Towards therapies for Parkinson's disease: treatment-related complications and animal models

by

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Table of Contents

List of tables

List of abbreviations

5-HT	5-hydroxytryptamine
5-HT_{1A}	5-hydroxytryptamine type 1A
5-HT_{1B}	5-hydroxytryptamine type 1B
5-HT_{2A}	5-hydroxytryptamine type 2A
5-HT_{2C}	5-hydroxytryptamine type 2C
5-HT_{3}	5-hydroxytryptamine type 3
6-OHDA	6-hydroxydopamine
AIMs	abnormal involuntary movements
AMPA	amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
cAMP	cyclic adenosine monophosphate
COMT	catechol-O-methyltransferase
DAT	dopamine transporter
DDCI	dopa decarboxylase inhibitor
FDA	Food and Drug Administration
fMRI	functional magnetic resonance imaging
GABA	γ-aminobutyric acid
GP	globus pallidus
GPe	globus pallidus pars externa
GPi	globus pallidus pars interna
IPMDS	International Parkinson and Movement Disorders Society
L-DOPA	L-3,4-dihydroxyphenylalanine
LRRK2	leucine-rich repeat kinase 2
MAO	monoamine oxidase
MAO-B	monoamine oxidase B
MAPT	microtubule-associated protein tau
MDS	Movement Disorders Society
mGlu	metabotropic glutamate
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	magnetic resonance imaging

NIMH	National Institute of Mental Health
NINDS	National Institute of Neurological Diseases and Stroke
NMDA	N-methyl-D-aspartate
PD	Parkinson's disease
PET	positron emission tomography
PFFs	pre-formed fibrils
RBD	rapid eye movement sleep behaviour disorder
SERT	5-hydroxytryptamine transporter
SPECT	single-photon emission computed tomography
SN	substantia nigra
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
SSRI	selective 5-hydroxytryptamine reuptake inhibitor
STN	subthalamic nucleus
TH	tyrosine hydroxylase
UPDRS	Unified Parkinson's Disease Rating Scale

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Abstract

Parkinson's disease (PD) is an increasingly prevalent neurodegenerative disorder with the global burden of disease expected to double in the next few decades. L-3,4-dihydroxyphenylanaline (L-DOPA) remains the mainstay symptomatic treatment for motor features. Despite its benefit in early disease, long-term L-DOPA therapy is accompanied by side effects, with most patients eventually developing L-DOPA induced dyskinesia. Our lab has focussed on the investigation of new therapeutic targets for treatment-related complications in neurotoxin-based animal models of PD. Preclinical and clinical studies have implicated an aetiological role for the dysregulated release of striatal dopamine in dyskinesia. Evidence from in vitro and in vivo studies has found that blockade of the serotonin type 3 (5-HT_3) receptor dampens the release of striatal dopamine, which may provide anti-dyskinetic benefit. Recent behavioural studies suggest that the 5-HT_3 antagonist ondansetron alleviates dyskinesia in the hemi-parkinsonian rat model. The objective of the current thesis was to examine the efficacy of 5-HT_3 blockade and its mechanism of action in treatment-related complications in PD, as well as to develop the methodology to construct a new animal model of PD to evaluate disease-modifying therapies. Neurotoxin-based experimental models of PD have led to the failure of several high-profile clinical trials that examined the effect of therapeutics at slowing disease progression, in part because they do not replicate the processes at play in the human disease, *e.g.*, the abnormal propagation of alpha-synuclein within the brain. Chapter 1 describes brain and plasma levels of ondansetron associated with doses that provide therapeutic effects in the rat, contextualising the literature over a range of indications. Chapter 2 expands the profile of 5-HT_3 blockade in dyskinesia with granisetron, another 5-HT_3 antagonist, which also significantly improved

dyskinesia in the hemi-parkinsonian rat without impairing the therapeutic efficacy of L-DOPA. Chapter 3 extends the anti-dyskinetic findings of ondansetron obtained in the rat to the parkinsonian non-human primate, while also demonstrating an improvement in global parkinsonism and psychosis-like behaviours severity. In Chapter 4, novel psychosis-like behaviours are described in L-DOPA treated parkinsonian marmosets, enriching the existing behavioural paradigm to characterise the anti-psychotic potential of experimental drugs. Chapter 5 provides the first investigation into the mechanism underlying the anti-dyskinetic efficacy of 5-HT_3 antagonists. Compared to sham-lesioned animals, there was a regional selective upregulation of 5-HT_3 receptors in L-DOPA treated hemi-parkinsonian rats, predominantly in the subthalamic nucleus, and to a lesser extent in the globus pallidus and ventral anterior/ventral lateral thalamus. Moving away from symptomatic therapies in toxin-based models and towards models that permit the evaluation of disease-modifying therapies, Chapter 6 describes the methodology that couples subject-specific registration with frameless stereotaxic neuronavigation to localise and inject alpha-synuclein fibrils in the putamen of two marmosets. Evidence of nigrostriatal denervation suggestive of accurate targeting was obtained, with a novel procedure that enhanced the accuracy of stereotaxic neurosurgeries in marmosets. Taken together, these results provide support for the therapeutic potential of 5-HT_3 antagonism in L-DOPA induced dyskinesia and PD psychosis, providing compelling data to move such drugs to clinical trials. Moreover, the development of a new alpha-synuclein based model in the marmoset represents an important step towards the testing of experimental molecules with disease-modifying potential in PD with higher chance of success upon translation to the clinic.

Résumé

La maladie de Parkinson (MP) est un trouble neurodégénératif de plus en plus répandu avec un fardeau global qui doublera au cours des prochaines décennies. La L-3,4-dihydroxyphénylanaline (L-DOPA) reste le principal traitement symptomatique des troubles moteurs. Malgré ses bénéfices, le traitement à long terme à la L-DOPA est accompagné d'effets secondaires et les patients présentent des dyskinésies. Notre laboratoire se concentre sur des nouvelles cibles visant à réduire les complications liées au traitement de la MP. Des études ont mis en évidence le rôle étiologique de la libération dérégulée de dopamine striatale dans la dyskinésie. Des données ont montré que le blocage du récepteur de la sérotonine de type 3 (5-HT_3) atténue la libération de dopamine striatale, ce qui pourrait apporter un effet anti-dyskinétique. Des études suggèrent que l'antagoniste des récepteurs 5-HT_3 ondansétron soulage la dyskinésie dans le rat hémi-parkinsonien. L'objectif de cette thèse était d'examiner l'efficacité du blocage des récepteurs 5-HT_3 et son mécanisme d'action dans les complications liées au traitement de la MP, ainsi que de développer la méthodologie pour construire un modèle animal de la MP afin d'évaluer les thérapies modificatrices de la maladie. Les modèles neurotoxiques de la MP ont mené à l'échec des essais cliniques qui examinaient des médicaments pour ralentir la progression de la maladie, en grande partie parce qu'ils ne reproduisent pas les processus en jeu observés chez les patients, telle que la propagation anormale de l'alpha-synucléine dans le cerveau. Le chapitre 1 décrit les niveaux de l'ondansétron associés aux doses qui procurent des effets thérapeutiques chez le rat, mettant en contexte la littérature. Le chapitre 2 élargit le profil du blocage des récepteurs 5-HT_3 dans la dyskinésie avec l'antagoniste des récepteurs 5-HT_3 granisétron qui a amélioré la dyskinésie chez le rat hémi-parkinsonien sans diminuer l'efficacité

thérapeutique de la L-DOPA. Le chapitre 3 étend les résultats anti-dyskinétiques de l'ondansétron obtenus chez le rat au primate non-humain, tout en démontrant une amélioration de la sévérité du parkinsonisme et des comportements de type psychose. Le chapitre 4 décrit de nouveaux comportements de type psychose chez des ouistitis parkinsoniens traités à la L-DOPA, enrichissant le paradigme comportemental pour caractériser le potentiel antipsychotique des médicaments. Le chapitre 5 présente la première étude sur le mécanisme qui sous-tend l'efficacité anti-dyskinétique des antagonistes des récepteurs 5-HT_3. Par rapport aux animaux ayant subi une lésion factice, on observe une augmentation régionale sélective des récepteurs 5-HT_3 chez les rats hémi-parkinsoniens traités à la L-DOPA, principalement dans le noyau sous-thalamique et, dans une moindre mesure, dans le globus pallidus et le thalamus ventral antérieur/ventral latéral. S'éloignant des thérapies symptomatiques dans les modèles basés sur les toxines et s'orientant vers des modèles qui permettent l'évaluation de thérapies modificatrices de la maladie, le chapitre 6 décrit la méthodologie qui couple l'enregistrement spécifique au sujet avec la neuronavigation stéréotaxique sans cadre pour localiser et injecter des fibrilles d'alpha-synucléine dans le putamen de deux ouistitis. La preuve de dénervation nigrostriatale suggère un ciblage précis avec cette nouvelle procédure qui a amélioré la précision des neurochirurgies stéréotaxiques chez les ouistitis. Dans l'ensemble, ces résultats soutiennent le potentiel thérapeutique de l'antagonisme des récepteurs 5-HT_3 dans la dyskinésie et la psychose dans la MP, fournissant des données convaincantes pour amener ces médicaments jusqu’aux essais cliniques. Le développement d'un nouveau modèle basé sur l'alpha-synucléine chez le ouistiti représente une étape importante vers l'essai de molécules ayant un potentiel de modifier la MP, avec de meilleures chances de succès lors de leur passage en clinique.

Chapter 1 - Introduction

1. General Introduction

1.1. Parkinson's disease

1.1.1. Epidemiology

1.1.1.1. Prevalence and incidence

Depending on the design of epidemiological studies, measures of the incidence and prevalence of Parkinson's disease (PD) have varied. The prevalence of PD is estimated between 100 to 300 current cases per 100,000 persons, whereas the incidence of PD is estimated at 10 to 50 new cases per 100,000 persons [1, 2]. By 2040, the global burden of PD is projected to exceed 17 million due to growth of the aging population and environmental factors [3].

1.1.1.2. Age and sex

The risk of developing PD increases sharply around the age of 65 with a mean age of onset of 60 years of age [4], although some patients under 50 also develop the disease [5], termed young-onset PD. In general, epidemiological studies have found an increased male to female ratio incidence of 1.5:1 of developing PD [6-8]. There is some evidence suggesting that biological sex differences, such as neuroprotective effects of oestrogen in women [9, 10] and sex chromosome genes [11, 12], may underlie differences in disease susceptibility. These findings remain controversial, however, as other studies reported an absence of sex-linked effects [1, 13] or female predominance [14, 15], and as such, the precise nature of sex differences in PD remains unclear.

1.1.1.3. Geographical variation

Studies have reported geographical differences in the frequency of PD with a slightly lower prevalence rate in Asia and Africa compared to Western countries [2, 16, 17], whereas other studies obtained similar estimates [18, 19]. Moreover, the prevalence rate of PD was higher for African Americans than Africans in Nigeria [20], which was similarly observed in Japanese Americans compared to Japanese [21], and suggests a role for environmental factors. Due to the inconsistent findings, it remains difficult to parcellate whether differences are due to genetic factors, environmental factors or methodological differences in sampling population or patient characteristics.

1.1.1.4. Risk factors

Environmental risk factors

Smoking and caffeine intake are well-established protective factors that are inversely associated to the risk of PD [22, 23], while pesticide exposure is closely linked [24]; the mechanism of action underlying their effects are unclear. Brain injury, urate levels and consumption of dairy products are also associated with risk of PD [25-27], whereas results from studies on intake of dietary fats and body mass index have been inconsistent [28-30].

Genetic risk factors

Although the majority of PD cases are sporadic, a positive family history has been reported in 10% of patients [31] and is associated with a 3-4% increased risk of developing PD [30]. These findings suggest a genetic contribution to the disease, which is further supported by the discovery of monogenic forms of PD [32]. The gene that encodes alpha-synuclein, *SNCA*, was the

first gene associated with inherited PD [33]. Mutations in the leucine-rich repeat kinase 2 (*LRRK2*) gene are the most frequent cause of dominantly inherited PD, whereas parkin gene mutations are the most common cause of recessively inherited PD [34]. Mutations in *GBA*, which encodes β-glucocerebrosidase, the lysosomal enzyme deficient in Gaucher disease, are the greatest genetic risk factor for developing PD [35]. Despite advances in genome-wide association studies involving large case-control cohorts of PD and control patients that have led to the identification of robust association signals [32, 36], including *SNCA*, microtubule-associated protein tau (*MAPT*) [37] and *LRRK2* loci [38], the associated loci do not account for all the genetic variance underlying PD [39], which suggests that gene-environment interactions may also contribute to disease susceptibility [36, 40].

1.1.2. Genetics

Over the last two decades, significant strides in the genetics of PD have led to an enhanced understanding of the pathogenesis of PD and new therapeutic approaches. Several genes have been proposed to mediate autosomal dominant and recessive forms of PD, however, convincing evidence only links *SNCA*, *LRRK2*, *VPS35*, *PRKN*, *PINK1*, *GBA* and *DJ-1* with typical PD [36]. The role of these genes extends beyond the scope of the present review and has been excellently presented previously [41, 42]. Age of onset of autosomal dominant PD is comparable to sporadic PD [32, 43], whereas recessively inherited parkinsonism is more frequently associated with early onset (before age 40 years) [44]. Analysis of genes linked to PD has consistently implicated several biological pathways in disease pathogenesis, including autophagy, mitochondrial quality control, endocytosis, lysosomal function and immune response, although the pathogenesis of PD likely culminates from multiple dysfunctional

pathways [41]. Whereas the majority of PD genetics research have studied European populations [38], it is unclear whether findings of genetic risk can be extended across populations [36], and concerted efforts to build genetic databases with participants from diverse ancestry will be informative for genetic risks associated with diverse populations [36].

1.1.3. Neuropathology

At the macroscopic level, reports have revealed mild atrophy of frontal lobe [45] and ventricular dilation [46] in some PD patients. In contrast, morphological changes are more apparent in the brainstem with the loss of dark pigment in the substantia nigra (SN) pars compacta (SNc) and locus coeruleus [47], an effect that correlates with the death of dopaminergic neurons in the SNc and noradrenergic neurons in the locus coeruleus [48]. Neuronal loss in the SN also correlates with severity of motor symptoms and disease duration [49-51]; while 30% of dopamine neurons in the SNc are lost by the onset of motor symptoms [52-54], neuronal loss increases to 60% or higher after the appearance of motor features [50, 55]. This neuronal loss leads to the denervation of the nigrostriatal pathway and reduced striatal dopamine levels [56], and this diminished dopaminergic signaling is a likely cause for the manifestation of motor features in PD, particularly bradykinesia and rigidity [57]. In addition to degeneration of dopaminergic neurons in PD, cell loss has been reported in the pedunculopontine nucleus, locus coeruleus, dorsal motor nucleus of the vagus, nucleus basalis of Meynert, and to a lesser extent the raphe nucleus [58]. This widespread neurodegeneration implicates a role for cholinergic, glutamatergic, γ-aminobutyric acid (GABA)-ergic, and serotonergic systems, and may explain the appearance of non-motor symptoms in PD [59, 60].

At the microscopic level, a hallmark of PD pathology is the presence of Lewy bodies, which consist of abnormal deposits of the protein alpha-synuclein in the cytoplasm of neuronal cell bodies [56, 61]. Lewy bodies are often accompanied by dystrophic neurites, termed Lewy neurites, which are mainly found in axons [62]. Lewy pathology can also extend outside of the brain and has been found in the spinal cord and peripheral nervous system [32], including the vagus nerve, enteric nervous system, and cardiac plexus [63-66]. From observation of post-mortem brains of PD subjects, Braak and colleagues formalised a hypothesis to describe the stereotypical spreading of Lewy body pathology based on the correlation between neuropathological findings with pre-clinical and clinical phases of the disease [67, 68]. The Braak hypothesis was revised to propose that Lewy pathology may be initiated in the olfactory bulb and enteric cell plexuses and enter the brain through nasal and gastric routes, respectively, forming the dual-hit hypothesis [69]. In early stages, Lewy bodies were confined to the brain stem and olfactory bulb, before spreading to the midbrain, and by later stages, throughout the lower forebrain and cortex. Evidence in support of the Braak hypothesis came from clinical trials where embryonic dopaminergic neurons were grafted into the brains of PD patients [70, 71]. Post-mortem analysis of the brains of PD patients revealed pathological inclusions in the healthy donor neurons over 10 years after transplantation, which suggests that Lewy pathology can spread from host to donor and that aggregated alpha-synuclein can seed the misfolding of endogenous alpha-synuclein [72]. However, Braak staging has also been controversial for a few reasons [56]: 1) some PD brains do not follow the stereotyped caudo-rostral propagation of alpha-synuclein pathology [73, 74]; 2) the lack of correlation in clinico-pathologic studies [75]; and 3) poor link between Lewy pathology and neuronal loss [75, 76].

1.1.4. Pathophysiology

Several mechanisms have been proposed to underlie the pathophysiology of PD, including the misfolding and aggregation of alpha-synuclein, mitochondrial dysfunction, impairment of protein clearance, and neuroinflammation. Although all these mechanisms may promote apoptosis or necrosis [77], a remaining issue that warrants further investigation is the relationship between these pathogenic factors. It remains unclear whether they converge on a common downstream pathway to neuronal death [78] or more controversially, if they represent separate pathogenetic pathways that define separate forms of PD [79].

1.1.4.1. Alpha-synuclein

Although the function of alpha-synuclein is unclear, studies implicate its involvement in synaptic plasticity and neurotransmitter release [80, 81]. Under physiological conditions, the native structure of alpha-synuclein is in dynamic equilibrium between unfolded monomers and α-helical folded tetramers [82, 83] but in PD, it adopts a β-sheet rich structure that is prone to aggregation into oligomers, protofibrils, and insoluble fibrils that accumulate in Lewy bodies [84]. Moreover, post-translational modifications, including serine 129 phosphorylation [85] and ubiquitination [86], as well as mutations, imbalance between synthesis and degradation of alpha-synuclein, and environmental factors, lead to conformational changes in alpha-synuclein that render it more prone to aggregation [84]. Although the mechanisms underlying the initiation and spreading of pathological alpha-synuclein in PD are not well understood, converging evidence suggests that a prion-like propagation may explain this phenomenon [87]. Once monomeric alpha-synuclein becomes misfolded, it can act as a seed to recruit endogenous synuclein, convert it into insoluble pathological polymers and, ultimately, Lewy bodies [88]. This pathological alpha-

synuclein then spreads throughout the brain in interconnected and neighbouring areas, eventually propagating to the entire brain [89]. As discussed in Section 4.3, paradigms that involve injection of fibrillar alpha-synuclein in the brain or gut produced Lewy-like pathology in the brains of rodents and non-human primates, similar to Braak staging observed in post-mortem PD brains [67].

1.1.4.2. Mitochondrial dysfunction

Defective mitochondrial function plays a crucial role in the pathogenesis of sporadic and familial forms of PD [90]. In particular, deficiency of mitochondrial complex I has been reported in the SNc [91] and skeletal muscle [92] of PD subjects. In further support for the role of mitochondrial dysfunction in PD, compounds that inhibit mitochondrial complex I activity, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [93], the prodrug to the neurotoxin 1-methyl-4-phenylpyridinium (MPP+) [94], and the pesticide rotenone [95, 96], have caused parkinsonian features in animal models [97, 98] and humans [99, 100]. Genetics research also confirms the importance of mitochondrial dysfunction in PD [32]; mutations in genes involved in mitochondrial health, such as *PRKN* and *PINK1* [101], lead to impaired mitochondrial quality control and cause autosomal recessive forms of PD [102].

1.1.4.3. Dysfunctional protein clearance

The ubiquitin-proteasome system and the autophagy-lysosomal pathway are two protein degradation systems that may be compromised in PD [103, 104]. Studies have found that inhibition of proteasomal activity [105, 106] leads to the accumulation of alpha-synuclein or production of ubiquitinated alpha-synuclein aggregates [107-109], and results in inefficient degradation of

misfolded proteins in sporadic PD [103]. Protein quality control is also regulated by autophagy [104] and studies have revealed an accumulation of autophagy-related proteins with alpha-synuclein pathology in PD [110-112].

1.1.4.4. Neuroinflammation

Both abnormal innate and adaptive immunity responses have been implicated in PD [77], including increased levels of proinflammatory cytokines [113], presence of lymphocyte infiltrates [114] and microglial activation [115] in the brains of PD subjects. Moreover, rodent models of PD demonstrated that suppressing microglial activation inhibited degeneration of the SNc [116, 117]. These results implicate the contribution of microglial-induced inflammation to neurodegeneration in PD [56]; consistent with reports that alpha-synuclein can trigger neuroinflammation [118, 119]. Considering the importance of the cross talk between brain parenchyma and peripheral immune system in underlying the disease [120], regulation of aberrant neuroinflammatory processes may provide opportunities for the development of new immune-targeted therapies for PD [121].

1.1.5. Diagnosis

Clinical diagnosis of PD has been classically based on a defined motor syndrome, i.e., bradykinesia, in combination with rest tremor, rigidity, and postural instability [32]. However, motor symptoms are often preceded by prodromal and nonmotor phases of the disease [122], which has led to the identification of prodromal, preclinical, prodromal and clinical stages of PD [123] and ensuing changes to the diagnostic criteria of the disease [124]. Dopaminergic imaging with positron emission tomography (PET) or single-photon emission computed tomography

(SPECT) imaging is a validated non-invasive approach to measure reductions in striatal dopamine levels [32]. However, neither are required for PD diagnosis as they cannot distinguish causes of dopamine deficiency and cannot differentiate the disease from other disorders that present with degeneration of the SNc (e.g., progressive supranuclear palsy, multiple system atrophy and dementia with Lewy bodies) [32, 125]. The gold standard of PD diagnosis remains neuropathological confirmation of neuronal loss in the SNc with Lewy bodies in surviving neurons [126]. The high misdiagnosis rate of PD [127], as well as the delayed diagnosis and lack of disease-modifying therapies, underscore the urgent need to develop validated biomarkers [128]. The discovery of diagnostic and prognostic biomarkers in PD would facilitate distinguishing it from other conditions, monitoring disease progression, or provide an indicator of a positive response to a therapeutic intervention [129, 130]. Recent advances in PD biomarker research include clinical prodromal biomarkers, biochemical fluid biomarkers, and imaging biomarkers, which are comprehensively reviewed [128, 129, 131].

1.1.6. Symptoms

1.1.6.1. Motor symptoms

First recognised by James Parkinson and later refined by Jean-Martin Charcot [132], the cardinal motor features of PD consist of bradykinesia and at least one or more of the following: rigidity, rest tremor, and postural instability [133]. Compared to other motor symptoms, postural instability is more commonly associated with advanced disease [134]. Nonetheless, evidence from neuroimaging and pathological studies suggest that motor symptoms of PD appear only when degeneration of the SN reaches 50% to 70% [135]. PD symptoms are progressive but clinical presentation and the rate of motor progression are highly variable [136, 137]. Evidence from clinical

observations propose three motor subtypes of PD where tremor, rigidity or postural impairment dominate but there remains a lack of consensus on the classification of PD subtypes [138]. Importantly, subtypes may have distinct prognoses and aetiologies [32, 139], for example, tremor-dominant PD is linked to slower progression and less functional disability [140]. While motor symptoms are managed with symptomatic therapies, with disease progression, patient outcome is encumbered by issues such as treatment-resistant therapies [32] as well as the development of treatment-related complications [141, 142].

1.1.6.2. Non-motor symptoms

During their disease course, as many as 90% of patients with PD present with a wealth of non-motor symptoms [143] and the burden of these symptoms has a superior impact on quality of life compared to the burden associated with motor symptoms [144, 145]. Some non-motor symptoms often precede the onset of motor symptoms [146], including olfactory dysfunction, constipation, and sleep disorders, and have been grouped together to form the prodromal phase [123]. Identification of patients during this stage when the nigrostriatal pathway is relatively intact may provide an ideal window for disease-modifying therapies that prevent or delay disease progression [147]. On the other hand, some autonomic symptoms, such as constipation and urinary incontinence, are common features of late stage PD [141, 148]. Dementia and psychosis are also highly prevalent in PD patients and 20 years after disease onset, 83% and 74% of patients were afflicted with these conditions, respectively [148]. Unlike motor symptoms, non-motor symptoms (e.g., neuropsychiatric symptoms and sleep disorders) are often unresponsive to dopaminergic therapy, which implicates the involvement of other neurotransmitter systems in their aetiology [149].

1.1.7. Management

Pharmacological treatment is the most common therapy for PD and drugs that correct the dopamine deficiency causing motor symptoms [150] by enhancing intracerebral dopamine concentrations or stimulating dopamine receptors, remain the mainstay treatment [32]. While there are no disease-modifying therapies available, current treatments provide relief for the management of both motor and non-motor symptoms [151].

1.1.7.1. L-DOPA

The dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) is the most effective therapy for PD [152] and often administered with decarboxylase inhibitors such as benserazide or carbidopa [153]. When administered alone, L-DOPA is mainly decarboxylated peripherally and little remains available to cross the blood-brain barrier and penetrate the central nervous system [154]. Moreover, several side effects associated with L-DOPA, including nausea, vomiting, and orthostatic hypotension [155-157], are related to its peripheral conversion by DOPA decarboxylase [154]. Thus, the addition of peripherally acting decarboxylase inhibitors to L-DOPA prevents its peripheral conversion to dopamine and reduces both undesirable effects and dosage of L-DOPA [158, 159]. Nonetheless, long-term L-DOPA therapy is accompanied by the development of complications, such as L-DOPA-induced dyskinesia (discussed in Section 2) and motor fluctuations [160, 161]. These complications may be related to pulsatile stimulation of dopamine receptors in the striatum [32], which has led to efforts to develop long-acting formulations [162] and

other modes of delivery of L-DOPA [163] to provide more stable dopamine concentrations and, consequently, improved clinical benefit [164].

