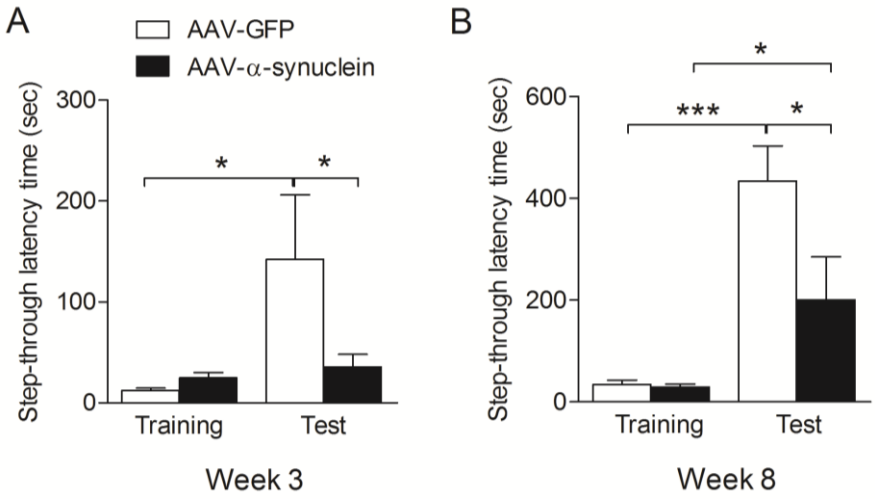


Figure 4.4 Passive avoidance performance in GFP and α-synuclein overexpressing rats 3 and 8 weeks following AAV injection. (A) 3 weeks after AAV injection only AAV-GFP injected animals learned the passive avoidance task, indicated as an enhanced step-through latency during the PA test phase compared with during the training phase. The AAV-GFP group also displayed a significantly prolonged step-through latency during the passive avoidance test phase compared with the AAV-α-synuclein group. (B) Upon re-exposure to the passive avoidance test 8 weeks following vector injection both groups learned the task. However, the step-through latency was still significantly prolonged in the AAV-GFP injected group compared with the AAV-α-synuclein group, indicating a better memory performance in this group. Two-way ANOVA followed by Fisher's LSD *post hoc* test, *p<0.05; ***p<0.001

Thus, the association of a unisensory cue with a fear response is mediated by the hippocampus when this cue is presented in a complex environment, making the passive avoidance task a measure of hippocampus-dependent contextual and emotional memory. Thus, the retention performance, i.e. the time it takes before the rodent enters the dark compartment again during the passive avoidance retention test, is a measure of emotional memory, and is sensitive to changes in serotonergic, glutamatergic and cholinergic neurotransmission (Madjid et al., 2006; Ogren, 1985). The Porsolt swim test, elevated plus maze and sucrose preference tests were performed to investigate depressive-like and anxiety-like behavior.

These experiments demonstrated that α-synuclein overexpression in dopaminergic neurons of the VTA induced a reduced number of TH+ neurons in the VTA, but not in the substantia nigra, 8 weeks after AAV-α-synuclein vector injection. Motor deficits appeared 3 weeks following AAV-α-synuclein vector injection and were limited to an increased number of stepping errors when crossing a beam, progressing in severity across time. Emotional memory impairments were revealed in the passive avoidance test 8 weeks following AAV-α-synuclein vector injection (Figure 4.4). However, no depressive- or anxiety-like behavior was found. These findings indicate that Lewy body accumulation in the VTA may play a role in some motor impairments related to body control, and in cognitive impairments related to emotional contextual memory.

4.5 GENERAL CONCLUSIONS

The theme of this thesis has been novel means to induce various aspects of PD in experimental animals to provide a deeper understanding of the disease processes and how it interacts with the symptomatology of PD. In conclusion, this work has revealed how both pathological and behavioural features related to early PD can be induced by ablation of the developmental transcription factors Nurr1 and Lmx1a/b, previously known to play elusive roles in adult dopaminergic neurons and PD in humans. Using the cNurr1^{DATCreER} KO model as a bilateral model of early PD, we also unveiled a potential role of TAAR1 in experimental PD. However, future studies will be warranted to understand the complex modulatory and sometimes discrepant properties of TAAR1 in Parkinsonian states. Finally, overexpression of α-synuclein in VTA was shown to induce cognitive and mild motor impairments paralleled by a loss of dopaminergic neuron integrity restricted to the VTA, but no anxiety- or depressive-like behavior, suggesting a role of α-synuclein aggregation in the VTA in the cognitive symptoms of PD. Hopefully these data will contribute to a better understanding of the early pathological disease stages, to improved experimental models and ultimately to better treatment regiments in PD.

5 ACKNOWLEDGEMENTS

I would here like to express my sincere gratitude to everyone who has contributed with support, encouragement and inspiration throughout these years. Many people have been involved directly or indirectly in this work. However, in particular I would like to thank:

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