1.1.7.2. Dopamine agonists

Dopamine agonists act directly on dopamine receptors and are classified as ergot (bromocriptine, pergolide, lisuride, and cabergoline) and non-ergot derivatives (apomorphine, pramipexole, and ropinirole) [165]. They offer several advantages over L-DOPA: 1) longer duration of action [166-168]; 2) no requirement for enzymatic activation [169]; 3) no production of toxic metabolites [170]; 4) no competition for active transport into blood and to cross blood-brain barrier; 5) no dependence on functional capacities of nigrostriatal neurons [150]. Although studies have reported a lower incidence of dyskinesia with dopamine agonists compared to L-DOPA [171-175], once L-DOPA was added, rate of dyskinesia development was comparable between both groups [176]. Compared to dopamine agonists, L-DOPA provides superior benefit for the treatment of motor symptoms, as well as fewer side effects [177-179]. Therefore, the practice guideline by the American Academy of Neurology (AAN) recommends L-DOPA as the initial preferential dopaminergic therapy for motor symptoms in patients with early PD [179]. Furthermore, dopamine agonists are also associated with impulse control disorders, which occur in 15% to 20% of PD patients taking dopamine agonists [177, 178]. Other adverse events include hallucinations, nausea and vomiting, and dry mouth [179-182]; dopamine withdrawal syndrome is another condition associated with PD patients that undergo a dopamine agonist taper [183].

1.1.7.3. COMT inhibitors

Catechol-O-methyltransferase (COMT) mediates a significant proportion of peripheral L-DOPA metabolism [184, 185] and the addition of a COMT inhibitor as an adjunctive therapy to L-DOPA and a decarboxylase inhibitor extends L-DOPA duration of action by prolonging its half-life and its delivery to the brain [186]. COMT inhibitors are often prescribed as adjunctive therapy [153] because they offer limited to no benefit for motor features when administered as monotherapy [151]. COMT inhibitors include entacapone and tolcapone [153] but entacapone is preferred due to rare cases of fatal hepatotoxicity [187]. Moreover, the third-generation COMT inhibitor opicapone was developed to lower the risk of liver toxicity and improve peripheral tissue selectivity [188], even significantly enhancing L-DOPA bioavailability, and as such, has been approved in Europe since 2016 as an adjunct to L-DOPA/dopa decarboxylase inhibitor (DDCI) for motor fluctuations [189]. In fact, a meta-analysis found that opicapone treatment was associated with fewer adverse events than both tolcapone and entacapone but its therapeutic effect was slightly inferior to that of tolcapone [190]. Adverse events associated with COMT inhibitors include potentiation of dyskinesia, nausea, and sleep disturbances [191-193].

1.1.7.4. MAO-B inhibitors

Monoamine oxidase B (MAO-B) is one of the main enzymes involved in the breakdown of dopamine to dihydroxyphenylacetic acid and hydrogen peroxide [194]; MAO-B inhibitors preserve endogenous levels of dopamine to increase dopaminergic activity within the striatum [151]. MAO-B inhibitors are beneficial during early PD in delaying the need for L-DOPA and are also used as an adjunct to L-DOPA for motor fluctuations [195-197] but most patients eventually require L-DOPA therapy [198]. Selegiline and rasagiline are the more frequently administered MAO-B inhibitors [161]; they are well tolerated and when administered with L-DOPA, the most

frequently reported adverse effects include nausea, dizziness, and insomnia [199, 200]. Furthermore, safinamide is a more recently developed MAO-B inhibitor approved as an adjunctive therapy to L-DOPA in mid- to late-stage PD with fluctuations [201]. In addition to its ability to inhibit MAO-B, safinamide also inhibits glutamate release by blocking voltage-gated sodium channels and modulating calcium channels [202, 203]. This dual mechanism of action is unique compared to other MAO-B inhibitors and may be responsible for its safety and tolerability in patients with few adverse events [201, 204].

1.1.7.5. Anticholinergic agents

Contrary to other drugs discussed so far that act on increasing striatal dopaminergic activity [151], anticholinergic drugs inhibit binding of acetylcholine through antagonism of cholinergic receptors [205, 206]. While studies reported that anticholinergic drugs (e.g., benzhexol and benztropine) improved rigidity and tremor in PD [207], evidence is lacking from randomised trials [161], likely because most studies were conducted prior to the introduction of randomised controlled trials in therapeutics [207]. Although the mechanism underlying anticholinergic drugs in PD is unclear, they may be restoring an imbalance between dopaminergic and cholinergic transmission [208].

1.1.7.6. Amantadine

Amantadine, initially developed as an anti-viral, has been used in PD for over 50 years [209]. The only drug approved to treat dyskinesia in PD by the Food and Drug Administration (FDA) [210], evidence for its use in controlling parkinsonian features has been mixed, while a

review found insufficient evidence for efficacy due to poor study quality [208], reports from the International Parkinson and Movement Disorders Society (IPMDS) and European Federation of Neurological Societies found that amantadine is likely efficacious for symptomatic monotherapy and adjunct therapy [152, 153]. The broad spectrum pharmacological profile of amantadine includes its action as a weak non-competitive NMDA receptor antagonist [214], sigma1 receptor agonist [214, 215], as well as modulation of dopamine release and reuptake [216]. On the other hand, despite its lack of anticholinergic activity in preclinical studies, in the clinic, amantadine exhibited anticholinergic side effects [217], such as dry mouth, blurred vision, and constipation [211, 218]. Nevertheless, the multiple pharmacologic actions of amantadine render it difficult to comment on the precise mechanism of action underlying its anti-parkinsonian and anti-dyskinetic effects [211, 212]. Side effects associated with amantadine include hallucinations, confusion and impaired concentration, and insomnia, which are more common with high doses [213].

1.1.7.7. Surgical treatment

Deep brain stimulation is an established treatment for motor features of PD [32] and largely favoured over lesioning procedures [214]. Two of the most common targets are subthalamic nucleus (STN) and globus pallidus (GP) pars interna (GPi) [215]. Randomised controlled trials have revealed that similar benefits are obtained with both targets [216-218] but subthalamic stimulation is associated with reductions in doses of dopaminergic drugs, while pallidal stimulation is accompanied by fewer mood and cognitive adverse effects [219]. Nonetheless, the restrictive patient selection criteria, variable risk for surgical complications, and development

of psychiatric and cognitive comorbidities limit the potential of deep brain stimulation as the treatment of choice for PD [214].

1.2. L-DOPA-induced dyskinesia

Since its initial usage in PD patients over 50 years ago [220], L-DOPA remains the most effective symptomatic therapy. With disease progression and chronic administration, its benefit is hindered by the development of complications, such as L-DOPA-induced dyskinesia and motor fluctuations. In a pivotal study, dyskinesia was first recognised after nearly 10 years of L-DOPA use in PD [220]. Thus, after 15 years of L-DOPA administrations, 94% of PD patients become afflicted with dyskinesia [141]. The reported incidence of dyskinesia in PD has varied from 3% to 94% [221-223], likely stemming from differences in demographic and clinical characteristics, including age, duration of disease and treatment, medical history, as well as changes in clinical practice and other methodological differences [224-226]. Dyskinesia is associated with impaired quality of life (e.g., activities of daily living, emotional wellbeing, bodily discomfort) [227-229] and increased socioeconomic burden [230-233].

1.2.1. Risk factors

1.2.1.1. L-DOPA dosage

The effect of L-DOPA on inducing dyskinesia appears to be dose-dependent [234] with higher cumulative dose of L-DOPA as a risk factor for the development of dyskinesia [235]. Earlier studies administered relatively higher doses of L-DOPA to patients compared to the modern era, which may explain the earlier appearance of dyskinesia [236]. Moreover, in a randomised, double-

blind, placebo-controlled trial, 16.5% of PD patients who received 600 mg of L-DOPA daily after 40 weeks developed dyskinesia, whereas only 2.3% of patients receiving 300 mg of L-DOPA daily developed dyskinesia [237]. A retrospective study reported that higher initial L-DOPA dose was a risk factor for dyskinesia [238], consistent with findings from a cross-sectional study [239]. On the other hand, the DATATOP study revealed that while PD patients with dyskinesia were administered higher L-DOPA doses at the time dyskinesia appeared, cumulative daily dose of L-DOPA was comparable to patients without dyskinesia [240]; similar findings were obtained in a smaller retrospective study [241].

1.2.1.2. Timing of L-DOPA

The prevalence of dyskinesia increases with duration of disease and treatment [242], although the latter may reflect, in part, disease duration [221, 240] and severity [243, 244], which are highly correlated with dyskinesia rate [245, 246]. Nevertheless, dyskinesia occurs less often during early treatment [223, 247] and incidence increases with longer duration of treatment [141]. Prospective randomised controlled trials reported that 40% to 50% of PD patients developed dyskinesia after 5 years of L-DOPA treatment [173, 221, 248]. Similarly, retrospective analyses of clinic samples found that 54% to 56% of patients developed dyskinesia after 3 to 6 years of L-DOPA therapy [241, 249]. Long term studies found that after 10 years of L-DOPA, the incidence of dyskinesia reached 52% to 78% [250, 251], and after 15 years, 94% of patients in the Sydney Multicentre study of PD had developed dyskinesia [141, 222].

The optimal treatment strategy for PD has been contentious the past few decades [252]. The association between prolonged L-DOPA treatment and increased incidence of dyskinesia led to "L-DOPA phobia" in the 1990s to 2000s [253] and the practice of prescribing dopamine agonists

instead of L-DOPA early in the disease [254]. This was supported by large randomised trials [172, 255, 256] that reported delays in the development of dyskinesia with initial treatment of dopamine agonists over 3 to 5 years [252, 254]. Later studies, however, challenged the notion that delayed L-DOPA therapy had a protective role [257]. Post hoc analysis of the CALM-PD trial [256] revealed that after adjusting for disease duration and L-DOPA dose, the incidence of dyskinesia was comparable whether the initial treatment was L-DOPA or the dopamine agonist pramipexole [258]. An open-label trial [197] and a follow-up study of up to 14 years [259] also reported that dyskinesia prevalence and long-term outcomes were similar irrespective of L-DOPA or L-DOPA sparing therapies. Moreover, further evidence against withholding L-DOPA therapy comes from a recent study that found dyskinesia was associated with disease duration and levodopa daily dose but not with the duration of L-DOPA therapy [257]. This body of evidence, coupled with side effects associated with dopamine agonists, including pathological behaviours [260] and psychosis [173, 256, 261], led to a shift in treatment approach towards early use of L-DOPA [254, 262, 263].

1.2.1.3. Sex differences

Whereas male sex is a risk factor for PD, some studies have found that female sex is associated with an increased risk for dyskinesia [264-266] but not in other reports [239, 245]. Furthermore, a prospective study found that the latency until the onset of dyskinesia in females was significantly shorter than in males [267]. The higher prevalence in females may be confounded by their relatively lower body weight [268] and adjusting for body weight revealed that females are exposed to significantly higher L-DOPA plasma levels than males [269-271]. Moreover, pharmacokinetic parameters of L-DOPA are inversely correlated with body weight [269, 270], which

may affect the onset of dyskinesia [272]. Logistic regression analysis of the REAL-PET trial found that a higher L-DOPA dose per kilogram body weight was the most significant factor for developing dyskinesia, whereas female gender, absolute L-DOPA dose and body weight were not significant [273].

1.2.1.4. Age of onset of Parkinson's disease

Younger age of onset of PD (< age 50) is a risk factor the development of dyskinesia [245, 274] that also tends to result in a more severe phenotype [222]. In patients with onset before 40 years of age, up to 90% develop dyskinesia after 5 years of treatment [275], whereas the risk is 26% with an age of onset in the 60 to 69 years group and 16% in the 70 years group [274]. Although it is unclear why younger age of onset is associated with a higher propensity for dyskinesia [224], genetic expression may contribute, in part, to this susceptibility [222, 276]. Autosomal recessive forms of PD associated with early onset of the disease are linked to earlier [277, 278] and a higher risk for developing dyskinesia [279-282]. Age-related differences in L-DOPA dynamics, such as alterations in dopamine turnover and consequent fluctuations in synaptic levels, may also be implicated [283]. A retrospective study also found that for PD patients with onset < 50 years, the increased risk for dyskinesia was concentrated in the first few years of L-DOPA therapy, which suggests that underlying disease progression is a primary determinant of dyskinesia risk rather than cumulative L-DOPA exposure [284].

1.2.1.5. Comorbidities

Dyskinesia and motor fluctuations may be interrelated as a retrospective study found that presence of one is linked to the earlier development of the other [285]. In contrast, PD patients that

present with resting tremor during early disease have a lower probability of developing dyskinesia [235, 286].

1.2.2. Disease subtypes

Based on the timing of symptoms with L-DOPA dosage, dyskinesia can be generally classified as on or peak-dose dyskinesia, diphasic dyskinesia and off dyskinesia [226]. Peak-dose dyskinesia is the most common form of dyskinesia, accounting for 75% to 80% of cases [287], and occurs during peak plasma L-DOPA levels [288]. Symptoms manifest as restless and continuous involuntary movements and frequently affect the extremities, although the head and respiratory muscles can be affected as well [289]. Peak-dose dyskinesia resolves with dose reduction but often, at the expense of worsening parkinsonism [224]. Diphasic dyskinesia occurs when L-DOPA levels are rising or falling [224], and usually manifests as dystonia or ballism [226]. Diphasic dyskinesia occurs more often in the legs than arms [290] and involves repetitive rapidly alternating dystonic foot movements or stereotyped leg kicking [289, 291]. Off dyskinesia accounts for the remainder of cases [292, 293] and occurs when L-DOPA levels are low (e.g. at night or prior to the first morning dose) or the patient is transitioning from the off to on period [289]. Predominantly manifesting as dystonia, it is also referred to as off dystonia [294], and tends to affect the lower limbs, usually ipsilateral to the side first affected by PD [295]. On time refers to the period of time where patients are receiving optimal benefit from medication [161] and symptoms have been reduced or abated by treatment [296], whereas off time refers to the period where medication is not optimally effective and a patient is exhibiting parkinsonian symptoms [161]. With disease progression, the progressive loss of dopaminergic terminals leads to suboptimal levels of L-DOPA between

doses [289] and the duration of the therapeutic benefit of each successive L-DOPA dose becomes shorter [297].

1.2.3. Symptomology

Dyskinesia is heterogeneous in manifestation and encompasses chorea, ballism, dystonia, and athetosis [287]; in clinical practice, it most often presents as chorea and dystonia but ballism, athetosis, and myoclonus also occur occasionally [288]. Dyskinesia tends to afflict the side of the body where PD first occurs and symptoms are often more severe on this side but with disease progression, symptoms eventually affect both sides of the body [289]. Chorea refers to involuntary, irregular and purposeless movements that appear to flow from one body part to another [298]. Chorea often involves the extremities and tends to occur ipsilateral to the side of the body where PD first occurs [226]. Dystonia is the second most common form of dyskinesia and consists of sustained, involuntary and patterned muscle contractions that cause twisting movements and/or abnormal postures [224, 289], including great toe extension, toe curling and inversion of the foot at the ankle [226]. Dystonia accounts for greater disability than chorea [224] and is associated with functional disabilities and pain [225]. Less commonly reported forms of dyskinesia include ballism, which refers to movements performed with maximal velocity and acceleration, mainly affecting proximal muscles [299] as well as athetosis, slow writhing movements of the extremities [289].

1.2.4. Pathophysiology

1.2.4.1. Basal ganglia circuitry

The basal ganglia comprise a group of interconnected subcortical nuclei that include the striatum, GP pars externa (GPe) and GPi, STN, as well as SNc and SN pars reticulata (SNr) [300]. These structures form a highly organised and complex network and are engaged in control of movement, executive functions and behaviour, and emotions [301]. The classical model of basal ganglia was developed in the late 1980s and early 1990s [302-304] and has been used to explain movement disorders, including PD and dyskinesia [300]. Input to the basal ganglia primarily arises from glutamatergic afferents from the cortex and thalamus to the striatum [305-307], whereas output is sent to the thalamus and brainstem [308-310].

The striatum is the main input structure of the basal ganglia and predominantly consists of medium spiny neurons (~ 90% to 95%) with relatively fewer interneurons (~ 5% to 10%) [311-313]. However, the ratio of striatal projection neurons to interneurons varies between species, for instance, in rats, it is 9:1 and in non-human primates, it is 3:1 [314, 315]. In the classical model, striatal medium spiny neurons are divided into the direct and indirect pathways, based on their projection targets [316]. Direct pathway medium spiny neurons express D1-like dopamine receptors [317-319] and project to output structures of the basal ganglia, the SNr and GPi [320]. Medium spiny neurons of the indirect pathway medium spiny neurons express D2-like dopamine receptors [317-319] and indirectly project to the SNr and/or GPi via the GPe and STN [320]. Activation of the direct pathway decreases basal ganglia output, which disinhibits the thalamus, stimulates the cortex, and facilitates movement, whereas activation of the indirect pathway increases basal ganglia output, which inhibits the thalamus and cortex, and inhibits movement [321].

Dopamine has opposing actions on the pathways, increasing the activity of the direct pathway while decreasing the activity of the indirect pathway [320]. In physiological conditions,

dopaminergic signalling in the classical model dampens output from the GPi to favour movement. In PD, degeneration of SNc dopaminergic neurons disrupts the equilibrium between the two pathways, leading to hypoactivity of the direct pathway and hyperactivity of the indirect pathway [320]. The excessive GPi output results in inhibition of the thalamus and cortex that causes a hypokinetic state, which may account for the appearance of parkinsonian symptoms [320]. In contrast, dyskinesia [303] may be mediated by changes in basal ganglia activity that are functionally opposed to PD [322, 323]. Thus, L-DOPA administration enhances striatal dopamine levels and favours activity of the direct pathway over the indirect pathway [316]. This reduction in GPi output results in disinhibition of the thalamus and cortex and induces the expression of dyskinesia [316].

1.2.4.2. Models of basal ganglia dysfunction

Three major hypotheses have been proposed to underlie the pathophysiology of L-DOPA-induced dyskinesia: 1) firing rate model, 2) firing pattern model, and 3) ensemble model [316]. The firing rate model suggests that decreased firing of the GPi and SNr may induce dyskinesia by disinhibition of the thalamic-cortical pathway [324]. Indeed, in PD patients, firing rate in GPi and STN shifted to a hypoactive state during dyskinesia expression [325]; similar results were obtained in animal models [326, 327]. However, a few inconsistencies with the firing rate model are difficult to reconcile, including its failure to account for some types of neural dynamics [328], the strong connection between the basal ganglia and motor cortex [329], and contrary findings on GPi lesions or stimulation [322]. Thus, the firing pattern model proposes that changes in bursting firing and neuronal oscillations within cortico-basal ganglia-thalamic loop may underlie dyskinesia [316]. Reports found that L-DOPA application increased the number of

bursting cells in the GPi and SNr of hemi-parkinsonian rats [330, 331]; increased bursting in the GPi and STN has also been reported in PD patients with dyskinesia [332]. However, it has been difficult to directly link bursting with dyskinesia [316] as only a single study has found that increased bursting alleviated dyskinesia in hemi-parkinsonian rats [333]; further research is required to clarify this relationship. In general, dyskinesia is also associated with reductions in β oscillations [334] but enhanced γ oscillations [335-337]; the link remains controversial [338-340]. Last, the recent ensemble model posits that distributed impairment in neurons across multiple brain regions may mediate dyskinesia expression [316]. A study demonstrated an association between neurons in the primary motor cortex and striatum with dyskinesia [341], while another found that a subpopulation of medium spiny neurons of direct pathway correlated with dyskinesia [342]. These results suggest that regulating the ensemble neural activity of the direct and indirect pathways may be another target for therapies that treat dyskinesia rather than correcting activity imbalances [328].

1.2.4.3. Molecular mechanisms

The pathophysiology of dyskinesia has predominantly focused on changes that occur at the striatum, including pulsatile stimulation of dopamine receptors [343], excessive swings in synaptic dopamine concentrations that lead to increased receptor occupancy [344], discordance between low levels of dopamine in the striatum and high plasma levels of L-DOPA [345], downstream changes in post-synaptic signalling, and changes in non-dopaminergic systems [329]. The majority of these processes are related to the dopaminergic system and are discussed in Section 2.6.1. but have also been reviewed previously [346, 347]. Nonetheless, these events lead to alterations in the firing and oscillatory activity in the basal ganglia circuitry that culminate in disinhibition of the thalamic-cortical pathway [329] and appearance of dyskinetic symptoms.

1.2.5. Imaging

In addition to evidence of structural changes in the brains of PD patients [348-350] and parkinsonian animals [351-353], dyskinesia may also be linked to structural and functional changes in the brain. One group reported that alterations in the inferior frontal cortex and supplementary motor area [354-356], both implicated in executive motor control [357, 358], were associated with dyskinesia development. These findings were consistent with those obtained by other imaging studies [359-361]. Moreover, the localisation of brain abnormalities appears dependent on the age of onset of L-DOPA-induced dyskinesia, whereby nigral pathology is observed in early onset, while cortical pathology is observed in late onset dyskinesia [362]. Reports of altered connectivity between the frontal lobe and striatum in dyskinesia in PD patients [360, 361, 363] and animal models of PD [364, 365] collectively provide support for the involvement of cortico-striatal projections in dyskinesia pathogenesis [366]. Besides changes in grey matter structure and activity in the dyskinetic brain, there is evidence of changes in white matter. A magnetic resonance imaging (MRI) study in PD patients revealed that increased white matter structural integrity and connectivity within the fronto-striato-pallido-thalamic regions was predictive of dyskinesia development and that the left superior frontal gyrus is a potential hub for neural substrates [367]. A previous study also linked the superior frontal gyrus and dyskinesia [368] and its role in inhibitory control [369, 370] may imply a similar role in the pathophysiology of dyskinesia [367]. Moreover, a recent diffusion tensor imaging study that assessed white matter in PD detected less microstructural white matter impairment in dyskinetic patients than non-dyskinetic patients, especially in the temporal lobe [366]. These novel results may suggest that the relative fibre preservation in young onset PD patients [371] may contribute to enhanced plasticity [372], which is

implicated in the pathophysiology of dyskinesia [373]. Finally, PET molecular imaging studies have predominantly implicated abnormalities in the dopaminergic and serotonergic systems in L-DOPA-induced dyskinesia [374-376] but other neurotransmitter systems, such as the glutamatergic, adenosinergic, and cholinergic systems, may also play a role in its development [374, 377].

1.2.6. Neuropharmacology

1.2.6.1. Dopaminergic system

Once dyskinesia is induced by L-DOPA therapy, it is irreversible or at least persistent [378] and subsequent administration of dopaminergic drugs will elicit its expression [379, 380]. This phenomenon suggests that dopaminergic drugs have modified the brain's response to dopamine [378]. Dopaminergic receptors are divided into five different subtypes of G protein-coupled receptors that regulate cyclic adenosine monophosphate (cAMP)-protein kinase A [381]. D1 class (D1 and D5) receptors mainly couple to $G\alpha_s/G\alpha_{olf}$ and stimulate cAMP production and protein kinase A activity, while D2 class (D2, D3 and D4) receptors couple to $G\alpha_i/G\alpha_o$ and negatively regulate cAMP production [382]. D1 and D2 receptors are the most studied in PD and dyskinesia [378], so the present review will focus on these subtypes. Both D1 and D2 receptors are highly expressed on striatal medium spiny neurons but lowly expressed in the cortex [383, 384]. D1 receptors are localised to dopaminergic neurons that contain substance P and dynorphin that project to the SNr and GPi, which form the direct pathway [385]. On the other hand, D2 receptors are mainly found on dopaminergic neurons that express enkephalin, which project to the GPe, forming the indirect pathway [386, 387]. D2 receptors are also localised on presynaptic nigrostriatal dopaminergic terminals [388, 389], presynaptic cortico-striatal terminals [390] and SNc neurons [391].

Two factors crucial for the development of dyskinesia are related to the dopaminergic system: 1) dopaminergic denervation in the nigrostriatal pathway [392-394]; 2) short half-life of oral L-DOPA [395]. The condition of severe nigrostriatal denervation is independent of duration of L-DOPA therapy; if denervation is severe, an initial dose of L-DOPA can trigger dyskinesia [346, 380, 396, 397] but the converse does not, i.e., chronic administration of L-DOPA does not generally lead to dyskinesia if the nigrostriatal pathway is intact [321, 397-399]. Another factor that contributes to dyskinesia development is related to the pharmacokinetics of oral L-DOPA [168, 400, 401] as its short half-life leads to non-physiological pulsatile stimulation of dopamine receptors [395]. Along with the progressive neuronal loss of dopaminergic terminals in PD [51, 402], this leads to altered dynamics in dopamine conversion, release, and uptake [329]. Indeed, PET studies revealed that PD patients with dyskinesia showed larger swings in dopamine levels compared to their non-dyskinetic counterparts [344, 403]. Further support arises from the observation that long-acting dopamine agonists [172, 173, 248] and continuous infusion of L-DOPA [404-406] are generally associated with a lower incidence of dyskinesia, although a recent meta-analysis found that continuous dopaminergic stimulation was linked to increased incidence of dyskinesia (Xie et al., 2014a). The combination of severe nigrostriatal denervation in PD and short half-life of L-DOPA cause changes in dopamine signalling and cortico-striatal synaptic plasticity [329].

Denervation-induced supersensitivity of D1-like and D2-like dopamine receptors has been posited as a mechanism underlying dyskinesia [407]. This supersensitivity may be attributed to changes in receptor levels and cellular distribution or changes in downstream signalling [407]. Autoradiographic binding studies have revealed increased striatal D2 receptor binding sites in the post-mortem brains of parkinsonian animals [408-410] and untreated PD patients [411]. Although hypersensitivity of D2 receptors would be expected in drug naïve PD patients [378], dyskinesia is

not induced with the first dose of L-DOPA but rather, it develops progressively with chronic administration of L-DOPA [224]. D2 receptor expression does not mimic this pattern [407] as some studies found that chronic L-DOPA administration led to decreased levels in PD patients [411] and MPTP-lesioned non-human primates [410, 412] but remained unchanged in 6-hydroxydopamine (6-OHDA)-lesioned rats [317]. PET studies also found that the initial upregulation of striatal D2 receptors was normalised by L-DOPA treatment [413, 414]. On the other hand, studies on D1 receptor binding have been highly variable [412, 415-417] but no change in striatal D1 density has been reported [411, 413]. While the association between D1 receptor expression and dyskinesia has been unclear, D1 receptor sensitivity as measured by GTPγS binding is linearly related to dyskinesia severity [418]. Moreover, this relationship appears dependent on subcellular localisation [419] as D1 receptors are internalised into the cytoplasm of PD patients previously treated with L-DOPA compared to healthy controls [420]; similar results were found in the hemi-parkinsonian rat [419]. The inconsistencies with the hypersensitivity hypothesis suggest that dyskinesia is more complex than an upregulation in striatal dopamine receptors [378]. Other mechanisms are likely at play, including L-DOPA-induced sensitisation to dopaminergic response [421] and changes in the activity and expression of dopamine receptors in the basal ganglia and downstream signalling [407, 422].

Therapeutic agents

Compared to L-DOPA, dopamine agonists have a lower propensity to induce dyskinesia [172, 173, 248] but at the expense of providing less relief for parkinsonian symptoms [423]. Furthermore, even if PD patients are initially treated with dopamine agonist monotherapy, with disease progression, they will require L-DOPA [32]; over the long term, motor complications appear

similar to PD patients initially treated with L-DOPA [197]. As mentioned earlier, there is also a dose-dependent effect of L-DOPA on dyskinesia development, where higher doses are associated with increased incidence [424, 425], whereas duration of therapy is also predictive of dyskinesia [245].

D1 class receptors

Compared to D2 agonists, D1 agonists have demonstrated comparable efficacy in alleviating parkinsonian symptoms but a lower propensity to trigger dyskinesia in non-human primate models [426-429]. Furthermore, inhibition of D1 receptor activity by pharmacological blockade reduced dyskinesia severity in hemi-parkinsonian rats [430-432] while genetic knockout in mice completely suppressed dyskinesia [433]. At odds with results obtained in preclinical studies, in the clinic, the D1 agonist prodrug ABT-431 produced similar anti-parkinsonism action and dyskinesia as L-DOPA [434]. However, D1 agonists are not in clinical use because of dose-limiting effects reported in studies conducted in PD patients with dyskinesia, including low bioavailability, poor tolerability, and short half-life [437-439]. At the post-mortem level, chronic treatment with No new investigation of D1 agonists was undertaken in PD for over 20 years [440, 441] until a Phase I study published in 2018 found that the D1/D5 agonist PF-06649751 was safe and well tolerated in PD patients [442], further investigation is required to determine its anti-parkinsonian potential.

Studies have found that D5 receptors are also expressed in the striatum, albeit to a lesser extent than D1 receptors [435, 436]. Thus, in a hemi-parkinsonian rat model, dyskinesia correlated with abnormal expression of D5 receptors in the striatum, whereas genetic knock down of

striatal D5 receptors downregulated D5 receptor expression and reduced dyskinesia [437]. Moreover, a D5 receptor mouse knockout model revealed elevated dyskinesia scores and impairments in locomotion in response to L-DOPA [438]. These recent findings may implicate a role for D5 receptor in dyskinesia, mediated in part by activity in the striatum or on cholinergic interneurons.

D2 class receptors

Once dyskinesia has been induced by L-DOPA, D2 receptor agonists can trigger dyskinesia in rat [439, 440] and non-human primate models of PD [441, 442] as well as in patients with PD [443]. Moreover, in parkinsonian monkeys with established dyskinesia, administration of D2 agonists led to more severe dyskinesia than D1 agonists [426]; D2 agonists also accelerated the development of dyskinesia in the same animal model [444]. While the D2 agonists bromocriptine [175, 259], ropinirole [173, 251, 445], and pramipexole [248, 446] induced less dyskinesia in PD patients within the first 2 to 5 years, in the long-term (10 to 14 years follow-up), the rate of dyskinesia was similar once L-DOPA was added [447]. Furthermore, the long-acting D2 agonist cabergoline is associated with similar rates of dyskinesia as shorter-acting dopamine agonists [171, 448], which suggests that longer duration of action cannot fully account for its ability to reduce dyskinesia development [447].

The role of the D3 receptor in L-DOPA-induced dyskinesia has been less studied compared to D1 and D2 receptors [378], in part due to the low expression in the striatum [449], and behavioural studies report conflicting results. Administration of a D3 agonist in parkinsonian monkeys elicited dyskinesia that was comparable in severity to that induced by apomorphine [450]. Moreover, blockade of the D3 receptor with the antagonist S33084 reduced the development

of dyskinesia but had no effect on established dyskinesia [451]. In contrast, the D3 antagonist nafadotride attenuated established dyskinesia but at the expense of impairing L-DOPA anti-parkinsonian action [452]. These inconsistencies suggest that further work is required to unravel the contribution of the D3 receptor in the development and expression of dyskinesia [378]. Although the D3 receptor has not been studied in dyskinetic PD subjects, a PET study found reduced D3 receptor binding in the ventral striatum and GP of drug naïve PD patients compared to healthy controls [453].

D4 receptors have been comparatively neglected in dyskinesia but there is evidence that they are located in the striatum [454] and GP [455], key structures implicated in PD and dyskinesia [324, 385]. The selective D4 receptor antagonist L-745,870 significantly reduced dyskinesia severity in a monkey model of PD at brain levels suggestive of D4 antagonism [456]. Similar results were obtained with L-745,870 in a rat model but at the cost of worsening L-DOPA anti-parkinsonian benefit [457], which suggests a narrow therapeutic window for the compound [458]. A more recently developed D4 antagonist VU6004461 was also effective at improving dyskinesia in a mouse model of PD [459]. These promising results encourage further research into the mechanism of action underlying D4 receptor blockade and the development of drug candidates for clinical testing.

1.2.6.2. Serotonergic system

The serotonergic system innervates virtually all regions of the central nervous system [460], including modulation of the cortico-basal ganglia-thalamic circuitry, notably by providing serotonergic inputs to the striatum, SNr and GP [461, 462]. Despite evidence showing a loss of serotonergic markers or terminals in the frontal cortex, hippocampus, and caudate nucleus [463,

[464], there is a lack of correlation with dyskinesia [465]. 5-hydroxytryptamine (5-HT) receptors comprise seven subfamilies (5-HT_{1-7}) with at least 14 subtypes [466] and are all G protein-coupled receptors [467] except for the 5-HT type 3 (5-HT_3) receptor, which is a ligand-gated cation channel [468]. These G protein-coupled receptors couple to $G\alpha_i$, $G\alpha_{q/11}$, and $G\alpha_s$ to mediate excitatory or inhibitory neurotransmission [469]. The present review will be limited to those studied in the context of dyskinesia: 5-HT type 1A (5-HT_{1A}), type 1B (5-HT_{1B}), type 2A (5-HT_{2A}), type 2C (5-HT_{2C}), and 5-HT_3 receptors [460].

In early stages of PD, the remaining dopaminergic terminals mediate the conversion of L-DOPA to dopamine and its physiological release [470]. Dopamine levels are well regulated by D2 autoreceptors and the dopamine transporter (DAT), which leads to optimal and sustained therapeutic responses with L-DOPA [470]. However, by late stages of PD, nigrostriatal denervation becomes more severe, which leads to reliance on non-dopaminergic terminals to convert and release dopamine [471]. Serotonergic neurons possess the same enzymatic machinery as dopaminergic neurons (i.e., aromatic amino acid decarboxylase enzyme and vesicular monoamine transporter 2), and are also able to synthesise, store, and release dopamine [472-474]. Whereas the contribution of serotonergic terminals may be beneficial during early disease due to the presence of spared dopaminergic terminals, by late disease stages, this contribution may become detrimental [460]. Unlike dopaminergic neurons, striatal 5-HT neurons lack the regulatory mechanisms to finetune dopamine release mediated by D2 autoreceptors [475]. The uncontrolled release of dopamine from serotonergic terminals leads to fluctuations in synaptic dopamine levels [476, 477], and in turn, pulsatile stimulation of postsynaptic receptors and the development of dyskinesia [478]. The ability of 5-HT neurons to convert L-DOPA to dopamine extends beyond the lesioned striatum and occurs in other brain regions that receive 5-HT innervation [479, 480].

Consistent with microdialysis studies in the rat [481, 482], a PET study in patients with PD found that one hour after L-DOPA administration, [^{11}C]raclopride binding was higher in dyskinetic patients compared to non-dyskinetic ones, which is indicative that changes in extracellular dopamine levels were more pronounced in the former group [344]. Similarly, a subsequent study also found that [^{11}C]raclopride binding was markedly higher in PD patients with dyskinesia but administration of the 5-HT$_{1A}$ agonist buspirone reduced increased [^{11}C]raclopride binding [403]. Importantly, this effect was accompanied by a reduction in dyskinesia severity, suggesting that PD patients exhibit greater striatal synaptic dopamine levels, and activation of 5-HT$_{1A}$ receptors restores their synaptic dopamine levels to those observed in patients with stable responses to L-DOPA, improving their dyskinesia [403].

Therapeutic agents

5-HT$_{1A}$ and 5-HT$_{1B}$ receptors

In line with these studies, lesion to the 5-HT system attenuated dyskinesia in rat [481, 483] and non-human primate [484] models of PD. Moreover, decrease in 5-HT neuron activity by activation of 5-HT$_{1A}$ and 5-HT$_{1B}$ autoreceptors, which control glutamate and GABA release [485-487], also significantly reduced dyskinesia in animal models of PD [488-491]. These results are in line with a study conducted in the post-mortem macaque brain that found changes in 5-HT$_{1A}$ levels in the cortex and striatum of dyskinetic parkinsonian animals [504], which is suggestive that alterations in cortico-striatal 5-HT$_{1A}$-mediated neurotransmission underlie dyskinesia [505]. On the other hand, for the less studied 5-HT$_{1B}$ receptor, chronic L-DOPA treatment to 6-OHDA-lesioned rats increased receptor levels and the adaptor protein p11 but selective activation of the receptor attenuated dyskinesia expression in 6-OHDA-lesioned animals [506]. Similarly, a

subsequent study found that MPTP-lesioned macaques treated with L-DOPA had elevated binding levels in the caudate nucleus, putamen, GPi, and SNr but co-administration of the kynurenine hydroxylase inhibitor Ro 61-8048 attenuated this increase in binding [507]. However, some studies also found that the anti-dyskinetic efficacy of 5-HT_1 agonists was accompanied by worsening L-DOPA anti-parkinsonian action in preclinical models [492-495] as well as in patients with PD [496, 497]. Of note, 5-HT_{1A} or 5-HT_{1B} receptor binding levels have not been examined at the post-mortem level in PD dyskinesia. Contrary to its success in an open-label trial [498], two large-scale placebo-controlled trials with the 5-HT_{1A} agonist sarizotan failed to demonstrate improvements in dyskinesia compared to placebo [499, 500], although there was a strong placebo response [501]. The discrepancy in findings may be related to the antagonist action of sarizotan at the D2 receptor [502], while others argue that trial design, continued administration of adjunct therapy or the predictive validity of the hemi-parkinsonian rat model contributed to the discrepancies [503]. On the other hand, therapeutic agents with improved pharmacological profile, such as agonists with dual 5-HT_{1A} and 5-HT_{1B} affinity without antagonism of dopamine receptors, may be more suited for clinical development [475]. Combined activation of 5-HT_{1A} and 5-HT_{1B} receptors with 8-OH-DPAT and CP-94253, respectively, had a synergistic effect on reducing dyskinesia in rat [481] and monkey models [504]. Furthermore, the mixed 5-HT_{1A}/5-HT_{1B} agonists eltoprazine and anpirtoline significantly reduced dyskinesia severity in preclinical models but worsened L-DOPA anti-parkinsonian action at higher doses [492, 495, 505]. Nonetheless, a double-blind randomised placebo-controlled trial revealed that eltoprazine improved dyskinesia without interfering with the therapeutic benefit of L-DOPA [506]. Despite these promising findings, the clinical development of eltoprazine remains unclear; the status of a Phase II trial is unknown and has not been updated since 2016 (NCT02439125) [507]. These

studies collectively suggest that while 5-HT_{1A} and 5-HT_{1B} agonists have demonstrated anti-dyskinetic efficacy, the narrow therapeutic window may encumber further clinical development of these therapeutic agents.

5-HT_{2A} receptor

Evidence from preclinical and clinical studies provides support for the involvement of the 5-HT_{2A} receptor in dyskinesia. An autoradiographic binding study revealed an increase in 5-HT_{2A} binding in the motor cortex and striatum of dyskinetic primates compared to non-dyskinetic animals [508]; another study only found an increase in the striatum [509]. These findings suggest that the dyskinetic state may be mediated by altered 5-HT_{2A} neurotransmission [510] and, in turn, it can be inferred that targeting the 5-HT_{2A} receptor can treat dyskinesia [460]. Thus, blockade of 5-HT_{2A} receptors with clozapine and quetiapine significantly improved dyskinesia in the hemi-parkinsonian rat [511] and parkinsonian primate [512, 513]. Contrary to these findings, selective antagonism of 5-HT_{2A} receptors with volinanserin and EMD-281,014 failed to attenuate dyskinesia in hemi-parkinsonian rats [514, 515]. Whereas EMD-281,014 led to a reduction in dyskinesia severity in the parkinsonian marmoset [516], there was a ceiling to its anti-dyskinetic efficacy as higher doses failed to confer greater therapeutic benefit [517]. The discrepancy between studies may be related to: 1) the selectivity of 5-HT_{2A} antagonists – volinanserin and EMD-281,014 are both highly selective antagonists that demonstrate ≈ 300 to 2000-fold selectivity for the 5-HT_{2A} receptor [518, 519], respectively, while clozapine and quetiapine demonstrate affinity for other serotonergic and non-serotonergic receptors [478] and, consequently, it is difficult to conclude the exact contribution of these targets to the anti-dyskinetic benefit of these compounds [510]; 2) differences in species neurochemistry and striatal anatomy may account for

variable findings between rat and monkey models [515] – expression of the 5-HT_{2A} receptor differs in the 6-OHDA-lesioned rat [520] compared to the MPTP-lesioned marmoset [508], as well as the organisation of striatal neurons between species [314]. On the other hand, the 5-HT_{2A} partial agonist R-3,4-methylenedioxymethamphetamine [521] and inverse agonist pimavanserin [522] both led to reductions in dyskinesia in the parkinsonian non-human primate. Although it remains unclear how the variable actions of these 5-HT_{2A} ligands contribute to anti-dyskinetic efficacy [517], the association of high doses with ceiling effects may point to a limit of the therapeutic potential of 5-HT_{2A} receptor blockade. Compared to 5-HT_1 agonists, 5-HT_{2A} antagonists have not been well-studied for dyskinesia-related endpoints in clinical settings. Open-label trials in PD subjects found a modest anti-dyskinetic benefit of pimavanserin [523] and a significant anti-dyskinetic effect of ritanserin [524]. A single double-blind placebo-controlled study found that clozapine was effective in the treatment of dyskinesia in severe PD [525], whereas one single-blind crossover study revealed that ritanserin improved peak dose dyskinesia in PD [526]. Importantly, the anti-dyskinetic benefit conferred by 5-HT_{2A} antagonists was at the cost of worsened parkinsonian symptoms [524, 526], which suggests that the use of 5-HT_{2A} antagonists may have a narrow therapeutic window in the clinic. For example, ritanserin significantly reduced dyskinesia severity but at the expense of exacerbating parkinsonian symptoms in clinical trials [524, 526]. Given its strong affinity for D2 receptors [527], it is possible that blockade of D2 receptors may be responsible, in part, for its anti-dyskinetic efficacy [516]. Of note, no autoradiographic binding study has assessed 5-HT_{2A} binding levels in the post-mortem PD brain, and as such, alterations in binding levels or the mechanism of action underlying the anti-dyskinetic efficacy of 5-HT_{2A} antagonists remain largely unexplored [505]. Nonetheless, further clinical testing of selective 5-HT_{2A} antagonists in L-DOPA-induced dyskinesia, particularly regarding ceiling

effects with high doses and L-DOPA anti-parkinsonian action, will clarify the therapeutic potential of their utility.

5-HT_{2A} receptors are abundantly expressed in the neocortex, striatum, and nucleus accumbens [528, 529]. The mechanism underlying the anti-dyskinetic efficacy of 5-HT_{2A} antagonists is unclear but may be related to their localisation in the basal ganglia, notably by modulation of nigrostriatal dopamine release and corticostriatal glutamate release [508, 530]. Thus, an in vivo microdialysis study found that activation of the 5-HT_{2A} receptor increased dopamine release in the rat striatum [531]. As blocking the 5-HT_{2A} receptor with antagonists would be expected to decrease nigrostriatal dopamine release, this effect could lead to impaired L-DOPA anti-parkinsonian action [510]. Indeed, quetiapine [532] and ritanserin [524, 526] interfered with the therapeutic benefit of L-DOPA in patients with PD. On the other hand, 5-HT_{2A}-mediated neurotransmission increased presynaptic glutamate release [510], an effect that was blocked by the selective antagonists volinanserin and SR 46549B [533]. Furthermore, local perfusion of volinanserin into the dorsal striatum of MPTP-lesioned mice reduced striatal glutamate levels [534]. It remains to be demonstrated whether reductions in striatal glutamate levels induced by 5-HT_{2A} antagonists is a mechanism whereby they improve dyskinesia [510].

5-HT_3 receptor

Compared to other 5-HT receptors, the 5-HT_3 receptor has received considerably less interest as a therapeutic target in L-DOPA-induced dyskinesia in PD. As the sole ionotropic receptor in the 5-HT family, the receptor is a hetero-pentamer that forms a cation channel permeable to Na^+ and K^+ [535, 536]. Largely localised to pre-synaptic terminals, activation of the 5-HT_3 receptor modulates the release of neurotransmitters such as dopamine [537], glutamate [538] and

5-HT [539]. The role of the 5-HT_3 receptor in controlling the release of dopamine, which plays aetiological roles in the development of dyskinesia [540], suggests that it can exert an anti-dyskinetic effect. Thus, in rat striatal slices, application of the 5-HT_3 agonists 2-methyl-5-HT and phenylbiguanide stimulated endogenous release of dopamine [541, 542], an effect that was attenuated with application of 5-HT_3 antagonists [537, 543]. At the behavioural level, blockade of the 5-HT_3 receptor has diminished dopaminergic transmission-mediated motor responses such as rotations [544], stereotypies [545] and oro-facial dyskinesias [546]. Moreover, in PD subjects and animal models, ligands with antagonistic action at the 5-HT_3 receptor [547-554], including clozapine [511, 525, 555], mirtazapine [556], quetiapine [512], AQW051 [557], and AZD0328 [558], alleviated dyskinesia. Although these ligands are non-selective, it is conceivable that blockade of the 5-HT_3 receptors may have contributed to their anti-dyskinetic action, although the extent is unclear.

Only two recent studies have assessed the anti-dyskinetic efficacy of selective 5-HT_3 receptor blockade. In the 6-OHDA-lesioned rat, treatment with the antagonist ondansetron (0.04 and 0.08 mg/kg) led to a ≈ 27% and ≈ 54% reduction of dyskinesia compared to vehicle [559]. In the same animal model, a similar magnitude of reduction in dyskinesia severity (≈ 64%) was obtained with administration of ondansetron 0.0001 mg/kg [560]. Of note, both studies administered the 5-HT_3 antagonist ondansetron, and as such, it is unclear whether its therapeutic benefit can be attributed to blockade of the 5-HT_3 receptor or the specific effect of ondansetron. Moreover, as several selective 5-HT_3 antagonists are clinically available as anti-emetics with variable pharmacokinetic profiles, it would be of interest to assess whether these differences may alter the anti-dyskinetic efficacy of these ligands (see Chapter 2). While in vitro and in vivo studies provide indirect support the role of the 5-HT_3 receptor in L-DOPA-induced dyskinesia, results of the behavioural studies with ondansetron in parkinsonian animal models are

compelling, although insight into possible mechanism(s) of action remains lacking. Indeed, only two autoradiographic binding studies investigated 5-HT_3 receptors in PD but neither have focused on dyskinesia. One study using post-mortem putamen tissue failed to detect a significant difference in 5-HT_3 binding between PD patients and healthy controls [561]. The medical history of patients including medication, co-morbid conditions (i.e., dyskinesia) was not disclosed, which precludes drawing conclusions about a specific involvement of 5-HT_3 receptors in PD. In another study performed in the 6-OHDA-lesioned rat, 5-HT_3 binding levels were reduced in the entorhinal and prefrontal cortices ipsilateral to lesion [562]. However, this study was limited for two reasons: 1) measurement of binding levels was limited to limbic forebrain and cortical regions and excluded basal ganglia; and 2) animals were not administered L-DOPA to induce expression of dyskinesia. Therefore, studies that investigate altered 5-HT_3-mediated transmission L-DOPA-induced dyskinesia may further our understanding about the relationship between the 5-HT_3 receptor and the condition (see Chapter 5).

5-HT transporter

The 5-HT transporter (SERT) has also been implicated in the pathophysiology of dyskinesia, notably by studies that assessed SERT binding as a measure of 5-HT innervation in the basal ganglia [475]. Compared to non-dyskinetic subjects, SERT binding was increased in dyskinetic PD patients, rats, and macaques [563]. Following these studies, PET imaging revealed increased SERT to DAT binding ratio in the putamen [564] and GP [565, 566] of dyskinetic PD patients. In support of these findings, inhibition of SERT with selective 5-HT reuptake inhibitors (SSRIs) suppressed dyskinesia in rodent and non-human primate models. Both acute [567] and chronic [568, 569] administration of the SSRIs citalopram and paroxetine improved dyskinesia in the hemi-

parkinsonian rat without compromising L-DOPA anti-parkinsonian action. Contrary to these positive results, a single study found that acute administration of fluoxetine failed to significantly reduce dyskinesia in the same animal model [570]. The clinical benefit of SSRIs on dyskinesia remains unclear as the few studies that were conducted reported conflicting results. Daily administration of fluoxetine over 2 weeks improved apomorphine-induced dyskinesia compared to baseline [571], whereas a 4-week randomised controlled trial revealed that paroxetine had no significant effect on intravenous L-DOPA-induced dyskinesia [572]. While a retrospective study found that exposure to SSRIs may delay the onset and severity of dyskinesia in PD subjects [573], further clinical investigations assessing the use of SSRIs in dyskinesia as an end-point are warranted [460].

The findings described above provide evidence of striatal 5-HT hyperinnervation in the dyskinetic state, which may contribute to the dysregulated release of L-DOPA-derived dopamine [403, 563]. However, the lack of selectivity of SSRIs raises questions about the mechanism mediating the anti-dyskinetic efficacy of SSRIs; some suggest that it may be a combination of mechanisms [460]. Therefore, SERT modulation by SSRIs may alleviate dyskinesia by: 1) activation of presynaptic 5-HT_1 receptors to reduce dopamine release [510, 574]; 2) reduced fluctuations in synaptic dopamine levels by blocking dopamine reuptake in serotonergic neurons [575]; and 3) activation of postsynaptic 5-HT_1 receptors [460].

1.2.6.3. Glutamatergic system

Evidence suggests that both dopamine depletion and dopaminergic replacement therapy with L-DOPA can contribute to secondary changes in glutamatergic transmission within the basal ganglia [576]. Changes in corticostriatal plasticity have been reported in animal models of

PD and dyskinesia, notably the 6-OHDA-lesioned rat and MPTP-lesioned macaque [380, 577-579], culminating in an inability to form long-term depression [580] and to reverse previously induced long-term potentiation [581]. Furthermore, dyskinesia is associated with increased extracellular glutamate levels in the striatum [487] and SNr [582, 583], as well as altered expression of glutamate transporters in the same structures [583-585].

Glutamate receptors are divided into two major types: ionotropic and metabotropic receptors. Ionotropic glutamate are ligand-gated ion channels that mediate fast excitatory synaptic transmission and consist of N-methyl-D-aspartate (NMDA), amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptors [586]. On the other hand, metabotropic glutamate (mGlu) receptors are divided into three subtypes of G-protein coupled receptors based on sequence homology, G-protein coupling, and selectivity for ligands [587, 588]. Group I includes mGlu 1 and 5 receptors, which mainly couple to G_q and stimulate phosphoinositide hydrolysis [579, 589]. Group II includes mGlu 2 and 3 receptors and Group III includes mGlu 4, 6, 7, receptors [587]; both groups couple to $G_{i/o}$ and inhibit adenylate cyclase [579, 589].

Therapeutic agents

Ionotropic glutamate receptors

Amongst ionotropic glutamate receptors, NMDA receptors have been the most extensively studied therapeutic target for dyskinesia and, particularly, converging evidence provides support for the role of the NR2B subunit. Indeed, autoradiographic binding studies have reported increased levels of NR2B subunit-containing NMDA receptors in the striatum of dyskinetic monkeys [590], as well as PD patients with dyskinesia [591]. Moreover, altered subcellular

distribution of NMDA receptor subunits has been associated with the development of dyskinesia in the hemi-parkinsonian rat [592-594] and the parkinsonian monkey [595]. Further support for enhanced glutamatergic activity in dyskinesia arises from a study that found that, following L-DOPA administration, dyskinetic PD patients showed high striatal uptake of [^{11}C]CNS 5161, a PET tracer that binds to activated NMDA receptors [596]. Consistent with these post-mortem studies, behavioural studies have demonstrated the anti-dyskinetic efficacy of the NMDA antagonists amantadine and memantine in rodents [511, 570, 597] and monkeys [598], although the benefit was modest [576]. This preclinical success has been reproduced in the clinic as amantadine alleviated dyskinesia without interfering with L-DOPA anti-parkinsonian action [599-601], which led to the approval of extended-release amantadine as the sole medication approved for dyskinesia in PD by the FDA [602-604]. However, the efficacy of amantadine was moderate [605, 606] and associated with the development of hallucinations [602-604], and tolerance also mitigates its long-term therapeutic efficacy [607, 608]. For their part, NR2B-selective NMDA antagonists demonstrated inconsistent efficacy in preclinical models, including exacerbating dyskinesia [609, 610], no improvement [611], and alleviating dyskinesia [612-615]. Although a randomised placebo-controlled trial found that the NR2B-selective antagonist CP-101,606 improved dyskinesia without affecting anti-parkinsonism [616], there has been no further clinical investigations of NR2B-selective antagonists in dyskinesia.

In contrast, the involvement of AMPA and kainate receptors in dyskinesia remains uncertain [510]. While one study found an increase in AMPA binding levels in the posterior striatum but a decrease in the anterior striatum of L-DOPA naïve and L-DOPA treated MPTP-lesioned macaques [632], another study reported that AMPA binding levels remained unchanged in dyskinetic MPTP-lesioned macaques compared to non-parkinsonian and non-dyskinetic

MPTP-lesioned animals [633]. In PD patients with dyskinesia, there was a decrease in AMPA binding levels in the caudate nucleus but not the putamen, when compare with healthy controls [606]. On the other hand, there is evidence of altered distribution of AMPA receptor subunits within glutamatergic synapses of dyskinetic animals [617], which may suggest abnormal insertion of GluR2 subunit lacking AMPA receptors at corticostriatal synapses [618]. Moreover, a greater anti-dyskinetic effect was obtained by combination of AMPA and NMDA antagonists in the MPTP-lesioned primate [598, 619], although ligands also demonstrated considerable affinity for other targets [620, 621]. Despite the anti-dyskinetic efficacy of AMPA/kainate antagonists reported in preclinical studies [622, 623], the selective AMPA antagonist perampanel failed to alleviate dyskinesia in a Phase II trial [624]. Similarly, the AMPA antagonist topiramate worsened dyskinesia and was poorly tolerated in a randomised placebo-controlled trial with PD subjects [625]. Another AMPA antagonist, talampanel, also underwent testing for safety and efficacy in dyskinesia in Phase I and II trials, respectively, but results have not been updated since 2011 (NCT00036296).

Metabotropic glutamate receptors

In addition to ionotropic glutamate receptors, an increasing body of literature has examined the role of mGlu receptors in L-DOPA-induced dyskinesia. Targeting mGlu receptors offers notable advantages over ionotropic receptors [579]: 1) mGlu receptors have a more limited anatomical distribution, which may lead to more specific activity of ligands and less adverse effects, and 2) they modulate glutamatergic signalling without affecting the excitatory action of glutamate on synaptic transmission. Amongst mGlu receptors, mGlu5 receptor antagonists have demonstrated the most therapeutic potential for dyskinesia [579]. Post-mortem studies in the

macaque found that mGlu5 binding levels were unaltered in the caudate nucleus, putamen, GPi, and GPe when parkinsonian animals were compared to control ones [643-645]. However, L-DOPA administration led to an increase of mGlu5 binding levels in these brain regions, and receptor levels even correlated with dyskinesia scores [643, 646]. On the other hand, the upregulation of mGlu5 binding levels in the caudate nucleus, putamen, GPi, and GPe was absent when parkinsonian macaques were co-administered L-DOPA and the mGlu5 NAM 2-nethyl-6-(phenylethynyl)pyridine [643, 647]. In line with these promising results obtained in post-mortem studies, the therapeutic potential of modulating mGlu5 receptors was evidenced by the anti-dyskinetic efficacy of mGlu5 antagonists in rodent and non-human primate models of PD [626-629] models as well as in patients with PD [630]. Following these favourable data, a Phase IIb/III trial with 140 PD patients is currently ongoing that will assess the effect of the mGlu5 receptor negative allosteric modulator dipraglurant on the Unified Dyskinesia Rating Scale (NCT04857359). The lack of further clinical investigation may be partly explained by the findings of a meta-analysis, which was unable to conclude if mGlu5 antagonists were beneficial for dyskinetic PD patients, in part due to discrepancies in the anti-dyskinetic efficacy depending on the primary outcome measure [631].

The mGlu4 receptor is the most studied target amongst Group III receptors [579] but its therapeutic potential is unclear. Whereas the mGlu4 positive allosteric modulators both ADX88178 and VU0364770 were devoid of anti-dyskinetic efficacy in the 6-OHDA-lesioned rat [632, 633], the orthosteric agonist LSP1-2111 attenuated the development of dyskinesia in the 6-OHDA-lesioned mouse, although it did not reduce established dyskinesia [634]. Contrary to the reported reduction in dyskinesia severity in the MPTP-lesioned macaque [635], foliglurax, an mGlu4 positive allosteric modulator, failed to demonstrate anti-dyskinetic efficacy in a Phase

IIa randomised placebo-controlled study (NCT03162874), which led to the termination of its clinical development [636]. For the mGlu2/3 receptor, results from autoradiographic binding studies are not suggestive that changes to binding levels in the caudate nucleus, putamen, GPi or GPe contribute to the development of dyskinesia in PD [659]; similar findings were obtained in a post-mortem examination of the macaque brain [660]. Despite an earlier report that mGlu2/3 activation with the agonist LY-379,268 did not improve dyskinesia [611], administration of another orthosteric agonist, LY-354,740 significantly reduced dyskinesia in the 6-OHDA-lesioned rat and MPTP-lesioned marmoset [637], although differences in experimental design may explain the discrepancies. Furthermore, mGlu2 positive allosteric modulation with LY-487,379 alleviated dyskinesia in rat and non-human primate models [638, 639], which suggests that the anti-dyskinetic efficacy of Group II compounds may be attributed to selective mGlu2 activation. Although there has been no clinical development of mGlu2/3 activation in dyskinesia, preclinical findings have been encouraging and await confirmation in PD subjects.

1.2.6.4. Other neurotransmitter systems

Evidence also implicates abnormalities in adenosinergic [640], opioid [641], cholinergic [642], noradrenergic [643], and cannabinoid [644] transmission in dyskinesia, which have been reviewed [374, 510, 645-647].

1.2.7. Management

According to an evidence-based report by the IPMDS, the only treatments considered efficacious for dyskinesia are: amantadine, clozapine, bilateral STN or GPi deep brain stimulation, and unilateral pallidotomy [153]. Management strategies for dyskinesia target either

continuous drug delivery, L-DOPA sparing or halting nigral loss [399]. Whereas these approaches are associated with a lower rates of dyskinesia [648-650], it is difficult to uncouple the effect of lowered total dosage of L-DOPA [651-654] as cumulative L-DOPA dose is a known risk factor for dyskinesia development [235].

1.2.7.1. Amantadine

The low-affinity and uncompetitive NMDA receptor antagonist amantadine [655] is the sole anti-dyskinetic agent approved by the FDA [210]. Whereas it demonstrated moderate efficacy in placebo-controlled trials [656], doses of immediate-release amantadine (100-200 mg daily) that conferred anti-dyskinetic benefit were associated with adverse events [399] including hallucinations, blurred vision, and dry mouth that affected 19% to 29% of subjects [657, 658], as well as tachyphylaxis [654]. These concerns led to the development of an extended-release formulation of amantadine (ADS-5102) that has been approved by the FDA [653], which effectively reduced dyskinesia at 12 weeks in two randomised double-blind placebo-controlled trials [602, 603], an effect that was maintained at 14 weeks [602]. An interim report from an open-label study found that at 64 weeks, ADS-5102 was well tolerated and was effective in reducing dyskinesia but adverse events including falls, hallucinations and constipation, led 14% of patients to discontinue the study [607]. However, insight into the mechanism of action governing the anti-dyskinetic efficacy of amantadine is still lacking [212]. Other NMDA receptor antagonists failed to demonstrate anti-dyskinetic efficacy [659-662] and/or compromised the therapeutic benefit conferred by L-DOPA [663]. Therefore, the action of amantadine at additional sites [211], including antagonism at nicotinic acetylcholine receptors [664, 665] at therapeutically relevant concentrations [211], may contribute to its anti-dyskinetic efficacy [212, 666].

1.2.7.2. Clozapine

The atypical antipsychotic clozapine is designated “clinically useful” for the treatment of dyskinesia in PD but requires specialised monitoring due to the risk (<2%) of agranulocytosis [153, 667]. In a single randomised double-blind placebo-controlled trial, clozapine (39.4 ± 4.5 mg/daily) treatment led to a significant reduction in on time with dyskinesia at 10 weeks compared to placebo [525]. Whereas agranulocytosis was not reported as an adverse event, 12% of patients developed eosinophilia [525], and the risk of serious adverse events has limited the use of clozapine in clinical practice [399].

1.2.7.3. Delivery of L-DOPA

Based on the hypothesis that pulsatile administration of L-DOPA triggers dyskinesia [343], several treatments that change L-DOPA formulation to deliver a more sustained release and/or administration of dopamine have been developed [668]. These novel formulations seek to improve the pharmacokinetics of L-DOPA and bypass the gastrointestinal tract [669, 670] and some are currently under investigation [399]. Whereas open-label studies reported a reduction of off time and dyskinesia with continuous dopaminergic stimulation [404-406, 671], a recent meta-analysis found that continuous dopaminergic stimulation therapies provided benefit for both on and off time but at the expense of increased incidence of dyskinesia [672]. It remains unclear whether this approach can reduce the development of dyskinesia [672] as additional factors, such as young age of onset, higher L-DOPA dose, and low body weight, also contribute to pathogenesis [266].

1.2.7.4. Device-assisted therapies

Apomorphine was first used in PD in 1951 to treat tremor and rigidity [673, 674] but its use was limited by its low bioavailability, cost and side effects (e.g. nausea and hypotension) [675]. However, changes to the formulation of apomorphine to continuous subcutaneous administration via a pump system has led to more rapid bioavailability by avoiding gastro-intestinal transit time and first pass hepatic metabolism [653, 676]. The therapeutic benefit conferred by apomorphine may be attributed to its profile as a broad spectrum dopamine agonist on D_1-like and D_2-like receptors [652, 677]. In addition, apomorphine exhibits affinity as an antagonist to 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} receptors and adrenergic α_{2A}, α_{2B} and α_{2C} receptors and agonist to 5-HT_{1A} receptors [652, 677], which have also been implicated in the pathogenesis of dyskinesia [510]. Continuous apomorphine infusion may reduce dyskinesia induction compared to oral dopaminergic therapy [678-680] by providing continuous delivery of drug to the basal ganglia [652, 681]. A substantial number of open-label trials have assessed the efficacy of apomorphine infusion and while most reported that apomorphine reduced off time [682-686], the effect on dyskinesia was variable. Studies reported that dyskinesia was attenuated by 14% to 65% [678, 687, 688] but two studies failed to detect a significant benefit [674, 689]. The discrepancy in results may be attributed to differences in clinical pattern of dyskinesia across studies, as well as the observation that dyskinesia reduction is associated with dose reduction of dopaminergic medication [652]. The TOLEDO study was the first randomised, placebo-controlled, double-blind multi-centre trial to assess apomorphine infusion in PD [690]. Conducted in 107 patients, subcutaneous infusion of apomorphine (3-8 mg/h) significantly increased on time without troublesome dyskinesia at 12 weeks. This benefit was maintained up to 64 weeks in the open-label addition of the TOLEDO study [691]. In line with these findings, multicentre observational studies also reported that subcutaneous apomorphine infusion reduced dyskinesia measured by the Unified PD Rating

Scale (UPDRS) part IV [692, 693]. The good long-term results of apomorphine infusion, however, are marred by high drop-out rates related to adverse events [222], such as skin nodules that affected 44% of patients, as well as nausea and somnolence [674]. In addition, subcutaneous and sublingual administration of apomorphine are indicated for the treatment of off episodes in subjects with PD, often for those with more advanced stages of disease [694, 695].

The development of gastro-intestinal infusion of L-DOPA has permitted its continuous delivery into the intestine, which avoids the erratic absorption of L-DOPA in PD [696]. In the form of an intestinal gel, L-DOPA is administered continuously via a pump system into the proximal jejunum [696], the principal site of L-DOPA absorption [697]. In a randomised placebo-controlled double-blind trial in PD subjects, gastro-intestinal infusion was superior to immediate release oral L-DOPA in increasing ON time without troublesome dyskinesia at 12 weeks [698]. In the open-label extension that followed 258 PD patients, intestinal gel L-DOPA led to sustained improvements in dyskinesia over two years [699]. Moreover, observational studies found that gastro-intestinal L-DOPA infusion alleviated dyskinesia [692, 693]. However, treatment is associated with concerns about polyneuropathy (5% of patients) [696, 700], as well as additional adverse events such as impulse control disorders and procedure or device related adverse events [701]. While a pooled analysis initially reported that the incidence of device or procedure related adverse events was ~75% of patients [702], a more recent study only reported an incidence of 16% [703]; this may be suggestive of improvements in device positioning and follow-up. Nonetheless, efforts are underway to develop new delivery modes of L-DOPA [669], a few are under investigation, including subcutaneous L-DOPA infusion [704] and an intestinal gel that contains entacapone with L-DOPA [705].

1.2.7.5. Surgical interventions

Non-pharmacological treatments that have demonstrated efficacy for dyskinesia only include deep brain stimulation of the bilateral STN, GPi and unilateral pallidotomy in an evidence-based medicine report by the IPMDS taskforce [153]. The choice of target for deep brain stimulation, however, remains contentious. A meta-analysis found that while STN deep brain stimulation was associated with a greater reduction in L-DOPA equivalent daily dose than targeting the GPi, at 12-months follow-up, the latter led to superior reductions in dyskinesia [706]. While dyskinesia severity improved by 21% to 70% with STN deep brain stimulation in PD patients [707, 708], there were also significant reductions in L-DOPA equivalent daily dose [653]. As L-DOPA dosage is a risk factor for the development of dyskinesia [709], it is difficult to parcellate the extent of the anti-dyskinetic action of targeting the STN. In contrast, deep brain stimulation of the GPi is thought to reduce dyskinesia directly [226]. Despite its success as a therapeutic approach in dyskinesia, the mechanism of action underlying the efficacy of deep brain stimulation is unclear [710, 711]. Moreover, the procedure is invasive, and limited by post-operative and long-term hardware-related complications, affecting approximately 8% of patients [712, 713], and adverse events that affect up to 25% of patients [711].

Amongst lesion surgeries, only unilateral pallidotomy is “efficacious” and “clinically useful” for dyskinesia [153] but it is seldom performed in clinical practice, due to the risks associated with surgical lesion and the availability of deep brain stimulation [399].

As iterated above, despite effective treatment options for dyskinesia in PD, efficacy is partial and associated with undesirable side effects. Thus, there remains a significant unmet need in the treatment of L-DOPA-induced dyskinesia.

1.3. Parkinson's disease psychosis

Psychosis is recognised as one of the most common and disabling neuropsychiatric disorders encountered in PD [141] and associated with impaired quality of life, caregiver burden [714], and early mortality [715]. Present in 26% to 74% of PD patients [148, 716-718], the large variation in prevalence may be attributed to differences in sample populations, symptom definition, and study design across studies [719]. PD psychosis tends to manifest during late disease stages [720], which suggests a multifactorial interplay between underlying disease and pharmacology of drugs [719, 721].

1.3.1. Risk factors for PD psychosis

Amongst risk factors associated with developing psychosis in PD, disease duration, disease severity, and presence of other co-morbidities are the most established [719]. Longer disease duration is associated with increased risk for PD psychosis [722, 723] with prevalence of visual hallucinations increasing from <4% in the first 5 years [724] to 74% by 20 years of follow-up [725]. Moreover, longer disease duration also implies greater disease severity [719], particularly with respect to deficits in motor symptoms and daily living [722, 726]. While PD psychosis is more frequently linked with late stages of disease, it has also been reported in 16% to 42% of drug naïve PD patients, although most experience minor hallucinations [727-729]. On the other hand, it is difficult to parcellate the contribution of medication to the development of psychotic symptoms in PD as psychosis is more common in advanced stages, when patients are treated with dopaminergic drugs [719]. Importantly, PD patients that exhibit visual hallucinations have a similar total daily dosage of dopaminergic drugs compared to those without hallucinations [722, 730-732]. In a prospective outpatient study with PD patients, there were stronger associations

between visual hallucinations and patient characteristics, such as disease severity and dementia, than with dopaminergic medication dosage [733]. These results collectively suggest that dopaminergic drugs alone are insufficient to induce psychosis [734, 735] but may facilitate milder forms of psychosis [736]. The risk of developing PD psychosis is variable with dopaminergic medication type – dopaminergic agonists are associated with a higher propensity to induce PD psychosis than L-DOPA [261], a discrepancy that was also revealed by two meta-analyses [737, 738].

The presence of co-morbidities, particularly rapid eye movement sleep behaviour disorder (RBD) and cognitive impairment, has been identified as a risk factor for PD psychosis. Longitudinal studies have found higher risks of developing visual hallucinations in PD patients with RBD after 2 [739] and 12 years [718] follow-up. The interplay between RBD and visual hallucinations may explain the presence of visual hallucinations in early PD [719], as well as a common neurobiological process that underlies both conditions [740]. Since the association between visual hallucinations and subsequent emergence of PD dementia was first reported [741], cognitive impairment has been regarded as one of the most important independent risk factors for developing psychotic symptoms in PD [722, 742-744]. In addition to increased prevalence of psychosis in PD dementia [730, 731, 745] to as high as 5-fold in 12 years [718], the severity and types of symptoms also differ [745] with more frequent reports of complex hallucinations in PD dementia [746]. On the other hand, two studies failed to find a significant association between cognitive dysfunction and development of PD psychosis [718, 747]; visual hallucinations have also been reported in untreated PD patients without dementia [748]. The discrepancy in findings may suggest that hallucinations and cognitive decline are conditions that share common neuropathologic processes that exist on a continuum rather than separate phenomena [719]. Mood disorders, particularly depression [726, 744], have been correlated with psychotic symptoms in PD, although

multivariate analyses found that the effect was inconsistent [722, 749]. While there is evidence that links visual disorders [746, 750], autonomic [732] and olfactory dysfunction [751] as risk factors for PD psychosis, the effect is weak to moderate and lacks support as independent risk factors [719].

1.3.2. Symptomology

The National Institute of Neurological Diseases and Stroke (NINDS)/National Institute of Mental Health (NIMH) working group proposed a unified diagnosis criteria set for PD psychosis that includes 1) presence of at least one psychotic symptom; 2) a primary diagnosis of PD that fulfils the United Kingdom brain bank criteria; 3) the symptoms occur after the onset of PD and are either recurrent or continuous for 1 month; 4) exclusion of other causes (i.e., dementia with Lewy bodies, psychiatric disorders, mood disorders with psychotic features, or a medical condition including delirium); 5) can occur with or without insight, dementia, or treatment for PD [752]. PD psychosis comprises a broad spectrum of symptoms, including hallucinations, delusions, illusions, and false sense of presence; presentation of symptoms is heterogeneous and idiosyncratic [752]. Amongst the psychotic features, visual hallucinations are the most common, affecting 14% to 50% of PD patients [753-755] but auditory, olfactory, gustatory and tactile hallucinations can also be present [717, 727, 754]. Multimodal hallucinations have also been reported in some patients but they receive comparatively little attention [756]. Minor symptoms, including feeling of presence and passage hallucinations [757], develop during early disease course and have gained interest as a possible biomarker for PD psychosis progression to more severe symptoms [727]. To date, the lack of a widely used and validated scale to evaluate psychotic features in PD has impeded understanding of the condition [719]. Scales have often been adopted from non-PD conditions, which do not represent actual symptomology, and no scale

has comprehensively covered the entirety of PD symptoms [758, 759]. Efforts to characterise the complete repertoire of psychotic symptoms in PD with validated scales will enhance our understanding and the development of therapies.

1.3.3. Pathophysiology

The pathophysiology of PD psychosis is poorly understood, as it is difficult to unravel the interaction between the underlying disease and drug-induced factors. Overall, functional MRI (fMRI) studies revealed that PD subjects with visual hallucinations showed greater activation of frontal and subcortical areas [760] but lower activation in the parietal lobe, lateral occipito-temporal cortex [760], and occipital cortex [761, 762], compared to those without hallucinations. In line with these results, PET and SPECT studies found that PD patients with visual hallucinations showed hypometabolism [763, 764] and hypoperfusion [765, 766] of occipito-parieto-temporal brain regions. The overlap with areas of reduced activation in MRI studies may be suggestive of similar functional changes or even a causal link [767]. In addition, structural MRI studies have reported atrophy in visual areas, such as the occipito-temporal and visual parietal cortices of PD patients with psychosis [768-770]. While these results provide support for the role of visual deficits in PD psychosis, symptoms were defined by visual hallucinations in most studies [767], and studies encompassing other psychotic symptoms are required to clarify the link. On the other hand, MRI and voxel-based morphometry studies of patients with visual hallucinations have revealed atrophy in the frontal and parietal cortices [770-772] and hippocampus [773], which is consistent with the association between cognitive deficits and visual hallucinations in PD [748].

1.3.4. Neuropathology

PD patients with visual hallucinations have increased accumulation of Lewy bodies in the superior and lateral frontal, inferior parietal and cingulate cortices, as well as in the amygdala and parahippocampal gyrus [774-777]. Moreover, higher densities of Lewy bodies in parahippocampal and inferior temporal cortices have been linked to shorter latency between time of PD onset and appearance of visual hallucinations [774]. In contrast, PD subjects with visual hallucinations but without dementia had increased Lewy body load in the amygdala but only sparse Lewy bodies in the cortex and hippocampus, compared to those with both visual hallucinations and dementia [775]. This discrepancy in Lewy body burden may indicate that distinct neuropathological changes may underlie visual hallucinations and dementia [778]. The paucity of studies assessing Lewy pathology and psychotic symptoms other than visual hallucinations [776, 777, 779] limits a more comprehensive understanding of Lewy bodies dissemination in PD psychosis and warrants further study.

1.3.5. Explanatory models

More recent models have proposed possible mechanisms underlying PD psychosis, particularly for visual hallucinations [780]. Whereas previous models have focused on neurotransmitter systems [781, 782], newer models integrate how multiple processes, such as modulation of attention by attentional and resting state brain networks [783-785], can contribute to deficits in perceptions, and lead to the presence of visual hallucinations. Dysfunction of these networks may have a biological basis, possibly related to neuropathological and structural changes in the brains of PD patients [776, 786]. The hypotheses put forth by these models have been reviewed elsewhere [780].

1.3.6. Neuropharmacology

1.3.6.1. Dopaminergic system

Early studies posited the role of the dopaminergic system in the pathogenesis of PD psychosis, notably the use of dopaminergic therapy [719]. In the majority of cases, psychotic symptoms in PD are induced by dopaminergic agents, such as L-DOPA and dopamine agonists [787, 788], as well as selective MAO-B inhibitors [789-791]. Moreover, psychotic symptoms in PD improved after lowering or withdrawal of dopamine stimulation [792, 793]. The "kindling" model proposed that chronic stimulation of mesolimbic dopamine receptors by dopaminergic drugs causes hypersensitivity of receptors [781, 794]. These changes result in the dysfunction of dopaminergic signalling within limbic structures that leads to a permissive environment for the development of psychosis [781, 793, 794]. Indeed, imaging studies revealed that drug naïve PD patients had a higher risk of developing visual hallucinations when they had a greater extent of caudate dopaminergic denervation [752, 795] or reduced DAT binding in the ventral and dorsal striatum [796]. These findings collectively suggest that damage to frontal-striatal circuits may be involved in the development of PD psychosis [784], possibly related to dysfunction in brain network activity [797, 798].

A few inconsistencies with the "kindling" model suggest that the relationship between dopaminergic therapy and PD psychosis is more complicated [721]. Psychotic symptoms have been reported in 16% to 42% drug naïve PD patients [727-729] and the dose of dopaminergic therapy is not associated with severity of PD psychosis [730, 799, 800]. While dopaminergic therapy is a risk factor for the development of PD psychosis, it does not appear to be a requirement, especially considering the patients that exhibit psychotic symptoms without being on any medication.

Therefore, later models posited that the interaction between multiple neurotransmitter systems [801] or the serotonergic-dopaminergic imbalance [802] may also be implicated in the complex aetiology of PD psychosis [803].

1.3.6.2. Serotonergic system

Evidence provides support for the involvement of the serotonergic system in PD psychosis [375, 719]. In addition to dopaminergic denervation in PD [48], there is also degeneration of the serotonergic system [804, 805], including a 56% loss of median raphe nucleus serotonergic neurons [806]. A model for psychotic symptoms in PD suggests that they are related to the compensatory upregulation of cortical 5-HT_{2A} receptors [782]. The link between the serotonergic system and PD psychosis was further supported by a PET study that revealed PD patients with visual hallucinations had increased 5-HT_{2A} receptor binding in the infero-lateral temporal cortex, ventral visual pathway, and dorso-lateral prefrontal cortex, compared to those without visual hallucinations [807]. In line with these results, an autoradiographic binding study also found increased 5-HT_{2A} receptor binding in the infero-lateral temporal cortex in PD patients with visual hallucinations [808]. While these studies provide evidence of increased 5-HT_{2A} receptor levels in PD psychosis, preclinical behavioural studies also found that 5-HT_{2A} ligands demonstrated antipsychotic efficacy. Agents with 5-HT_{2A} antagonist activity, such as clozapine [809], mirtazapine [810], and EMD-281,014 [519], significantly alleviated psychosis-like behaviours in the MPTP-lesioned marmoset [513, 516, 556]. The 5-HT_{2A} inverse agonist pimavanserin also attenuated spontaneous head twitches, amphetamine-induced hyperactivity, and prepulse inhibition deficits in the 6-OHDA-lesioned rat [811].

Despite their success in animal models, few 5-HT_{2A} ligands have been recommended by the IPMDS evidence-based medicine report for the treatment of PD psychosis [812]. Clozapine significantly improved psychotic symptoms without worsening parkinsonism in randomised placebo-controlled clinical trials [813-815] and is considered clinically useful but the need for specialised monitoring for agranulocytosis makes it unsuitable for some patients [812, 816]. Whereas quetiapine showed promising results in open-label [532, 817] and clozapine comparison studies [818, 819], only one out of five randomised controlled trials reported antipsychotic efficacy with a modest sample size of 16 patients [820-824]. Based on these conflicting findings, the antipsychotic efficacy of quetiapine is inconclusive [825] and it is considered possibly useful for the treatment of PD psychosis [812]. Mirtazapine and EMD-281,014 have not undergone testing in randomised placebo-controlled trials, although case reports found that mirtazapine reduced psychosis severity in PD subjects has been documented for both Mirtazapine [826, 827]. In contrast, pimavanserin demonstrated efficacy in a Phase III randomised, placebo-controlled trial [828], which led to its approval treat PD psychosis by the FDA [829] and its designation as clinically efficacious with an acceptable safety profile [812].

The increase in 5-HT_{2A} receptors in the infero-lateral temporal cortex of PD patients with psychosis [807, 808], along with overstimulation of these receptors by dopaminergic drugs may contribute to the development of visual hallucinations [830]. In PD psychosis, atypical antipsychotics may exert antipsychotic efficacy by blockade of 5-HT_{2A} receptors in these brain areas [831]. In contrast, the therapeutic benefit obtained with pimavanserin may be through its action as a 5-HT_{2A} inverse agonist [719], which leads to additional dampening of 5-HT_{2A} receptor-mediated neurotransmission in the same brain areas [832]. Despite the efficacy of atypical antipsychotics in PD psychosis, their use is hindered by concerns of agranulocytosis and long-

term monitoring with clozapine [667, 812, 833], and mild to no efficacy for pimavanserin [828, 834] and quetiapine [820-822].

1.3.6.3. Other systems

Evidence suggests that cholinergic deficits favour the emergence of visual hallucinations in PD [803], as well as a role for anticholinergic therapies in triggering them [207]. These studies remain outside the scope of the present review but have been comprehensively reviewed by others [835-837]. Emerging therapies for PD psychosis include targets in glutamatergic, GABAergic, and the cannabinoid system (EudraCT: 2019-003623-37) [55].

1.4. Animal models of Parkinson's disease

Animal models are crucial to preclinical research in drug discovery [840] and in PD research, they are broadly categorised as neurotoxic, genetic, and alpha-synuclein based models [841, 842]. Neurotoxin-based animal models reproduce the extensive dopaminergic denervation of the human condition, behavioural deficits but fail to produce Lewy body pathology [843]; these models have been reviewed previously [841, 844], On the other hand, genetic approaches, such as viral vector-mediated and transgenic models, allow the study of some key features of PD – alpha-synuclein pathology, cell loss and motor symptoms, and genes linked to monogenic causes of the disease [845]. Genetic models of PD extend beyond the scope of this review but comprehensive reviews have been published [845, 846].

1.4.1. 6-OHDA-lesioned rat

Amongst neurotoxin-based models in PD, the 6-OHDA-lesioned rat is amongst one of the most frequently used, partly due to its cost-effectiveness and minimal labour requirements [847]. Following intracerebral injection of the neurotoxin 6-OHDA [848], an analogue of dopamine, it is transported into cell bodies and fibres of both dopaminergic and noradrenergic neurons, where it accumulates in mitochondria [849]. Inhibition of mitochondrial respiratory enzymes [850] causes oxidative stress and mitochondrial damage [851], which is thought to be responsible for the neurotoxic effect of 6-OHDA [852]. A common strategy in studies using the 6-OHDA-lesioned rat model includes administration of desipramine, a noradrenaline transporter blocker, prior to 6-OHDA lesion [851]. This allows selective destruction of dopaminergic neurons by preventing uptake of the neurotoxin by noradrenergic fibres [853, 854]. 6-OHDA has poor penetration across the blood brain barrier, so it is typically injected intra-cerebrally into one of three target sites: the SNc, median forebrain bundle or the striatum [855], where it induces variable dopaminergic denervation [851]. Injection of 6-OHDA into the median forebrain bundle produces >97% dopamine depletion lesions [856, 857], which resembles more advanced stages of PD [50], whereas injection of 6-OHDA into the SNc results in more moderate dopamine depletion (88% dopaminergic cell loss in the SNc) [858]. In contrast, injections in the terminal field of the nigrostriatal pathway only produce a partial lesion that progresses more slowly [856, 857]; such a model more closely resembles earlier stages of the disease, and allows the investigation of neuroprotective interventions in PD. In addition to tissue injection, intracerebroventricular administration of 6-OHDA also induces both degeneration of mesencephalic dopaminergic neurons [859, 860] and deficits in motor behaviour [861-863]. However, this model has been seldomly used since the late 1970s [863], possibly due to the high risk of mortality associated with aphagia,

adipsia [864, 865], and epileptic seizures [866]. Furthermore, the 6-OHDA-lesioned rat model has been pharmacologically validated to assess dyskinesia [511, 867], which provides a paradigm to test drug candidates for symptomatic therapies.

1.4.1.1. Behavioural assessment

Tests of physiological motor behaviour have been performed to estimate the extent of dopamine-denervating lesions in the 6-OHDA-lesioned rat and are described below.

Cylinder test

First described by Schallert and Tillerson, the cylinder test measures rat forepaw use during spontaneous exploration [868], taking advantage of the rearing behaviour exhibited by rats in a novel environment, *i.e.*, standing on their hindlimbs and using their forepaws to make wall contacts [869]. Forelimb asymmetry is based on the number of independent weight bearing contacts an animal makes with each forelimb (ipsilateral, contralateral and both) and the score is expressed as the performance of each limb as a percentage of total wall contacts [849]. Whereas intact rats use both forepaws indiscriminately, rats subjected to a unilateral 6-OHDA lesion only use the forepaw contralateral to lesion in 10% to 30% of total contacts [511]. Moreover, use of the paw ipsilateral to lesion in ≥ 70% of rears is indicative of 88% striatal dopamine deficit [868] and has been used as a threshold to select animals for further behavioural analyses [457, 515]. Compared to other tests of physiological motor activity, the cylinder test offers several advantages [869], including: 1) a measure of spontaneous forelimb use in the testing cylinder that is identical to behaviour exhibited in its home cage; 2) the inter-rater reliability is very high ($r > 0.95$) [868]; 3) it is a simple and objective test that does not require pre-training or extensive manipulation of

animals; and 4) the cylinder test uses a drug-free paradigm to assess the extent of nigrostriatal lesion, which is crucial for study designs assessing the effects of therapies on the development of treatment-related complications [637, 870].

For paradigms assessing anti-dyskinetic therapies, it is crucial to determine whether their mode of action is due to suppressing motor behaviour, which would limit their value in PD because of further deterioration of parkinsonian symptoms [869]. Therefore, using forelimb asymmetry measured by the cylinder test, we can determine whether the benefit conferred by anti-dyskinetic agents compromises L-DOPA anti-parkinsonian action. However, while L-DOPA administration improves forelimb asymmetry to restore use of the forepaw contralateral to lesion in 6-OHDA-lesioned rats, the development of dyskinesia interferes with physiological limb use [511]. To limit the disruptive effect of dyskinesia on cylinder test performance, a subtherapeutic dose of L-DOPA is often administered, which is sufficient to assess forelimb asymmetry in animals without triggering dyskinesia [515, 637, 870].

Drug-induced rotations

Unilateral lesion of 6-OHDA in rats leads to asymmetrical motor behaviour, generally evidenced by preferential turning ipsilateral to lesion [848, 871]. If dopaminergic depletion is nearly complete, then there is slight recovery of this motor asymmetry [849]. When administered with dopamine agonists, rats rotate contralateral to lesion [872, 873] but when administered with drugs that increase dopamine levels, such as amphetamine, they exhibit ipsilateral rotations [871, 874]. Although the mechanism underlying rotational behaviour in unilateral 6-OHDA-lesioned rats has not yet been elucidated [874], the predominant hypothesis proposes that turning is the result of

an imbalance in striatal dopaminergic activity, whereby animals rotate away from the side of greater dopaminergic activity [875].

The relationship between rotation rates and degree of striatal denervation or dopaminergic cell loss in the SN has been well documented [858, 876] and consequently, drug-induced rotations are commonly used to assess the extent of nigrostriatal dopamine depletion in unilateral 6-OHDA-lesioned animal models [849]. Drug-induced rotations offer some notable advantages, including objective outcome measures, automation, and no pretraining of animals is required [869]. However, use of these tests is also hampered by their lack of clinical relevance [877], failure to distinguish between dyskinetic and anti-akinetic effects of drugs [511], and behaviours that are dependent on mesolimbic systems [878-880], which are not the primary brain regions involved in the pathophysiology of PD motor symptoms [881]. Moreover, some critics have argued that drug-induced rotations lack reliability as an estimator of dopaminergic denervation [882-884], for instance, when there is possibility of damage to the striatum [885].

Amphetamine

As mentioned earlier, when amphetamine is administered to unilateral 6-OHDA-lesioned rats, animals demonstrate ipsilateral rotational behaviour, which has been shown to correlate with the extent of dopamine denervation [858, 886, 887]. A cut-off of 6 turns/min over 90 min after amphetamine administration has often been used for inclusion in studies [396, 888]. Unlike rotations induced by apomorphine that require maximal lesion, those induced by amphetamine only require submaximal lesion (75-90%) [889], suggesting a relatively higher sensitivity to detect nigrostriatal dopamine depletion. While a single study found a positive correlation between amphetamine-induced rotation and development of dyskinesia, the study administered a

dextro(d)-amphetamine dose of 5 mg/kg [890] but most dyskinesia studies use a dose of 2.5 mg/kg to limit side effects [888, 891, 892]. When amphetamine-induced rotation used a d-amphetamine dose of 2.5 mg/kg, it demonstrated poor correlation with dyskinesia, as well as no correlation between amphetamine-induced rotations and tyrosine hydroxylase (TH) positive cell loss in the SNc [888]. These findings led the authors to conclude that the amphetamine-induced rotation test is a poor predictor of 6-OHDA-lesion success and dyskinesia development based on the d-amphetamine dose often used in studies. The test also has important pitfalls that limit the conclusions drawn from rotational data, such as non-linear recovery of rotations, overcompensation in rotational behaviour contralateral to lesion, and behavioural conditioning, emphasising the constraints of employing amphetamine-induced rotation test in the 6-OHDA-lesioned rat [893].

Apomorphine

In the 6-OHDA-lesioned rat, the dopamine agonist apomorphine induces rotational behaviour contralateral to lesion [872]. This phenomenon was first hypothesised as the result of the supersensitivity of postsynaptic dopaminergic receptors of the lesioned striatum [872], and was later corroborated by pharmacokinetic, gene expression, and receptor binding studies [894, 895]. Of note, partial dopamine denervating lesions do not result in apomorphine-induced rotations [896] as extensive lesions of the striatum (>90% loss of dopamine fibre density) and concomitantly SNc (>50% loss of dopaminergic neurons) are required for such turning behaviour [889]. While the cut-off value for apomorphine-induced rotations varies amongst studies in terms of the number of turns/min and timing of assessment [895, 897, 898], 60 turns/30 min in the test negatively correlated with survival of TH+ SNc neurons, suggesting that these parameters are appropriate as inclusion criteria for studies [898].

Rotarod

The rotarod test is widely used to assess physiological motor function in the rat [899, 900] and measures its ability to maintain itself on a rod that rotates with accelerated speeds [869]. The test can be used to detect deficits induced by 6-OHDA lesion, whereupon administration of dopamine agonists, e.g., L-DOPA, leads to improvements in performance [580, 899, 901]. The main disadvantages of the rotarod test are that it is dependent on the willingness of the animals to perform the task, which can be compromised by stressors, as well as the relatively lower accuracy and consistency of the investigator compared to other tests [869]. Moreover, improvements in rotarod performance induced by anti-parkinsonian treatments are compromised with the development of dyskinesia [580, 901]. Nonetheless, the test exhibits a wide dynamic range and demonstrates the sensitivity to detect subtle changes in motor function following interventions [869].

Abnormal involuntary movements

Preclinical studies on L-DOPA-induced dyskinesia were previously only conducted in non-human primates because of the belief that only these species could demonstrate the repertoire of movement disorders exhibited by patients with PD [407]. It was only in the late 1990s that a dyskinesia rating scale was published in the 6-OHDA-lesioned rat [902], the efforts of Cenci and collaborators to characterise the rat equivalent of dyskinesia, termed abnormal involuntary movements (AIMs). When hemi-parkinsonian rats are treated with L-DOPA, they exhibit abnormal postures and movements that affect the trunk, limbs, and orofacial muscles contralateral to lesion [902], and present functional and phenomenological analogies to dyskinetic

behaviour in PD patients [570]. Phenotypically, AIMs are complex movements that involve multiple muscle groups, including repetitive head movements and rapid flexion of the forelimb, which are similar to choreiform observed in PD patients [869]. Functionally, AIMs are disabling and involuntary movements [396, 511, 869], as are dyskinesia in subjects with PD [903]. Importantly, assessment of AIMs in the 6-OHDA-lesioned rat model has been pharmacologically validated with drugs of varying dyskinesiogenic potential [511]. Whereas non-dopaminergic compounds that demonstrated anti-dyskinetic efficacy in PD patients and/or non-human primates also attenuated AIMs, anti-parkinsonian therapies with low dyskinetic potential in primates also failed to induce AIMs in rodents [511, 904]. These studies demonstrated that clinically-relevant measures of akinesia and dyskinesia could be obtained in rats [511, 904]; a later study affirmed the high predictive value of the AIMs rating scale as a preclinical screen for novel anti-dyskinetic drugs [570]. Indeed, treatments with anti-dyskinetic effects in PD subjects and/or non-human primates, such as amantadine [600], clozapine [525], and buspirone [497], also improved AIMs severity in the 6-OHDA-lesioned rat [570].

During AIMs assessment, rats are placed in individual transparent cylinders and observed for two minutes every twenty minutes for three hours following L-DOPA administration [511, 570, 905]. AIMs are grouped into four subtypes based on their topographic distribution: 1) axial AIMs: dystonic postures or choreiform twisting of the neck and body towards the side contralateral to lesion; 2) limb AIMs: purposeless and abnormal movements of the forelimb and digits contralateral to lesion; 3) orolingual AIMs: empty jaw movements and tongue protrusions contralateral to lesion; and 4) locomotive AIMs: hyper locomotion with contralateral side bias [869]. Whereas locomotive AIMs are expressed by dyskinetic 6-OHDA-lesioned rats, they do not provide a specific measure of dyskinesia [511, 904], and instead, correlate

with contralateral turning behaviour [511]. In fact, locomotive AIMs may result from enhanced locomotor activity in rats with sensorimotor asymmetry as treatments that induce low dyskinesia severity may still induce marked contralateral rotations, and thus, high locomotive AIMs scores in 6-OHDA-lesioned rats [869]. Therefore, studies often evaluate the severity of axial, limbs, and orolingual AIMs but omit locomotive AIMs [906-908].

Presentation of AIMs subtypes is heterogeneous between animals but is fairly consistent in the same animal upon repeated testing [869]. In the original scale, the duration of AIMs severity was rated on a scale from 0 to 4, where 0 = absent and 4 = continuous and not suppressible by external stimuli (Table I, page I) [902]. However, by only assessing the frequency of AIMs, investigators failed to consider their intensity, which along with dyskinesia frequency, increases over time in PD patients with long-term L-DOPA therapy [407, 909]. This led the authors of the original AIMs rating scale to include an additional scale based on the amplitude of AIMs (Table II, page II) rated on a scale from 0 to 4 for each AIMs subtypes (axial, limbs, and orolingual) [396, 905], which allowed, for instance, differentiation between small forelimb movements and dystonic-like movements that involved shoulder muscles [396, 905, 909]. In fact, L-DOPA treatment in animals with partial dopaminergic denervation resulted in lower AIMs amplitude severity [396, 905]. Importantly, the duration and amplitude rating scales are scored simultaneously but the expression and analysis of AIMs scores varies depending on experimental design [905]. Lastly, large fluctuations in striatal dopamine levels following L-DOPA administration result in supersensitive dopamine receptors in the denervated striatum [330, 910], leading to the upregulation of immediate early genes and neurotransmitter-related genes [578, 851]. Rat AIMs severity correlates with the upregulation of genes and signalling molecules downstream of dopamine receptors [432, 902, 911, 912], which has also been reported in non-human primate models [441, 913] and

PD patients [914, 915], demonstrating the construct validity of AIMs rating in the 6-OHDA-lesioned rat [874].

1.4.2. 6-OHDA-lesioned mouse

Another neurotoxic model is the 6-OHDA-lesioned mouse model, although it is less commonly used than the 6-OHDA-lesioned rat and MPTP-lesioned non-human primate models [939]. Initial attempts to establish this model were challenging as animals were prone to high mortality rates and weight loss post-surgery [940, 941], as well as difficulty targeting smaller structures, such as the median forebrain bundle or SN [864]. Moreover, 6-OHDA-lesioned mice showed variable degrees of dopamine depletion [942] and expression of dyskinesia [943]. But modification of injection protocols [944] and more rigorous post-operative care and monitoring [939] have enhanced the survival rate of animals [945]. While 6-OHDA is typically injected in the mouse medial forebrain bundle, striatum or SN to induce parkinsonian motor deficits [939, 941, 943, 946, 947], each lesion site is associated with a distinct profile of degeneration in the nigrostriatal pathway [941]. Nonetheless, intrastriatal injection is the most common lesion site in mice and the slow retrograde degeneration of the nigrostriatal pathway in this model renders it suitable for investigation of neuroprotective therapies [948].

1.4.3. MPTP-lesioned non-human primates

MPTP-induced parkinsonism was first reported in 1983 when four drug addicts injected themselves with a derivative of meperidine and presented with severe bradykinesia and rigidity [916]. Although a case report of a man who injected himself with 4-propyloxy-4-phenyl-N-methylpiperidine and subsequently developed parkinsonism was likely the first report of MPTP-

induced parkinsonism in humans as the injection may have contained MPTP as an impurity [844, 917]. Following these case reports, it was demonstrated that MPTP administration in both Old World primates, e.g., *Macaca mulatta* (rhesus macaque) [918] and *Macaca fascicularis* (cynomolgus macaque) [919] and New World primates, e.g., *Saimiri sciureus* (squirrel monkey) [920] and *Callithrix jacchus* (marmoset) [921], induced a parkinsonian syndrome, see [844] for a more extensive review.

Injection of the neurotoxin MPTP in non-human primates resulted in the selective loss of dopaminergic neurons in the SNc with a concomitant deficit of striatal dopamine [918]. The mechanism underlying cell death is the conversion of MPTP to its toxic metabolite MPP+, which after uptake into dopaminergic neurons, induces toxicity by interfering with neuronal cell activity [93]. For instance, MPP+ impairs mitochondrial function [922, 923] by inhibition of Complex I [924, 925], leading to the production of reactive oxygen species [926, 927] and deficits in adenosine triphosphate formation [928]. Furthermore, MPTP-induced neurotoxicity is associated with microglial activation [929, 930], which furthers inflammatory processes through increased production of proinflammatory or neurotoxic factors [931], including nitric oxide [932], reactive oxygen species [933], chemokines [934], and cytokines [935]. In addition to neuropathological similarities, MPTP-lesioned non-human primates also exhibit a repertoire of motor symptoms reminiscent of PD, such as bradykinesia, rigidity, and postural abnormalities, and that is responsive to dopamine replacement therapy [936, 937]. Moreover, MPTP-lesioned non-human primates also develop motor complications associated with chronic L-DOPA administration, namely dyskinesia [938-940].

Amongst non-human primates species, cynomolgus and rhesus macaques, marmosets, and squirrel monkeys are the most frequently employed in studies assessing the anti-parkinsonian, anti-dyskinetic, and antipsychotic potential of drugs [941] and are further discussed below.

1.4.3.1. MPTP-lesioned macaque

The first MPTP-lesioned non-human primate model of PD was developed in the rhesus macaque in 1983 [918] and a few years later, MPTP-induced parkinsonism was reported in the cynomolgus macaque [919]. A range of regimens of MPTP administration have been described in the macaque, such as subcutaneous injection, intravenous, and intracarotid, leading to varying degrees of parkinsonism [844], although the severity of parkinsonian features depends on the individual sensitivity of animals [844], as well as the dose and frequency of administration [937]. Post-mortem analyses have revealed greater than 95% striatal dopamine depletion and reduction in DAT binding in MPTP-lesioned macaques [508, 942, 943]. Different scales have been developed to assess parkinsonism severity in MPTP-lesioned macaques [944-950] and despite some variation in the number of items scored, virtually all scales evaluate bradykinesia, rigidity, and postural abnormalities [951]. A review of the records of MPTP-lesioned macaques also found pronounced discrepancies in the behavioural phenotype across animals, which led to recommendations to optimise MPTP administration paradigms to produce consistent and stable parkinsonian features [952]. MPTP-lesioned macaques are sensitive to the main factors of dyskinesia [953], including loss of nigrostriatal dopaminergic projections [939], L-DOPA dosage [345, 510, 954], and increased dyskinesia incidence following long-term L-DOPA therapy [407, 851]. Accordingly, the repertoire and severity of dyskinesia in this model are reminiscent from clinical practice [452, 955] with animals exhibiting choreic-athetoid, dystonic, and ballistic movements [953]. Although rating

scales to assess dyskinesia severity have been developed in macaques [955-957], quantitative evaluation of dyskinesia has been lacking [626, 958]. This gap led to efforts to develop a highly sensitive and quantitative method to evaluate dyskinesia [959] but this novel scale has not been adopted by other groups, limiting its value. Whereas the MPTP-lesioned macaque is largely considered the gold standard animal model for dyskinesia with a higher positive predictive value than the MPTP-lesioned marmoset [941], its use is hampered by ethical and economic issues [960]. Although a single study has assessed psychosis-like behaviours in the MPTP-lesioned macaque, the rating scale was developed in the marmoset and lacked pharmacological and predictive validation [961]. Given that the behavioural repertoire of macaques does not permit the evaluation of antipsychotic drugs, it restricts the translational potential of this model as PD psychosis affects a significant proportion of patients [754, 962].

1.4.3.2. MPTP-lesioned squirrel monkey

In 1984, a parkinsonian syndrome was described in squirrel monkeys for the first time following systemic MPTP administration [920]. In addition to animals exhibiting akinesia, rigidity, and hypophonia, post-mortem examination of their brains revealed selective cell loss in the SNc [920]. In the squirrel monkey, MPTP is most frequently administered subcutaneously [963-965] and less commonly intraperitoneally [920]. Depending on the region of the striatum examined, traditional MPTP regimens generally lead to greater than 80% reduction in DAT binding levels, while milder regimens lead to more moderate reductions in striatal DAT binding with relative presentation of striosomes [844, 963]. Moreover, the role of monoamine oxidase (MAO) in the conversion of MPTP to MPP+ was also discovered in the squirrel monkey [844], as concomitant administration of the MAO inhibitor pargyline with MPTP prevented the parkinsonian

phenotype to develop [966]. This effect of pargyline may be related to its action opposing the neurotoxic effects of MPTP as squirrel monkeys co-treated with pargyline and MPTP did not show evidence of nigral cell loss [966]. In addition to a rating scale for assessment of parkinsonism in squirrel monkeys, a simple, sensitive and validated instrument has also been developed for assessment of dyskinesia [957]. However, compared to the macaque and marmoset, only a few pharmacological targets have been investigated in a limited number of studies in the squirrel monkey for their effects on parkinsonism and dyskinesia [967-970], and anti-psychotic drugs have not been examined at all, see review by [941]. Therefore, it is difficult to calculate its predictive value for these endpoints [941].

1.4.3.3. MPTP-lesioned marmoset

In recent years, the common marmoset has attracted interest as a model for neuroscience research, in part due to their closer genetic and anatomical relationship to humans and are preferred over rodent species [971]. In 1984, a parkinsonian syndrome was first reported in the common marmoset following MPTP administration [921]. Different MPTP regimens in the marmoset have led to varying degrees of nigrostriatal denervation [844]. For instance, a mild dose of MPTP injected subcutaneously twice a week over 5 to 10 months led to greater than 95% dopamine depletion within the striatum [972], while a mild dose of MPTP injected subcutaneously daily over 9 days also led to more than 95% striatal dopamine depletion [973, 974]. At the behavioural level, the MPTP-lesioned marmoset exhibits deficits that resemble clinical features of PD including bradykinesia, rigidity and postural instability [921]. Accordingly, rating scales to assess parkinsonism severity have been developed in the marmoset [623, 940, 975, 976]. Moreover, following dopamine replacement therapy, MPTP-lesioned marmosets also develop dyskinesia

[940] and psychosis-like behaviours [513, 977], suggesting that the species is suitable to model both dyskinesia and PD psychosis. Thus, variations of clinical rating scales for PD have also been established and validated to assess the severity of dyskinesia and psychosis-like behaviours in the marmoset [556, 977]. Dyskinesia encompasses both chorea and dystonia, which are both evaluated on a 0-4 severity gradient that distinguishes between disabling and nondisabling movements [517, 556], which is in accordance with part IV of the Movement Disorders Society (MDS)-UPDRS [978]. On the other hand, psychosis-like behaviours consist of visual hallucinations, hyperactivity, stereotypies, and excessive grooming, where each element is rated on a 0-4 severity gradient [513, 977]. The MPTP-lesioned marmoset demonstrates high face validity that has led to high predictive value in assessing clinical efficacy for dyskinesia, psychosis, and parkinsonism [941, 979], supporting its therapeutic value as a paradigm to test drugs for several conditions.

Compared to macaques, marmosets are evolutionarily further to humans [1009, 1010], and as phylogenetic differences increase between species, they are generally accompanied by behavioural, physiological and anatomical differences as well [970, 1011]. For instance, the brain volume of a marmoset is about 12 times smaller than that of the rhesus macaque, and 180 times smaller than the human brain [1012, 1013]. Furthermore, in terms of anti-dyskinetic efficacy of drugs, the macaque has higher positive predictive value than the marmoset, although both species are limited when predicting detrimental effects of drugs on dyskinesia [974]. In general, systemic MPTP treatment is also associated with a greater need for post-op care for animals and increased risk of inadvertent toxic exposure to researchers compared to local 6-OHDA administration [1014-1016]. Nonetheless, despite these drawbacks, as iterated above, the MPTP-lesioned marmoset has

demonstrated construct, face, and predictive validity for PD and therapeutic complications of the disease.

1.4.4. Limitations of toxin-based animal models

Despite their important contribution to the understanding and treatment of PD, there are limitations to the MPTP-lesioned non-human primate. For instance, neurotoxin-based animal models do not generally harbour alpha-synuclein inclusions in the form of Lewy bodies [980], which are a pathological hallmark of PD [56, 61], as seen above (Section 1.3). In addition, differences in the temporal profile of cell death, where toxin models produce acute severe nigrostriatal denervation [849] compared to the progressive neurodegeneration that occurs in the human condition [981], may also explain failures in the clinical development of disease-modifying therapies that showed benefit in toxin-based animal models. Whereas PYM50028, an inducer of brain trophic factors, provided neuroprotection against MPTP damage in mice [982] and macaques [983, 984], it failed to demonstrate beneficial effects in a Phase II clinical trial with PD subjects (NCT 18364399). For this reason, neurotoxin models may be more suited for the evaluation of symptomatic therapies [844], whereas development of disease-modifying therapies for PD may require an animal model that recapitulates core pathological processes of the human disease [147, 985].

1.4.5. Pre-formed fibrils injection models

Paradigms that involve the injection of fibrillar alpha-synuclein in the brain or gut also produce Lewy-like pathology in the brain of rodents and non-human primates, similar to Braak staging observed in post-mortem PD brains [67, 68]. Several lines of evidence suggest that

recombinant alpha-synuclein pre-formed fibrils (PFFs) act as seeds to template and induce the pathological conversion of endogenous alpha-synuclein, resulting in the propagation of PD [986, 987]. The accumulation of phosphorylated alpha-synuclein inclusions ultimately leads to neuronal dysfunction and degeneration [988]. Importantly, the toxicity can be directly linked to the recruitment of endogenous alpha-synuclein into inclusions [988] as PFFs fail to induce toxicity when applied to alpha-synuclein knockout primary neurons [986, 989]. Moreover, the PFF model demonstrates several advantages compared to other alpha-synuclein models [842, 988]. For instance, PFFs induce the conversion of endogenous alpha-synuclein into pathological aggregates [986, 990], while viral vector-based and transgenic models rely on the overexpression of human wild-type or mutated alpha-synuclein [991-993], leading to alpha-synuclein expression levels 2 to 20 times higher than normal endogenous expression [994] and exceeding those observed in the human condition. In addition to its physiological relevance, the time course of neurodegeneration and alpha-synuclein pathology induced by PFFs is similar to that in PD [842], whereby dysfunction of dopaminergic neurons precedes the presentation of motor symptoms [50]. Last, the PFF model allows the investigation of the progression of alpha-synuclein aggregation from their formation to neuronal death [842]. Taken together, the PFF model provides both high temporal and spatial resolution [990].

Towards this end, several models have been established to study the propagation of alpha-synuclein in rodents. In a seminal study by Luk et al., injection of PFFs in the mouse striatum resulted in the accumulation of pathological alpha-synuclein pathology in neural circuits ipsilateral to injection site, including SNc and amygdala, loss of dopaminergic neurons of the SNc accompanied by motor deficits after 6 months [995]. Importantly, the authors found that alpha-synuclein pathology was mainly limited to anatomical sites connected to the injection

site, suggesting that interneuronal connectivity and cell-to-cell propagation of pathogenic alpha-synuclein are major determinants of Lewy body pathology dissemination [987, 989, 995, 996]. Similar results have also been obtained following intrastriatal injection of PFFs in the rat [997-999]. In addition to the striatum, intracerebral PFF injection models also include the mouse SN [987], cortex [989, 1000] and hippocampus [1001], with variable patterns of aggregation spreading depending on the injection site. In line with Braak's hypothesis [1002], several PFF models have injected into the olfactory bulb and peripheral regions [842]. Injection of PFFs into the olfactory bulb of wild-type mice led to widespread propagation of pathological alpha-synuclein aggregates to more than 40 brain regions after 12 months [1003], as well as neuronal loss in the anterior olfactory nucleus and deficits in olfaction [1003, 1004]. Furthermore, PFF injection in the mouse enteric nervous system, such as the gastric wall [1005] and colon [1006] resulted in alpha-synuclein pathology in enteric neurons and brainstem after 12 months, although the brainstem pathology was minor and only observed at a single time point (1 month), suggesting clearance of pathological alpha-synuclein and lack of significant propagation [1006]. A later study that injected PFFs in the mouse muscularis layer of the pylorus and duodenum found widespread alpha-synuclein pathology in the brain, including the brainstem, amygdala, and SNc that was associated with both motor and nonmotor symptoms [1007]. Importantly, this pathological propagation of alpha-synuclein was prevented by truncal vagotomy and absent in alpha-synuclein knockout mice, modelling the gut to brain transneuronal propagation observed in PD [1008, 1009].

Considering the need for animal models to have closer neuro-anatomical and genetic proximity to humans, PFFs have also been injected in non-human primates. A pilot study in the common marmoset showed evidence of bilateral spreading of pathological alpha-synuclein following mouse PFF injection in the striatum after 3 months [1010]. A recent study found that

intrastriatal injection of PFFs in the macaque led to ipsilateral dissemination of pathological alpha-synuclein to the SNc with significant reduction of dopaminergic neurons after 15 months [1011]. Moreover, inoculations of PD-derived Lewy body extracts into the SN or striatum of macaques also led to progressive nigrostriatal degeneration after 14 months [1012]. However, Lewy body extracts were purified from post-mortem PD brains, and it remains unclear whether other components in the fractions other than alpha-synuclein were also present and possibly contributed to the pathology that was observed. In addition to intracerebral injections of PFFs, one study injected in the gastric walls and colon of macaques and after 12 months post-injection and reported uptake of recombinant alpha-synuclein and aggregate formation in enteric neurons [1006]. Contrary to findings obtained in mice [1005, 1006], alpha-synuclein pathology failed to spread to the central nervous system, which raises doubts about the gut to brain prior hypothesis of PD, at least in a non-human primate model [1006].

Important research avenues were also opened by these previous studies. For instance, while some PFF models characterised behavioural deficits, they were generally lacking regarding imaging biomarkers, which provide a measure of lesion development. Therefore, complementing in vivo monitoring of the rate of striatal denervation with behavioural analysis could facilitate greater understanding of what is occurring during early stages of the pathological process and remains to be performed. In addition, most studies have injected mouse alpha-synuclein PFFs and, given that the mouse sequence has an aggregation-prone mutation in familial PD [33], this may affect interpretation of their findings. Therefore, constructing a model that addresses these concerns could further understanding of the pathological processes occurring in PD. Developing the methodology that allows for accurate identification of the surgical target and subsequent injection of PFFs would be crucial first steps in establishing this

novel animal model of PD. Compared to neurotoxic models, alpha-synuclein based models show modest nigral dopaminergic cell loss. For instance, transgenic alpha-synuclein overexpression models rarely show significant degeneration despite widespread alpha-synuclein pathology [1050]. While PFF models only lead to modest impairments in neuronal function and survival [1051], these modest effects are more consistent with the slow progression of disease in patients [1050]. Moreover, in non-human primates, there is a lack of motor impairment following injection of alpha-synuclein PFFs [1047, 1048], although these studies did not include motor-related endpoints. Nonetheless, the relationship between neuropathology and behavioural dysfunction in rodent models becomes clearer with longer follow-up (e.g., 12 months follow-up) [1052]. Despite the more complex methods employed in PFF and viral injection models compared to neurotoxin-based ones, efforts to improve the reproducibility of the former models have led to publication of guidelines and standard practices [1053].

2. Objectives and hypotheses

As presented earlier, there is accumulating evidence that implicates the involvement of the 5-HT system in the development of dyskinesia and PD psychosis. We sought to expand upon earlier findings on the anti-dyskinetic efficacy of the 5-HT_3 antagonist ondansetron obtained in the 6-OHDA-lesioned rat. The present thesis seeks to examine the effect of 5-HT_3 receptor blockade on treatment-related complications in PD using the 6-OHDA-lesioned rat and the MPTP-lesioned marmoset. In addition to symptomatic therapies, efforts to engineer a novel alpha-synuclein propagation model in the marmoset led to the development of a frameless stereotaxic approach to localise and inject into brain structures, which is also described in the thesis. More specifically, we hypothesised that:

1. Ondansetron plasma and brain levels inform upon its behavioural effects reported in literature
2. 5-HT_3 receptor blockade reduces the severity of established L-DOPA-induced AIMs in the 6-OHDA-lesioned rat without compromising L-DOPA anti-parkinsonian action
3. 5-HT_3 receptor blockade reduces the severity of dyskinesia and psychosis-like behaviours in the MPTP-lesioned marmoset without impairing the therapeutic efficacy of L-DOPA
4. Characterisation of novel psychosis-like behaviours in the MPTP-lesioned marmoset are idiosyncratic and stereotyped
5. 5-HT_3 receptor levels are altered in the brains of post mortem dyskinetic 6-OHDA-lesioned rats
6. Co-registration of imaging modalities localises a surgical target in the marmoset brain for injection of biological material

To validate these hypotheses, we met the following aims:

1. To determine the rat pharmacokinetic profile of ondansetron in plasma and brain following administration of small doses to contextualise the literature on its behavioural effects.
2. In the 6-OHDA-lesioned rat:
   2.1. To determine the effect of acute challenges of the 5-HT_3 antagonist granisetron at alleviating established L-DOPA-induced AIMs;
   2.2. To assess whether the anti-dyskinetic benefit conferred by granisetron is achieved without hindering L-DOPA anti-parkinsonian action.
3. In the MPTP-lesioned marmoset:
   3.1. 5-HT_3 receptor blockade alleviates the severity of dyskinesia;
   3.2. 5-HT_3 receptor blockade alleviates the severity of psychosis-like behaviours;
   3.3. 5-HT_3 receptor blockade alleviates the severity of parkinsonism.
4. To expand upon the existing behavioural repertoire of psychosis-like behaviours in MPTP-lesioned marmosets following L-DOPA administration.
5. To determine the distribution of the 5-HT_3 receptor in brain areas implicated in L-DOPA-induced dyskinesia, including the motor loop of the basal ganglia, using autoradiographic binding in 6-OHDA- and sham-lesioned rats.
6. Test for correlations between the severity of L-DOPA induced dyskinesia and specific [^{3}H]GR65630 binding levels in different brain areas.
7. To describe the co-registration of imaging modalities (CT, MRI, and PET) to precisely locate a target in the marmoset brain for injection of alpha-synuclein pre-formed fibrils.

3. Contributions to Original Knowledge

The current thesis presents data that consist of original contribution to knowledge including:

1. The pharmacokinetic profile of ondansetron in the rat was determined following subcutaneous administration. This was the first report of ondansetron plasma and brain levels (e.g., primary motor cortex, striatum) in the rat following a subcutaneous administration regimen, providing some contextualisation of the vast preclinical findings on the central effects of ondansetron in this species.
2. Granisetron alleviated the severity of both the duration and severity of L-3,4-dihydroxyphenylalanine (L-DOPA)-induced abnormal involuntary movements (AIMs) in the 6-hydroxydopamine (6-OHDA)-lesioned rat. Importantly, this therapeutic benefit was achieved without hindering L-DOPA anti-parkinsonian action. Taken together with previous results obtained with ondansetron, these findings suggest that the anti-dyskinetic efficacy of selective serotonin type 3 (5-HT_3) antagonists may be attributed to 5-HT_3 blockade and support the development of a new therapeutic strategy to manage dyskinesia in Parkinson's disease (PD).
3. Ondansetron significantly improved the severity of dyskinesia, psychosis-like behaviours, and parkinsonism in the gold standard model of PD, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned marmoset. These results provide further support for the role of 5-HT_3 blockade in the treatment of dyskinesia and are amenable to the clinical testing of 5-HT_3 antagonists. Moreover, it was demonstrated that ondansetron effectively reduced psychosis-like behaviours in a randomised paradigm with controls, reaffirming findings reported in open-label trials and broadening the value of 5-HT_3 blockade to the treatment of PD psychosis.

4. Expanded the behavioural repertoire of psychosis-like behaviours in the MPTP-lesioned marmoset by characterising novel stereotyped behaviours that varied depending on the environment and animal. These behaviours in marmosets are reminiscent of punding observed in PD patients and illustrate the value of the MPTP-lesioned marmoset as a validated model to understand stereotypical behaviours.
5. 5-HT_3 receptor levels were upregulated in the subthalamic nucleus, and ipsilateral entopeduncular nucleus and motor thalamus of post-mortem dyskinetic 6-OHDA-lesioned rats but remained unaltered in five other brain regions studied, i.e., the primary motor cortex, dorsolateral striatum, globus pallidus, and substantia nigra pars reticulata. The severity of AIMs also negatively correlated with 5-HT_3 binding levels in the ipsilateral dorsolateral striatum and contralateral subthalamic nucleus. These results collectively suggest that a regionally selective upregulation of 5-HT_3 binding may contribute to the pathophysiology of L-DOPA-induced dyskinesia and potentially provide insight to the anatomical substrate(s) for the anti-dyskinetic efficacy of 5-HT_3 antagonists in PD.
6. Developed a frameless imaging-guided stereotaxic system by registering computed tomography, magnetic resonance imaging, and positron emission tomography (PET) data to identify the putamen, in two marmosets. After localisation, alpha-synuclein pre-formed fibrils were injected into the putamen using a robotic arm and four months post-injection, a PET scan found evidence of nigrostriatal denervation. This approach improves upon traditional methods to localise surgical targets in non-human primates and can also be used for studies that assess longitudinal endpoints.

The current thesis provides support for the translational potential of 5-HT_3 blockade in treatment-related complications in PD, namely L-DOPA-induced dyskinesia and PD psychosis. In addition to work conducted in neurotoxin-based animal models, the methods to develop an alpha-synuclein propagation-based model in the marmoset were also described, which will facilitate the discovery and advancement of disease-modifying therapies in PD.

4. Contributions of Authors

The following summarises my (**C.K.**) contribution along with those of co-authors.

Chapter 2 originally appeared as:

Kwan C, Bédard D, Frouni I, Gaudette F, Francis B, Hamadjida A, Huot P. Pharmacokinetic profile of the selective 5-HT_3 receptor antagonist ondansetron in the rat: an original study and a minireview of the behavioural pharmacological literature in the rat. *Can J Physiol Pharmacol*. 2020 Jul;98(7):431-440. doi: 10.1139/cjpp-2019-0551.

Contributions: **C.K.** first-authored this manuscript; **C.K.** and P.H. conceived and designed experiments while A.H. managed the organisation of experiments; **C.K.,** D.B., and F.G. performed experiments; **C.K.**, F.G. and F.B. analysed the data; **C.K.** and P.H. wrote the manuscript and managed the submission and editing process. D.B., I.F., F.G., F.B., and A.H. edited the manuscript.

Chapter 3 originally appeared as:

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Contributions: **C.K.** first-authored this manuscript; **C.K.** and P.H. conceived and designed experiments while A.H. managed the organisation of experiments; **C.K.,** D.B., and I.F. performed experiments; **C.K.** analysed the data; **C.K.** and P.H. wrote the manuscript and managed the submission and editing process. I.F., D.B., and A.H. edited the manuscript.

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Chapter 2 - Pharmacokinetic profile of the selective 5-HT_3 receptor antagonist ondansetron in the rat: an original study and a minireview of the behavioural pharmacological literature in the rat

Chapter 2. Pharmacokinetic profile of the selective 5-HT$_3$ receptor antagonist ondansetron in the rat: an original study and a minireview of the behavioural pharmacological literature in the rat

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Short title: Pharmacokinetic profile of ondansetron

Chapter 2 Abstract

The availability of agonists and antagonists to modulate the activity of the 5-hydroxytryptamine (5-HT) type 3 (5-HT_3) receptor has renewed interest in its role as a therapeutic target. Ondansetron is a highly selective 5-HT_3 receptor antagonist that is well tolerated as an anti-emetic for patients undergoing chemotherapy. Preclinical studies in rat have shown the effects of small doses of ondansetron on cognition, behavioural sensitisation, and epilepsy. However, the pharmacokinetic (PK) profile of ondansetron in rat has not been described, which limits the translational relevance of these findings. Here, we aim to determine, in the rat, the PK profile of ondansetron in the plasma and to determine associated brain levels. The plasma PK profile was determined following acute subcutaneous administration of ondansetron (0.1, 1, and 10 µg/kg). Brain levels were measured following subcutaneous administration of ondansetron at 1 µg/kg. Plasma and brain levels of ondansetron were determined using high-performance liquid chromatography-tandem mass spectrometry. Following administration of all three doses, measured ondansetron plasma levels (≈30–3000 pg/mL) were below levels achieved with doses usually administered in the clinic, with a rapid absorption phase and a short half-life (≈30–40 min). We also found that brain levels of ondansetron at 1 µg/kg were significantly lower than plasma levels, with brain to plasma ratios of 0.45 and 0.46 in the motor and pre-frontal cortices. We discuss our findings in the context of a minireview of the literature. We hope that our study will be helpful to the design of preclinical studies with therapeutic end-points.

Key words: rat, ondansetron, pharmacokinetics, 5-HT_3 receptor antagonist, HPLC-MS/MS.

La disponibilité d'agonistes et d'antagonistes permettant de moduler l'activité des récepteurs de la 5-hydroxytryptamine (5-HT) de type 3 (5-HT_3) a renouvelé l'intérêt dans son rôle comme cible thérapeutique. L'ondansétron est un antagoniste très sélectif des récepteurs 5-HT_3 avec une bonne tolérabilité en tant qu'antiémétique chez les patients sous chimiothérapie. Des études précliniques chez le rat ont montré les effets de l'ondansétron à faibles doses sur l'état cognitif, la sensibilisation comportementale et l'épilepsie. Cependant, le profil pharmacocinétique (PK) de l'ondansétron n'a pas été décrit chez le rat, ce qui limite la pertinence translationnelle de ces résultats. Ici, nous cherchons à établir le profil PK de l'ondansétron dans le plasma chez le rat et à établir les niveaux cérébraux associés. Le profil PK plasmatique a été établi après l'administration d'ondansétron (à 0,1, 1 et 10 µg/kg) par voie sous-cutanée. Nous avons mesuré les niveaux cérébraux à la suite de l'administration sous-cutanée d'ondansétron à 1 µg/kg. Nous avons établi les niveaux plasmatiques et cérébraux d'ondansétron à l'aide de la chromatographie liquide à haute performance couplé à la spectrométrie de masse en tandem. Après l'administration de chacune des trois doses, nous avons mesuré des niveaux plasmatiques d'ondansétron inférieurs à ceux obtenus avec des doses habituellement utilisées en clinique (≈30–3000 pg/mL), avec une phase d'absorption rapide et une courte demi-vie (≈30–40 min). Nous avons aussi observé que les niveaux cérébraux d'ondansétron administré à 1 µg/kg étaient nettement moins élevés que dans le plasma, avec des ratios cerveau à plasma de 0,45 et de 0,46 dans les cortex moteur et préfrontal, respectivement. Nous discutons de nos observations dans le contexte d'une courte synthèse de la littérature. Nous espérons que notre étude sera utile pour la conception d'études précliniques avec des paramètres d'évaluation thérapeutiques. [Traduit par la Rédaction]

Mots-clés: rat, ondansétron, pharmacocinétique, antagoniste des récepteurs 5-HT_3, chromatographie liquide à haute performance en tandem avec la spectrométrie de masse.

Chapter 2 Introduction

Initially described as the "M receptor" in the guinea pig ileum, the 5-hydroxytryptamine (5-HT) type 3 (5-HT_3) receptor is uniquely positioned in the 5-HT receptor family as the only ionotropic receptor (Barnes and Sharp, 1999). Along with the nicotinic acetylcholine and glycine receptors, the 5-HT_3 receptor is a member of the Cys-loop superfamily of ligand-gated ion channels that mediates fast synaptic neurotransmission and is thought to be involved in diverse functions (Sugita et al., 1992, Thompson, 2013). Autoradiographic binding studies using selective radioligands for the 5-HT_3 receptor have led to the identification and characterisation of 5-HT_3 receptor binding sites to discrete areas of the rat central nervous system, including the area postrema, lower brainstem nuclei and the substantia gelatinosa (Kilpatrick et al., 1987, Barnes et al., 1990a, Hewlett et al., 1998).

Ondansetron, a potent and selective antagonist of the 5-HT_3 receptor, is used in the clinic to treat nausea and vomiting induced by chemotherapy, radiotherapy, and surgery (Marty et al., 1990, Butcher, 1993, Macor et al., 2001, Mujtaba et al., 2013) and is well tolerated with minimal side effects. The pharmacokinetic (PK) profile of ondansetron has been previously characterised in young and elderly healthy volunteers (Colthup et al., 1991, Pritchard et al., 1992). In addition to its anti-emetic effect, pre-clinical studies in the rat have also assessed the efficacy of ondansetron in paradigms such as cognition and behavioural sensitisation (Costall and Naylor, 1992, Hodges et al., 1996, Davidson et al., 2002). However, because the PK profile of ondansetron in the rat has not been disclosed, the clinical relevance of these findings is unclear.

In the present study, we have determined the PK profile of ondansetron in the plasma and associated brain levels following administration of small doses in the healthy adult rat. We then

discuss our findings in light of the pharmacological literature reporting behavioural effects of ondansetron in rats.

Chapter 2 Materials and methods

Chemicals

Ondansetron hydrochloride was obtained from MilliporeSigma (Oakville, Canada) and $^{2}H_3$-ondansetron was purchased from Toronto Research Chemical (Toronto, Canada). Drug-free rat plasma containing K_3-EDTA as anticoagulant was purchased from BioIVT (Westbury, USA). Formic acid was purchased from MilliporeSigma. Other chemicals, including, ammonium formate, methanol, acetonitrile and water were purchased from Fisher Scientific (Fair Lawn, USA).

Animals

Female Sprague-Dawley rats (225–250 g) (Charles River, Saint-Constant, Canada) were group-housed under conditions of controlled temperature (21 ± 1ºC), humidity (55%) and light (12h light/dark cycle, 07:00 lights on) environment with unlimited to food and water. Upon arrival, rats were left undisturbed to acclimatise for one week before experiments. All procedures were approved by the Montreal Neurological Institute Animal Care Committee in accordance with the regulations defined by the Canadian Council on Animal Care.

Plasma pharmacokinetic study

Twelve rats (*N* = 4 per dose) were used for these studies. Following sub-cutaneous (s.c.) administration of ondansetron hydrochloride (0.1, 1 and 10 µg/kg free base), blood samples (150 µL) were collected from animals by jugular vein puncture at each of the following time points: baseline, 2 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, and 6 h, as previously described (Gaudette et al., 2017, Gaudette et al., 2018) and subsequently transferred to K_3-EDTA-coated tubes (Sarstedt, St-Leonard, Canada). An additional sample was collected 3 h following s.c. injection of ondansetron 0.1 µg/kg. To calculate derived PK plasma parameters, following intravenous (i.v.) administration of ondansetron 1 µg/kg, blood was collected at these time points: baseline, 2 min, 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, 4h, and 6 h. Samples were gently inverted and centrifuged at 1500*g* for 10 min at 4°C and plasma aliquots were stored at -80°C until analysis. Ondansetron plasma levels were determined by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). A separate paper detailing the methodology has been published (Gaudette et al., 2019).

Brain concentrations

Fifteen rats (*N* = 5 per time point) were used. Following administration of ondansetron 1 µg/kg s.c., blood and brain were collected 10 min, 30 min, or 1 h after drug administration. Blood samples were collected and processed as described above for plasma extraction. For brain collection, animals were quickly euthanised (10 min, 30 min, or 1 h after drug administration) by isoflurane anaesthesia (2%-4%; MilliporeSigma) and perfused trans-cardially with 0.9% NaCl. Then, brains were rapidly removed from the skull and pre-frontal cortex, motor cortex, striatum and cerebellum were dissected, flash-frozen in 2-methyl-butane at -56°C and stored at -80°C into

separate 1.5 mL sterile microcentrifuge tubes. The blood and brain of a control, ondansetron-naïve, animal was also collected.

Sample preparation

Ondansetron was extracted from rat plasma using protein precipitation as the sample preparation technique. Two hundred and fifty microlitres of internal standard solution (5 pg/mL $^{2}H_{3}$-ondansetron in methanol) were added to an aliquot of 25 µL of rat plasma. The sample was vortexed for approximately 5 s and left to stand for 10 min, then centrifuged at 16,000 × g for 10 min. The supernatant was transferred into a clean 13 × 100 mm borosilicate tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The dried extract was re-suspended with 50 µL of methanol and transferred to an injection vial for analysis.

For brain, tissue was rinsed with phosphate-buffered saline, and immediately frozen and stored at -80°C until further sample preparation. The frozen brain samples were accurately weighed into Precellys Tissue grinding CKMix tubes equipped with ceramic beads (Cayman Chemical, Ann Arbor, USA). Homogenates were prepared by adding PBS to the tissue sample at a ratio of 4:1 *v/w* (buffer/solid tissue). A Precellys-24 homogeniser (Bertin Technologies, Montigny-le-Bretonneux, France) was used to grind up samples. The temperature of samples was maintained between 0 and 10°C during homogenisation using a homogeniser equipped with the Cryolys cooling option. The program used to homogenise the samples consisted of two cycles of 25 s at a frequency of 6000 rpm with a 15 s pause between cycles. Ondansetron was extracted from brain tissue homogenates by adding 500 µL of internal standard solution (10 pg/mL $^{2}H_{3}$-ondansetron in methanol) to an aliquot of 100 µL of rat brain homogenate. The sample was vortexed for approximately 5 s and left to stand for 10 min and then centrifuged at 16 000*g* for 10

min. The supernatant was transferred into a clean 13 × 100 mm borosilicate tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The dried extract was re-suspended with 50 µL of methanol and transferred to an injection vial for analysis.

HPLC-MS/MS conditions

A Thermo Scientific TSQ Quantiva Triple Quadrupole mass spectrometer (San Jose, USA) was interfaced with a Thermo Scientific UltiMate 3000 XRS UHPLC system (San Jose, USA) using a pneumatic assisted heated electrospray ion source. MS detection was performed in positive ion mode, using multiple reaction monitoring (MRM). The MRM transitions were set to 294 → [170 + 184] and 297 → [173 + 187] for ondansetron and $^{2}H_{3}$-ondansetron, respectively. Isocratic elution was used with a Thermo Scientific Aquasil C18 analytical column (100 × 2.1 mm I.D., 5 µm) operating at 40°C. The mobile phase consisted of acetonitrile and 10 mM ammonium formate pH 3 at ratio 30:70, respectively. The flow rate was fixed at 300 µL/min and ondansetron and the internal standard eluted at 2 min. Five microlitres of the extracted sample was injected and the total run time was set to 4 min. The method met all requirements of selectivity, sensitivity, linearity, precision and accuracy, and stability generally accepted in bioanalytical chemistry (U.S. Department of Health and Human Services et al., 2018)

Statistical Analysis

Ondansetron plasma and brain levels are presented as the mean ± standard deviation (SD). Plasma PK parameters and brain concentrations were determined from the mean concentration value at each time point by non-compartmental analysis using PKSolver (Rowland M and TN., 1995, Zhang et al., 2010). The area under the curve (AUC) was calculated using the linear and log-

linear trapezoidal rule. AUC_{0-t}, $AUC_{0-\infty}$, maximal plasma concentration (C_{max}), time to C_{max} (T_{max}), terminal half-life ($T_{1/2}$), clearance (CL), bioavailability (F), volume of distribution (V_z) and mean residence time (MRT) were all calculated. For i.v. injection, the extrapolated concentration at T = 0 (C_0) and steady-state volume of distribution (V_{ss}) were also determined. The brain to plasma ratio of ondansetron was calculated by dividing the mean AUC of each brain region by the mean AUC_{plasma} after administration. Statistical analyses were performed using GraphPad Prism 8.0d (GraphPad Software Inc., San Diego, California, USA).

Chapter 2 Results

Pharmacokinetic profile

Plasma PK parameters obtained following i.v. administration of ondansetron 1 µg/kg in the rat are summarised in Table 1. As shown in Figure 1, ondansetron 1 µg/kg i.v. was rapidly detected in the plasma, with a C_0 of 898.3 pg/mL. The mean AUC_{0-t} values represented 93% of mean $AUC_{0-\infty}$; thus, the extrapolated AUC was relatively small compared to $AUC_{0-\infty}$. The $T_{1/2}$ and mean calculated MRT values were 42 min and 0.81 h, respectively. CL was 2.46 $L \cdot h^{-1} \cdot kg^{-1}$, while V_z and V_{ss} values of 2.58 L/kg and 2.08 L/kg were obtained, respectively, which suggests an interesting drug tissue distribution since the larger the V_z, the more likely that the drug will be found in the peripheral tissue of the animal (Urso et al., 2002).

Plasma PK parameters of ondansetron in the rat following s.c. injection are summarised in Table 2. As displayed in Figure 2, ondansetron (0.1, 1, and 10 µg/kg) was detected in the plasma as early as 5 min after s.c. administration. The T_{max} occurred 15 and 10 min after administration

of ondansetron (0.1 and 1 µg/kg) and 7 min after administration of ondansetron at 10 µg/kg, while $T_{1/2}$ was 39 and 32 min after administration of ondansetron (0.1 and 1 µg/kg) and 43 min after administration of ondansetron at 10 µg/kg. There was a dose-dependent concentration profile leading to a C_{max} of 30.6, 525.0, and 2987 pg/mL following administration of ondansetron at 0.1, 1, and 10 µg/kg, respectively. The mean $AUC_{0-\infty}$ values calculated in plasma were comparable to the mean AUC_{0-t} values, suggesting that the extrapolated AUC was relatively small. The MRT calculated was 0.87 h, 0.79 h, and 0.85 h, respectively, following injection of ondansetron (0.1, 1, and 10 µg/kg). The calculation of CL/F and V_z/F was estimated. The F observed was 94% when comparing s.c. and i.v. administration of ondansetron at 1 µg/kg, suggesting an almost complete drug absorption.

Brain to plasma ratio

Plasma and brain concentrations obtained following s.c. administration of ondansetron at 1 µg/kg to assess brain penetrance are presented in Table 3 and Fig. 3. Ten min after s.c. administration of ondansetron, plasma levels reached 260.0 ± 44.8 pg/mL, while brain tissue levels were 173.8 ± 160.6 pg/g in the pre-frontal cortex, 161.0 ± 68.7 pg/g in the motor cortex, 51.3 pg/g ± 15.07 in the cerebellum, and 56.6 ±12.4 pg/g in the striatum.

Plasma and brain tissue levels started to decline 30 min after administration of ondansetron, where the plasma concentration was 193.4 ± 29.4 pg/mL, while the concentration was 56.7 ± 21.1 pg/g in the pre-frontal cortex, 58.2 ± 19.2 pg/g in the motor cortex, 45.9 ± 30.9 pg/g in the cerebellum, and 59.4 ± 26.7 pg/g in the striatum. Finally, 1 h post-ondansetron administration, plasma levels had further declined to 98.0 ± 10.8 pg/mL. Brain tissue levels were 42.2 ± 13.7 pg/g

in the pre-frontal cortex, 45.7 ± 14.5 pg/g in the motor cortex, 16.6 ± 5.1 pg/g in the cerebellum and 26.0 ± 10.4 pg/g in the striatum.

Chapter 2 Discussion

In this study, we have assessed the plasma PK profile of ondansetron and determined its brain penetrance in the healthy female rat. We must emphasise that, because the experiments were conducted solely in female rats, our results, or part thereof, may not be applicable to male rats.

To the best of our knowledge, this is the first study reporting the plasma PK profile of ondansetron following s.c. or i.v. administration in the rat, as well as brain levels following s.c. administration, although the PK profiles following i.v. and oral administration were previously reported in human (Blackwell and Harding, 1989, Colthup et al., 1991, Roila and Del Favero, 1995, Simpson and Hicks, 1996).

A single s.c. administration of ondansetron (0.1, 1 and 10 µg/kg) showed rapid absorption with T_{max} values between 7 and 15 min and rapid elimination with $T_{1/2}$ that ranged from 28 to 43 min. Moreover, we observed that C_{max} values were dose-dependent, ranging from 30.6 to 2,987.2 pg/mL. These findings are coherent with clinical studies that administered a single 8 mg oral dose to healthy volunteers, in which C_{max} of 26.4-42.0 ng/mL was obtained within 1.25-2.1 h (Colthup et al., 1991, Baber et al., 1992). After a single i.v. administration of 8 mg ondansetron in subjects, $T_{1/2}$ ranged between 3.2 and 5.0 h, while plasma CL was approximately 0.26-0.44 $L \cdot h^{-1} \cdot kg^{-1}$ and Vz was 1.71-1.94 L/kg (Colthup et al., 1991, Baber et al., 1992, Roila and Del Favero, 1995). Thus, the PK profile of i.v. administration of ondansetron at 1 µg/kg described in our study is in

line with clinical studies that administered higher doses of ondansetron. In fact, we found that $T_{1/2}$ is shorter in rat than in humans, while CL and V_z values seem larger in rat than in humans, which suggests that this dose of ondansetron is efficiently eliminated and highly distributed in peripheral tissue in the healthy female rat compared to humans (Urso et al., 2002).

Moreover, our results indicate that ondansetron penetrates into the brain (Hitchcock and Pennington, 2006, Shaffer, 2010). The mean concentration-time profiles exhibited similar patterns of distribution, with a rapid peak within 15 min and brain levels below the limit of detection threshold after 1 h. In addition, the distribution of ondansetron in brain tissue may be consistent with its reported effects on cognition, behavioural sensitisation and sleep disorders discussed below (Barnes et al., 1990b, King et al., 1998, Radulovacki et al., 1998). However, the mechanism(s) underlying the different concentrations in different brain areas is unclear. A possible explanation may be the higher density of 5-HT_3 receptors in cortical areas compared to sub-cortical structures, as found in autoradiographic binding studies (Kilpatrick et al., 1987, Barnes et al., 1990a).

Minireview of the central effects of ondansetron in the rat

We will now discuss our findings in the broad context of previous pre-clinical and clinical studies conducted with ondansetron. The principal findings of pre-clinical studies assessing the central effects (excluding drug- and radiation-induced nausea and vomiting) of ondansetron in the rat are presented in Table 4. This minireview was conducted to compare plasma and brain concentrations of ondansetron obtained in the present study with previous studies that administered ondansetron with a similar dose range. Indeed, as we will see, a U-shaped dose-response curve has consistently been obtained in studies where a wide dose range of ondansetron was administered.

This U-shaped dose response curve does not appear to be specific to ondansetron and has also been described with other 5-HT_3 antagonists (Jones et al., 1988, Faerber et al., 2007).

To conduct this brief literature review of ondansetron in the rat, we limited the search for its behavioural effects on the brain following parenteral administration (intraperitoneal (i.p.) or s.c.)). It is noteworthy that few rat studies with ondansetron have been published recently and that, for this reason, several of the articles cited here were published in the 1980s or 1990s.

Cognition

As mentioned above, the effects of ondansetron are often depicted by a U-shaped dose-response curve, notably on learning and memory, where low (0.001 µg/kg – 1 mg/kg) (Barnes et al., 1990b, Fontana et al., 1995, Hodges et al., 1996), but not high (> 1 mg/kg) (Costall and Naylor, 2000), doses enhance performance. Thus, in studies where low doses were administered, an improvement in cognitive performance was generally observed, specifically in spatial navigation tasks where, following ondansetron administration (0.1 µg/kg – 1 mg/kg), animals spent less time searching for the platform in the Morris water maze task (Fontana et al., 1995, Hodges et al., 1996, Diez-Ariza et al., 2003) and had improved performance in object recognition tests (Staubli and Xu, 1995, du Jardin et al., 2014). Moreover, ondansetron (0.001–1 mg/kg) prevented deficits in spatial discrimination tasks (Carli et al., 1997), decreased working memory deficits in the runway apparatus (Kumar and Kela, 2004) and reversed latencies in the passive avoidance task (Balakrishnan et al., 2000). However, some studies reported that ondansetron (0.001 µg/kg – 0.3 mg/kg) did not attenuate or non-significantly improved cognitive impairments in learning and memory paradigms (Bratt et al., 1994, Boast et al., 1999, Diez-Ariza et al., 2003, du Jardin et al., 2014), notably the impairments induced by scopolamine in the Stone maze task (Bratt et al., 1994), which suggests that the cognitive effect of ondansetron may be task specific (Costall and Naylor,

2000). In contrast with preclinical studies, ondansetron did not elicit procognitive effects in healthy subjects (Little et al., 1995) and patients with Alzheimer's disease (Dysken et al., 2002).

Sleep apnoea

Ondansetron (0.01–1 mg/kg) also attenuated apnoea in rats during nonrapid eye movement (REM) and REM sleep, more so in the latter sleep stage (Radulovacki et al., 1998), as well as in anaesthetised rats (Carley and Radulovacki, 1999). To the best of our knowledge, the effects of ondansetron on sleep apnoea in human have not been reported.

Behavioural sensitisation

The action of ondansetron on behavioural sensitisation is unclear, with some groups finding that doses of 0.0 –1 mg/kg resulted in reductions of cocaine self-administration (King et al., 1997, King et al., 1998, King et al., 2000, Davidson et al., 2002), while others did not find any effect on such behaviour with doses of 0.001–3.3 mg/kg (Peltier and Schenk, 1991, Lane et al., 1992, Depoortere et al., 1993). The discrepancies may perhaps be attributed to the period of ondansetron administration and possibly relate to the rapid absorption and short $T_{1/2}$ of ondansetron, as sensitisation was inhibited when ondansetron was administered during acute or chronic cocaine withdrawal (Costall et al., 1990, King et al., 1998, King et al., 2000, Davidson et al., 2002), but not if ondansetron was administered 30 min prior to cocaine self-administration (Peltier and Schenk, 1991, Lane et al., 1992, Depoortere et al., 1993).

Anxiety

The effect of ondansetron on anxiety has varied across studies and differences in treatment regimen, methodology and evaluation of behavioural criteria, which may all have accounted for this variability (Olivier et al., 2000). For this reason, the anxiolytic potential of ondansetron is still undetermined, as no effects were obtained in the ultrasonic vocalisation model (0.001–0.1 mg/kg)

(Molewijk et al., 1995, Sanchez, 1996, Olivier et al., 1998, Schreiber et al., 1998), while increased activity was generally reported in the social interaction test with doses ranging from 0.01 μg/kg to 1 mg/kg (Costall et al., 1989a) and 0.3–3 μg/kg decreased latency to eat in the modified open-field test (Rex et al., 1998). Contradictory results were obtained in the light/dark exploration test with ondansetron 0.0001–1 mg/kg (Costall et al., 1989a, Morinan, 1989, Young and Johnson, 1991). Conflicting evidence surrounds the efficacy of ondansetron on benzodiazepine dependence and withdrawal, with reports ranging from no effect (0.01–1 mg/kg) (Goudie and Leathley, 1992, Prather et al., 1993) to symptom attenuation (0.01–0.1 mg/kg) (Goudie and Leathley, 1990, Valdman et al., 1996). It is possible that these various effects depend on the stage of withdrawal (Nowakowska et al., 1998), and further studies are required to clarify this possibility. In agreement with the conflicting reports in pre-clinical models of anxiety, mixed results have also been found in clinical studies. Earlier clinical trials demonstrated that the anxiolytic profile of ondansetron is comparable to diazepam (Greenshaw and Silverstone, 1997); however previous studies had reported that ondansetron did not reduce anxiety in patients with generalised anxiety (Mathew and Wilson, 1991, Wilde and Markham, 1996).

Pain

Inconsistent findings have been reported regarding the effect of ondansetron on pain, ranging from interference with antinociceptive response to drugs (0.5–2 mg/kg) (Bhargava and Saha, 2001, Baek et al., 2005, Scott et al., 2006), no effect (0.5–1 mg/kg) (Lopes et al., 2009, Erthal et al., 2013, Turtay et al., 2015) to (0.4 – 3 mg/kg) alleviating pain (Ye et al., 1997, Shen et al., 2013, Akiba et al., 2017). The studies reviewed here have been limited to systemic administration of ondansetron, while the majority of the literature employed intra-thecal administration of ondansetron to target 5-HT_3 receptors in the brain, without necessarily providing

corresponding plasma levels, which precludes comparison with the results we gathered here. Nonetheless, inconsistencies in antinociceptive effects have also been reported across these studies (Oatway et al., 2004, Peters et al., 2010). A possible reason for the variable results may be the numerous animal models of pain available as the source and mechanism underlying pain conditions differs (Mogil, 2009), such as between inflammatory pain and neuropathic pain, rendering it difficult to make comparisons between studies. Mixed results have also been reported in the clinic. A randomised placebo-controlled trial found that irritable bowel syndrome patients treated with ondansetron had fewer painful episodes (Goldberg et al., 1996). Likewise, i.v. injection of ondansetron had an analgesic effect on neuropathic pain (McCleane et al., 2003). However, a more recent trial found that ondansetron did not have a significant effect on pain but produced adequate relief for irritable bowel syndrome, suggesting a therapeutic effect independent of an antinociceptive process (Garsed et al., 2014). The variability in findings highlights the challenges posed by grouping diverse conditions of pain under one term (Raffaeli and Arnaudo, 2017) and emphasises the need for further studies to assess the efficacy, or lack thereof, of ondansetron in treating different subtypes of pain.

Epilepsy

A possible anticonvulsant activity has also been reported, with ondansetron (0.5–2 mg/kg) potentiating protection against maximal electroshock-induced seizures in a rat model of epilepsy (Balakrishnan et al., 2000). Contrary to this finding, single case reports indicated an association between ondansetron and seizures in humans (Sargent et al., 1993, Sharma and Raina, 2001, Mason et al., 2007) and a later study suggested that ondansetron administration could be rarely associated with seizures in humans (Singh et al., 2009). Due to the small sample size and

differences in medical history of patients, it is unclear if confounding factors contributed to the findings of these reports.

Considering the reported influence of relatively low doses of ondansetron on cognitive performance, behavioural sensitisation, and anxiety in the rat, we assessed the PK profile of such doses. The C_{max} observed in animals following s.c. administration of ondansetron (0.1, 1 and 10 µg/kg) ranged from 30.6 to 2,987.2 pg/mL, which was at least one order of magnitude below those observed in the clinic after oral administration (26.4 – 42.0 ng/mL) (Colthup et al., 1991, Baber et al., 1992). From this, we may infer that the PK plasma profile described may not reach target concentrations needed to obtain a pharmacological effect in the rat, which may explain some of the controversy surrounding the effects of ondansetron on behavioural sensitisation, anxiety, and pain. However, this possibility is mitigated by the fact that the brain to plasma ratio of ondansetron in human is unknown and may be higher than the one we found here, especially in areas where it was lower, such as the cerebellum and the striatum. Studies are thus warranted to assess the PK of s.c. administration of ondansetron in non-human primates to provide a better proxy of the brain to plasma ratio in human.

The mechanism underlying the U-shaped dose-response curve is not known and the questions remains as to whether it is due to a shared characteristic of 5-HT_3 antagonists or the pharmacology of individual compounds (Farber et al., 2004). The most favoured mechanism proposes that, at high concentrations of 5-HT_3 antagonists, there is mutual steric hindrance at the receptor (Faerber et al., 2007), which could inhibit binding of ondansetron to the receptor. Furthermore, ondansetron may exert additional effects due to low-affinity binding to other receptors to 5-HT_{1A}, 5-HT_{1B}, α-adrenergic and opioid receptors, although this appears negligible compared its binding to high-affinity 5-HT_3 receptor sites that is about 250- and 500-fold higher

that of the other receptors (Van Wijngaarden et al., 1990b). Alternatively, the density of 5-HT_3 receptors varies between different brain regions, so one density type may be completely inhibited at low concentrations while another type requires high concentrations of 5-HT_3 receptor antagonists (Faerber et al., 2007). This could explain contrary effects, for example, where blockade of 5-HT_3 receptors within the cortex that confers therapeutic benefit is offset when 5-HT_3 receptors from another brain region are completely antagonised, which could explain the lack of efficacy of higher doses of ondansetron.

We found that s.c. administration of small doses of ondansetron did not have a linear dose-dependent effect on $T_{1/2}$ and plasma levels, which suggests that elimination of ondansetron cannot solely explain the dose-response curve. Rather, we speculate that the U-shaped dose-response of ondansetron in this minireview, where maximum response is observed in the microgram dose range while higher doses are ineffective (Faerber et al., 2007), may be explained, in part, by the distribution of 5-HT_3 receptors in the brain. In addition to high levels of 5-HT_3 receptor binding in the area postrema and solitary tract nucleus (Kilpatrick et al., 1987, Waeber et al., 1988), the 5-HT_3 receptor has been localised to cortical and limbic regions by autoradiographic binding studies (Waeber et al., 1990, Gehlert et al., 1991), which suggests that the cognitive and anxiolytic effects of ondansetron may be mediated at these sites (Costall et al., 1989b, Hodges et al., 1996). Thus, relatively low doses of ondansetron may preferentially block 5-HT_3 receptors in these regions to suppress the excitation of gamma-aminobutyric acid (GABA)-ergic neurons (Morales and Bloom, 1997) and disinhibit pyramidal cells (Staubli and Xu, 1995) to provide therapeutic benefit. However, intermediate doses of ondansetron may saturate binding to 5-HT_3 receptors in cortical and limbic regions and lead to a compensatory response of GABAergic neurons that translates to an absence of response at the behavioural level. At high doses, ondansetron may be reach sufficient

levels in the brain to antagonise 5-HT_3 receptors present in regions of lower densities such as the nucleus accumbens and striatum (Kilpatrick et al., 1987), and may underlie its efficacy in inhibiting sensitisation to drugs of abuse (King et al., 2000). Moreover, the variable efficacy of ondansetron obtained with higher doses may be attributed to its low affinity for 5-HT_{1A}, 5-HT_{1B}, α-adrenergic and opioid receptors, which suggests that its action is not limited to blockade of the 5-HT_3 receptor. As these mechanisms have not been demonstrated experimentally and remain the subject of speculation, further studies are required to elucidate the mechanisms governing the U-shaped dose-response curve to guide attempts to determine optimal dosage of ondansetron.

Collectively, these results in preclinical models are encouraging the administration of ondansetron as a therapeutic compound in a variety of central nervous disorders. However, despite these promising preclinical results, the efficacy of ondansetron needs to be demonstrated in randomised-controlled clinical trials prior to its off-label for new indications.

Chapter 2 Conclusion

In summary, we have determined the plasma PK and associated brain concentrations of ondansetron in the rat. Ondansetron levels reported in the motor cortex, pre-frontal cortex, cerebellum and striatum provide support for its action on 5-HT_3 receptors in these brain regions. Further studies are required to confirm that these levels of ondansetron are sufficient to bind to and antagonise 5-HT_3 receptors and mediate its central effects. Moreover, the mechanism underlying its therapeutic efficacy remains to be elucidated, as its U-shaped dose-response curve still lacks explanation.

Conflicts of interest statement

None. PH has received payments from Valeo Pharma.

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Author contributions

CK: Conceptualization; Investigation; Writing – original draft; Visualization. DB: Methodology; Investigation; Writing – review & editing. IF: Investigation; Writing – review & editing. FG: Investigation; Validation; Formal Analysis; Writing – review & editing. FB: Formal Analysis; Writing – review & editing. HA: Project Administration; Supervision; Writing – review & editing. PH: Conceptualization; Funding Acquisition; Supervision; Writing – original draft

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Chapter 2 Tables and Figures

Table 1. Derived PK plasma parameters following i.v. administration of ondansetron at 1 µg/kg in the rat.

Parameter	1 µg/kg (mean ± SD)
$AUC_{0\text{-}t}$ ($pg \cdot mL^{-1} \cdot h^{-1}$)	384.1±52.4
$AUC_{0\text{-}\infty}$ ($pg \cdot mL^{-1} \cdot h^{-1}$)	411.1±49.3
C_0 (pg/mL)	898.3±163.5
$T_{1/2}$ (min)	42±16
CL ($L \cdot h^{-1} \cdot kg^{-1}$)	2.46±0.30
V_z (L/kg)	2.58±1.29
MRT (h)	0.81±0.45
V_{ss} (L/kg)	2.08±1.42
F (%)	100

Note: AUC, area under the curve; C_0, extrapolated concentration at T = 0; CL, clearance; F, bioavailability; MRT, mean residence time; $T_{1/2}$, terminal half-life; V_{ss}, steady-state volume of distribution; V_z, volume of distribution. *N* = 4 per dose.

Table 2. Derived PK parameters in the plasma following s.c. administration of ondansetron in the rat.

Parameter	0.1 µg/kg (mean ± SD)	1 µg/kg (mean ± SD)	10 µg/kg (mean ± SD)
AUC_{0-t} (pg/mL•h)	32.6±7.9	432.0±43.8	2,891.3±115.7
$AUC_{0-\infty}$ (pg/mL•h)	35.2±8.4	435.5±43.7	2,911.0±128.1
C_{max} (pg/mL)	30.6±5.5	525.0±293.0	2,987.2±764.7
T_{max} (min)	15±0	10±6	7±6
$T_{1/2}$ (min)	39±3	32±7	43±20
CL/F (L/h/kg)	2.95±0.6	2.31±0.24	3.4±0.15
V_z/F (L/kg)	2.7±0.4	1.79±0.48	3.5±1.5
MRT (h)	0.87±0.1	0.79±0.10	0.85±0.20

AUC, area under the curve; CL, clearance; C_{max}, maximal plasma concentration; F, bioavailability; MRT, mean residence time; $T_{1/2}$, terminal half-life; T_{max}, time to maximal plasma concentration; V_z, volume of distribution. N = 4 per dose.

Table 3. Plasma and brain PK parameters following s.c. administration of ondansetron at 1 µg/kg in the rat.

	1 µg/kg (mean ± SD)				
Parameters	Plasma	Motor cortex	Pre-frontal cortex	Cerebellum	Striatum
$AUC_{0\text{-}t}$ (pg· h^{-1}·mL^{-1})	170.0± 27.7	75.9±32.4	77.6±32.4	36.1± 32.4	45.4± 32.4
Ratio (vs. plasma)		0.45	0.46	0.21	0.27

Note: AUC, area under the curve.

Table 4. Literature review of the central effects of ondansetron in the rat.

Indication	Administered dose(s) (mg/kg)	Effective dose(s) (mg/kg)	Route of administration	Effect	Reference
Drug sensitisation	0.01, 0.1, 1	0.01, 0.1, 1	s.c.	Inhibited locomotor and behavioural sensitisation to cocaine withdrawal	King et al. 1997
	0.01, 0.1, 1	0.01, 0.1, 1	s.c.	Inhibited sensitisation and tolerance to cocaine withdrawal	King et al. 1998
	0.01, 0.1, 1	0.01, 0.1, 1	s.c.	Blocked sensitisation to cocaine	King et al. 2000
	0.2	0.2	s.c.	Inhibited sensitisation and self-administration of cocaine sensitisation	Davidson et al. 2002
	0.01, 0.1, 1		i.p.	No effect on cocaine self-administration	Peltier and Schenk 1991
	0.001–3.3		i.p.	No effect on cocaine self-administration	Lane et al. 1992
	0.001–1		i.p.	No effect on cocaine self-administration	Depoortere et al. 1993
Cognition	1×10^{-5} [a]	1×10^{-5} [a]	i.p.	↑ Correct responses in T-maze reinforced alternation task	Barnes et al. 1990b
	0.003–3	0.03–1	i.p.	↓ Latency to find platform in the Morris water maze in young rats	Fontana et al. 1995
	0.1	0.1		↓ latency to find platform in the Morris water maze in age-impaired rats	
	0.01-5	0.01, 0.5, 1	i.p.	↑ Frequency of hippocampal theta rhythm	Staubli et al. 1995
	0.1 and 0.5	0.1 and 0.5		↑ duration and intensity of LTP	
	0.1	0.1	i.p.	↑ Correct choices in olfactory delayed match-to-sample task	
				↑ Correct entries and ↓ re-entry errors in the radial maze (spatial task)	
	0.001, 0.01, 0.1	0.001, 0.01, 0.1	s.c.	↓ Latency to find platform in the Morris water maze	Hodges et al. 1996
				↓ Searching time for correct quadrant and circling periphery	
				↑ Retention of platform position during probe trial	
	0.0001, 0.001	0.001	s.c.	Prevented deficits in the spatial discrimination task	Carli et al. 1997
	0.25–4	0.25 and 0.5	i.p.	↑ Retention latencies in the passive avoidance task	Balakrishnan et al. 2000

Table 4 (*continued*)

Indication	Administered dose(s) (mg/kg)	Effective dose(s) (mg/kg)	Route of administration	Effect	Reference
Cognition	0.0001	0.0001	i.p.	Reversed learning and retention deficits in the Morris water maze when administered in combination with flumazenil or tacrine [b]	Diez-Ariza et al. 2003
	0.01 – 1	1	i.p.	↓ Working memory deficits in the runway apparatus	Kumar and Kela 2004
	1×10^{-6}, 0.003, 0.3	1×10^{-6}, 0.003, 0.3	s.c.	Reversed memory deficits in novel object recognition test	du Jardin et al. 2014
	1×10^{-6} [a] – 0.001 [a]		i.p.	Did not attenuate impairments in the Stone maze task	Bratt et al. 1994
	1×10^{-6}, 0.003, 0.3		s.c.	No effect on Y-maze spontaneous alternation (spatial working memory)	du Jardin et al. 2014
	0.3	0.3	i.p.	Attenuated impairment in radial maze performance in rats	Boast et al. 1999
	0.0001	0.0001	i.p.	Partially prevented learning deficit in the Morris water maze	Diez-Ariza et al. 2003
Pain	0.4, 0.8, 1	0.4, 0.8, 1	s.c.	↑ Tail flick latency	Ye et al. 1997
	3	3	i.p.	Potentiates anti-nociceptive response to duloxetine and morphine [c]	Shen et al. 2013
	3	3	i.p.	↓ Duodenal injury	Akiba et al. 2017
	0.5		i.p.	Did not interfere with anti-nociceptive effect of topiramate in formalin test [d]	Lopes et al. 2009
	1		i.p.	Did not interfere with laser acupuncture-induced anti-nociception	Erthal et al. 2013
	1		i.p.	Attenuated analgesic effect of apelin-13 [e]	Turtay et al. 2015
	0.5		i.p.	↓ Anti-nociceptive response to imipramine [f]	Bhargava and Saha 2001
	0.5		i.p.	Blocked analgesic effect of electroacupuncture	Baek et al. 2005
	2		i.p.	↑ Pain sensitivity in P-glycoprotein knockout mice [g]	Scott et al. 2006
Anxiety	0.01, 0.1, 1 [a]	0.01 and 0.1 [a]	i.p.	↓ Tolerance to diazepam or benzodiazepine withdrawal	Goudie and Leathley 1990
	0.05	0.05	i.p.	↓ Tolerance to diazepam or benzodiazepine withdrawal	Valdman et al. 1996
	0.0001–0.003	0.0003–0.003	i.p.	↓ Latency to eat in the modified open-field test	Rex et al. 1998

Table 4 (*concluded*)

Indication	Administered dose(s) (mg/kg)	Effective dose(s) (mg/kg)	Route of administration	Effect	Reference
Anxiety	1×10^{-5}–0.001	1×10^{-5}–0.001	i.p.	Increased social interaction	Costall et al. 1989
	0.001–0.1	0.001–0.1	i.p.	Increased light/dark exploration	Costall et al., 1989
	0.0001–1	0.0001–1	i.p.	Increased light/dark exploration	Young and Johnson 1991
	0.01, 0.1, 1		i.p.	No effect on benzodiazepine dependence or withdrawal	Goudie and Leathley 1992
	0.01, 0.1, 1		i.p.	No effect on benzodiazepine dependence or withdrawal	Prather et al. 1993
	0.08		i.p.	No effect on foot shock-induced ultrasonic vocalisation in adult male rats	Sanchez 1996
	0.001, 0.01, 0.1		i.p.	No effect on ultrasonic vocalisation in adult male rats	Molewijk et al. 1995
	0.1		i.p.	No effect on ultrasonic vocalisation in adult rats	Schreiber et al. 1998
	0.001, 0.01, 0.1		i.p.	No effect on ultrasonic vocalisation in rat pups	Olivier et al. 1998
	0.001–1		i.p.	No effect on light/dark exploration	Morinan 1989
Sleep	1	1	i.p.	Suppressed non-rapid eye movement (REM) and REM sleep apnoea in freely moving rats	Radulovacki et al. 1998
	0.1	0.1	i.p.	Attenuated REM sleep apnoea in freely moving rats	Carley and Radulovacki 1999
Epilepsy	0.25–4	0.5–2	i.p.	↑ Protection against maximal electroshock-induced seizures	Balakrishnan et al. 2000

[a]bis in die (b.i.d.).

[b]Flumazenil: benzodiazepine receptor antagonist (Hoffman and Warren, 1993); tacrine: anticholinesterase drug (Nair and Hunter, 2004).

[c]Duloxetine: noradrenaline and 5-HT transporter inhibitor (Wong et al., 1993); morphine: mu opioid receptor agonist (Bowen et al., 2002).

[d]Topiramate: anticonvulsant; sulfamate-substituted monosaccharide {Perucca, 1997 #4048}.

[e]Apelin-13: endogenous ligand of the orphan G protein-coupled receptor APJ (Tatemoto et al., 1998).

[f]Imipramine: tricyclic antidepressant (Klerman and Cole, 1965).

[g]P-glycoprotein: drug efflux pump (Sharom, 1997).

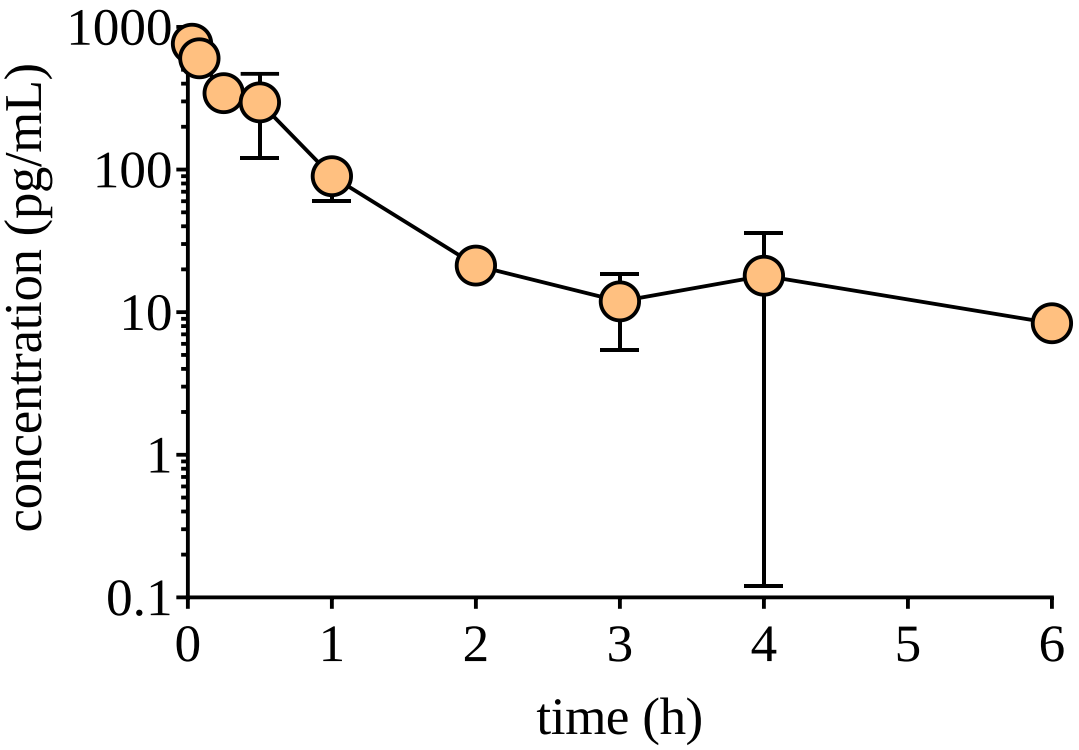

Fig. 1. Mean ± SD plasma concentration time profile of ondansetron at 1 µg/kg (*N* = 4) following i.v. administration in the rat. [Colour online.]

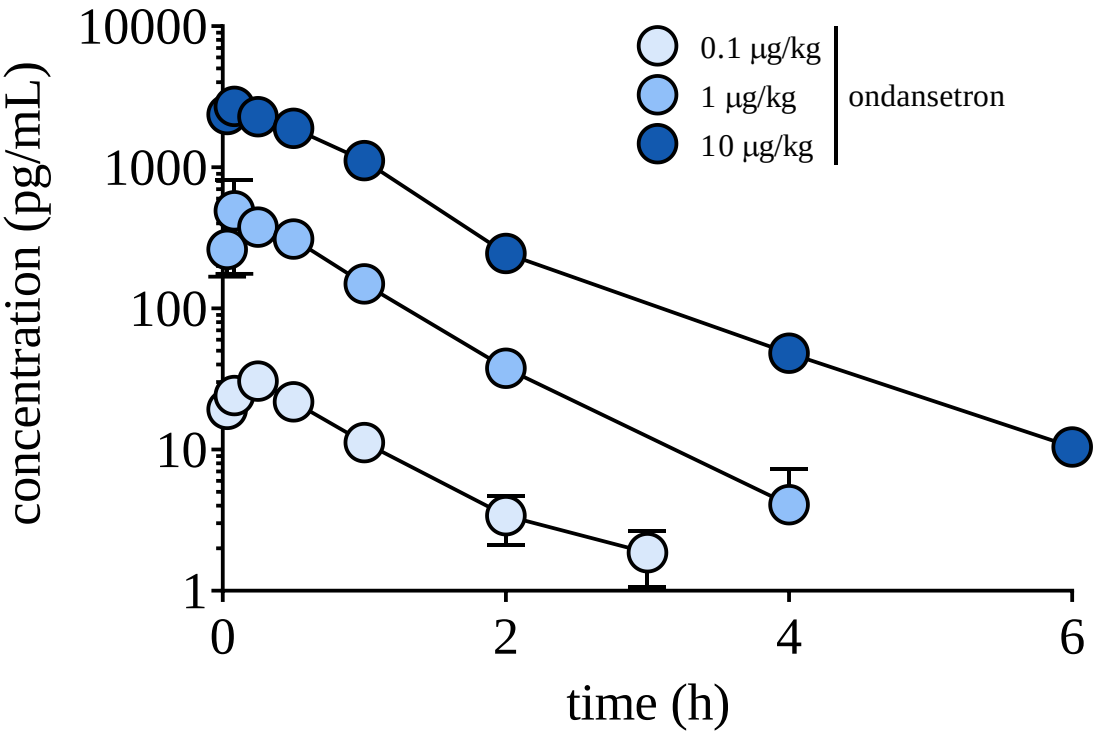

Fig. 2. Mean ± SD plasma concentration time profile of ondansetron (0.1, 1 and 10 µg/kg) following s.c. administration in the rat (*N* = 4 per dose). [Colour online.]

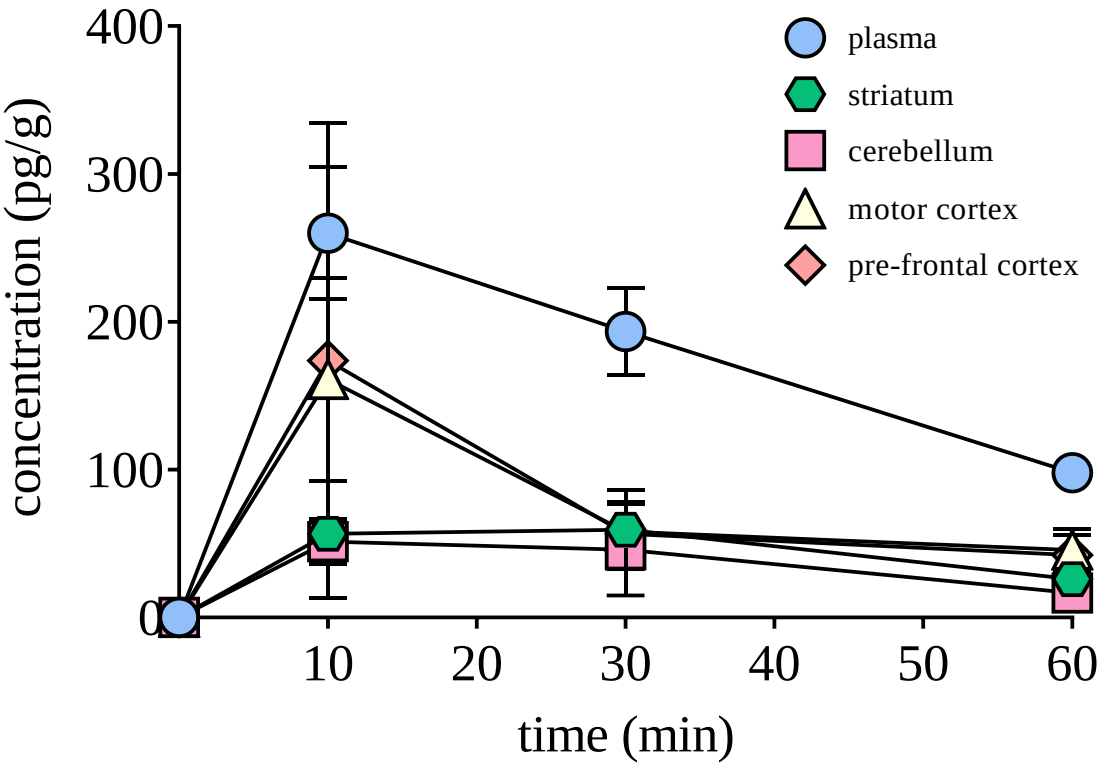

Fig. 3. Mean ± SD plasma and brain concentration time profile of ondansetron at 1 µg/kg (*N* = 4 per timepoint) following s.c. administration in the rat. Time course of ondansetron levels in plasma, cortical and sub-cortical areas of the brain. [Colour online.]

Transition 1: Expanding the profile of 5-HT_3 receptor antagonists in L-DOPA induced dyskinesia

Recent behavioural studies in the 6-OHDA-lesioned rat have demonstrated 5-HT_3 receptor blockade with the prototypical antagonist ondansetron diminished the severity of established dyskinesia by 25% and attenuated the development of dyskinesia by as much as 64% [559, 870]. However, it is unclear whether the anti-dyskinetic benefit obtained with ondansetron represents a class effect and may also be elicited by other 5-HT_3 antagonists.

In addition to ondansetron, there are several highly-selective 5-HT_3 receptor antagonists such as granisetron, dolasetron and palonosetron that are clinically-available as anti-emetics [1013]. Besides a shared mechanism of action, these antagonists exhibit different receptor binding affinities and pharmacokinetic profiles [1014], which may potentially translate to varied anti-dyskinetic potential.

In Chapter 3, we evaluated the anti-dyskinetic efficacy of granisetron in the 6-OHDA rat and found that granisetron significantly reduced dyskinesia parameters by as much as 45%. Moreover, its anti-dyskinetic effect did not compromise L-DOPA anti-parkinsonian action, which is consistent with reports that investigated the effects of ondansetron in the same model [559, 870]. Taken together, these results suggest that the anti-dyskinetic benefit conferred by ondansetron and granisetron may be attributed to blockade of 5-HT_3 receptors. Moreover, the clinical availability of 5-HT_3 receptor antagonists may facilitate testing in Phase II clinical trials for repositioning as an adjunct therapy to L-DOPA in the treatment of dyskinesia in PD.

Chapter 3 - Granisetron, a selective 5-HT_3 antagonist, reduces L-3,4-dihydroxyphenylalanine-induced abnormal involuntary movements in the 6-hydroxydopamine-lesioned rat

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Short title: Granisetron reduces dyskinesia in the rat

Note: The journal incorrectly linked the supplementary materials in the published text and on the article website. The correct supplementary materials are presented in the Figures section of this Chapter.

Chapter 3 Abstract

Administration of L-3,4-dihydroxyphenylalanine (L-DOPA) provides Parkinson's disease patients with effective symptomatic relief. However, long-term L-DOPA therapy is often marred by complications such as dyskinesia. We have previously demonstrated that serotonin type 3 (5-HT_3) receptor blockade with the clinically available and highly selective antagonist ondansetron alleviates dyskinesia in the 6-hydroxydopamine (6-OHDA)-lesioned rat. Here, we sought to explore the antidyskinetic efficacy of granisetron, another clinically available 5-HT_3 receptor antagonist. Rats were rendered hemi-parkinsonian by 6-OHDA injection in the medial forebrain bundle. Following induction of stable abnormal involuntary movements (AIMs), granisetron (0.0001, 0.001, 0.01, 0.1 and 1 mg/kg) or vehicle was acutely administered in combination with L-DOPA and the severity of AIMs, both duration and amplitude, was determined. We also assessed the effect of granisetron on L-DOPA antiparkinsonian action by performing the cylinder test. Adding granisetron (0.0001, 0.001, 0.01, 0.1 and 1 mg/kg) to L-DOPA resulted in a significant reduction of AIMs duration and amplitude, with certain parameters being reduced by as much as 38 and 45% ($P < 0.05$ and $P < 0.001$, respectively). The antidyskinetic effect of granisetron was not accompanied by a reduction of L-DOPA antiparkinsonian action. These results suggest that 5-HT_3 blockade may reduce L-DOPA-induced dyskinesia without impairing the therapeutic efficacy of L-DOPA. However, a U-shaped dose-response curve obtained with certain parameters may limit the therapeutic potential of this strategy and require further investigation.

Keywords: dyskinesia, granisetron, Parkinson's disease, 5-HT_3 receptor, 6-hydroxydopamine-lesioned rat

Chapter 3 Introduction

Parkinson's disease is the second most common neurodegenerative disorder, and in the next few decades, prevalence is expected to grow with an increasingly ageing population (Manson et al., 2012). The main treatment to provide symptomatic relief for Parkinson's disease is the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) (Tarakad and Jankovic, 2017). However, with prolonged administration of L-DOPA, as many as 95% of patients become encumbered by abnormal involuntary movements (AIMs), dyskinesia (Hely et al., 2005). Current pharmacological management of dyskinesia includes modification of dopaminergic therapy and the *N*-Methyl-D-aspartate (NMDA) receptor antagonist amantadine, but the efficacy achieved is partial and often marred by side effects (Kong et al., 2017, Oertel et al., 2017).

The serotonin (5-HT) system has received considerable interest in the pathophysiology of dyskinesia (Huot and Fox, 2013), particularly through the role of serotonergic terminals in converting exogenous L-DOPA into dopamine and controlling its release in the denervated Parkinson's disease striatum (Carta et al., 2007). Much of the focus has been directed towards 5-HT type 1A (5-HT_{1A}) and type 1B (5-HT_{1B}) agonists and 5-HT type 2A (5-HT_{2A}) antagonists, which effectively reduced dyskinesia in the 6-hydroxydopamine (6-OHDA)-lesioned rat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned non-human primate models (Bibbiani et al., 2001, Iravani et al., 2006, Hamadjida et al., 2018d, Kwan et al., 2019). In light of these promising results, clinical trials tested the 5-HT_{1A} agonists tandospirone and sarizotan in treating dyskinetic Parkinson's disease patients, but these agents only produced modest antidyskinetic efficacy and/or worsened parkinsonian symptoms (Kannari et al., 2002a, Olanow et al., 2004, Goetz et al., 2008a).

The 5-HT type 3 (5-HT_3) receptor is the only ligand-gated receptor amongst 5-HT receptors and several clinically-available antagonists used to treat chemotherapy-induced nausea can modulate its activity (Walstab et al., 2010). It was previously shown that blockade of 5-HT_3 receptors with ondansetron diminished dopamine release within the basal ganglia (Koulu et al., 1990), which suggests that antagonising 5-HT_3 receptors may alleviate dyskinesia, by dampening the excessive release of L-DOPA-derived dopamine that occurs in the dyskinetic state (Carta et al., 2007). Moreover, although we acknowledge that this is not their primary mechanism of action, some compounds that alleviated dyskinesia in animal models of Parkinson's disease (Durif et al., 2004, Hamadjida et al., 2017) also antagonise the 5-HT_3 receptor (Ashby and Wang, 1996, Anttila and Leinonen, 2001), which suggests that 5-HT_3 blockade may play a minor role in their antidyskinetic effect.

Two recent studies in the hemi-parkinsonian rat found that the 5-HT_3 receptor antagonist ondansetron diminished the severity of established, and prevented the development of, dyskinesia (Aboulghasemi et al., 2018, Kwan et al., 2020c). However, it is unclear if the antidyskinetic efficacy obtained with ondansetron represents a class effect and might also be elicited by other 5-HT_3 antagonists. Indeed, 5-HT_3 antagonists exhibit different pharmacokinetic profiles and affinity for various receptors (Gan, 2005), which could potentially translate to varied antidyskinetic action. Ondansetron displays low affinity for 5-HT_{1A}, 5-HT_{1B}, α-adrenergic and opioid receptors (van Wijngaarden et al., 1990a), whereas the 5-HT_3 receptor antagonist granisetron has minimal to no affinity for other receptors (Blower, 1990). In the present study, we evaluated the antidyskinetic efficacy of 5-HT_3 blockade with the highly selective antagonist granisetron in the hemi-parkinsonian rat.

Chapter 3 Methods

Subjects

Experiments were conducted on adult female Sprague-Dawley rats (225–250 g, Charles River, Canada). Rats were group housed in three under temperature (21± 1°C), humidity (55%), light (12-h light/dark cycle, lights on at 07:00) controlled conditions with ad-libitum access to food and water. Upon arrival, rats remained undisturbed to acclimatise to housing conditions for at least 5 days before experiments. All procedures were approved by the Montreal Neurological Institute Animal Care Committee, in accordance with the Canadian Council on Animal Care guidelines.

Drug treatments

Desipramine hydrochloride, pargyline hydrochloride, 6-OHDA hydrobromide, L-DOPA methyl ester hydrochloride, benserazide hydrochloride were purchased from MilliporeSigma, Canada (Etobicoke, Ontario, Canada). Granisetron hydrochloride was purchased from Cedarlane Laboratories, Canada (Burlington, Ontario, Canada). All drugs were dissolved in 0.9% NaCl unless otherwise specified. 6-OHDA was dissolved in 0.9% saline with 0.02% ascorbic acid, L-DOPA was dissolved in 0.9% NaCl with 0.1% ascorbic acid. All solutions were administered sub-cutaneously (s.c.) in a volume of 1.0 mL/kg body weight.

All drug doses are expressed as free base weights.

Induction of parkinsonism

Rats (*N*=35) were pre-treated with desipramine (10 mg/kg subcutaneously) and pargyline (5 mg/kg subcutaneously) to protect noradrenergic neurons (Ungerstedt, 1968). Then, they were deeply anaesthetised and placed into a stereotaxic frame (David Kopf Instruments, Tujunga, California, USA) 30 min later, after which they were injected with 2.5 µL of 6-OHDA (7 µg/µL) in the right medial forebrain bundle, as previously described (Huot et al., 2015, Frouni et al., 2018), at the following coordinates: antero-posterior: − 2.8 mm, medio-lateral: − 2.0 mm, dorso-ventral: − 9.0 mm) relative to Bregma, with the incisor bar set 3.3 mm below ear bars (Paxinos and Watson, 2007). Throughout surgery, rats were anaesthetised with isoflurane (2-4%; MilliporeSigma, Canada) in 100% oxygen (1 L/min).

Evaluation of parkinsonism

Three weeks after 6-OHDA injection, the degree of parkinsonism was assessed using the cylinder test (Schallert et al., 2000, Frouni et al., 2018, Frouni et al., 2019). Rats (*N*=22) were placed in a transparent cylinder (14 cm diameter × 28 cm height), recorded for 10 min and behaviours were analysed post hoc. Only animals that demonstrated preferential use of the un-lesioned forelimb in ≥70% of the rears were selected for inclusion in behavioural studies, a score that is indicative of ≥88% dopamine depletion in the striatum (Schallert et al., 2000, Frouni et al., 2018, Frouni et al., 2019).

Assessment of axial, limbs and oro-lingual abnormal involuntary movements

Axial, limbs and oro-lingual (ALO) abnormal involuntary movements (AIMs) were assessed by an observer blinded to treatment, according to a scale previously described (Cenci and Lundblad, 2007), which encompasses both time-based, ‘duration,’ and severity-based, that is ‘amplitude,’

assessment of abnormal movements. The scales utilised are presented in Supplementary Tables 1 and 2, Supplemental digital content 1, *http://links.lww.com/BPHARM/A61*. Briefly, axial AIMs affect the neck and upper trunk of the animal and results in torsional movement towards the side contralateral to lesion (Cenci and Lundblad, 2007). Limbs AIMs are defined by hyperkinetic and/or sustained contraction of muscles of the forelimb contralateral to lesion, whereas oro-lingual AIMs affect facial, tongue and masticatory muscles. On days of behavioural scoring, following administration of L-DOPA/granisetron or L-DOPA/vehicle, rats were put in individual glass cylinders and ALO AIMs were rated for 2 min starting at baseline (prior to treatment administration) and every 20 min over a 3 h testing session thereafter. Both ALO AIMs duration and amplitude were rated on a scale from 0 to 4 in each monitoring interval, as we have previously done (Frouni et al., 2018, Frouni et al., 2019, Kwan et al., 2020c). The maximum obtainable axial, limbs or oro-lingual AIMs score per session was 36. The 3 AIMs subtypes were summed to obtain the cumulative ALO AIMs score with a maximum score of 108 per session.

Acute challenge study

Rats (*N*=22) that displayed severe rearing asymmetry following assessment of parkinsonism underwent daily priming with L-DOPA/benserazide (10/15 mg/kg) for 14 days to induce ALO AIMs (Frouni et al., 2018, Frouni et al., 2019) and animals (*N*=13) that exhibited stable and reproducible AIMs were retained for further testing. On days of behavioural testing, rats were administered L-DOPA (6/15 mg/kg, hereafter referred to as L-DOPA) in combination with granisetron (0.0001, 0.001, 0.01 0.1 and 1 mg/kg) or vehicle, and the severity of ALO AIMs was assessed as described above. Treatments were randomised according to a within-subjects design,

in which all animals received all treatments, in a random order and behavioural testing sessions were separated by at least 72 h of drug washout.

After a 3-day washout period, rats were administered a low dose of L-DOPA/benserazide (3/15 mg/kg subcutaneously), sufficiently high to produce an antiparkinsonian effect but without triggering AIMs, in combination with granisetron (0.0001 0.001, 0.01, 0.1, 1 mg/kg) or vehicle. Forty-five minutes later, corresponding to peak antiparkinsonian action, animals underwent the cylinder test to determine whether granisetron compromised the therapeutic action of L-DOPA.

High-performance liquid chromatography-tandem mass spectrometry

At the end of the experiments, rats were anaesthetised with isoflurane (2-4%; MilliporeSigma, Canada) prior to trans-cardial perfusion with 0.9% NaCl. Brains were then removed, flash frozen in isopentane at -56 °C and the left and right striata were dissected from the rest of the brain and stored at -80ºC until further analysis. High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was performed to quantify the striatal levels of dopamine, 5-HT, and their metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA), according to previous protocols (Huot et al., 2012b, Frouni et al., 2018, Frouni et al., 2019). Tissue monoamine concentrations of are expressed as ng/mg wet tissue. The dopamine turnover rate was calculated from DOPAC/dopamine and (DOPAC+HVA)/dopamine ratios, whereas the 5-HT turnover rate was calculated from the 5-HIAA/5-HT ratio (Smith et al., 2003).

Statistical Analysis

Data from the cylinder test to assess hemi-parkinsonism are graphed as the mean ± SEM: standard error of the mean and were analysed using one-way analysis of variance (ANOVA) with the Greenhouse-Geisser correction; post hoc comparisons were performed using Tukey's post hoc test. Tissue concentrations of the biogenic amines dopamine, 5-HT and their metabolites DOPAC, HVA, 5-HIAA are presented as the mean ± SEM and were analysed by unpaired Welch's unequal variances *t* test. The turnover rates of dopamine and 5-HT were assessed for significant differences using unpaired Welch's unequal variances *t* test. Cumulative AIMs scores are expressed as the median with semi-interquartile interval and were analysed using nonparametric Friedman test followed by Dunn's post hoc test. AIMs time course data are presented as the median and, following data ranking in ascending order, were analysed by two-way repeated measures (RM) ANOVA followed by Dunnett's post hoc test (Howell, 2011). The effect of granisetron on L-DOPA antiparkinsonian action is presented as the mean ± SEM and was analysed using one-way RM ANOVA followed by Tukey's post hoc test. Statistical significance was assigned when $P < 0.05$. Statistical analyses were performed with GraphPad Prism 8.2.0 (GraphPad Software Inc, San Diego, California, USA).

Chapter 3 Results

Extent of dopaminergic lesion

In the cylinder test, animals demonstrated a mean of 48 ± 14 rears over the 10 min observation period. As shown in Fig. 1a, animals selected for inclusion in behavioural studies exhibited rearing asymmetry ($F_{(2, 36)} = 155.9$, $P < 0.001$, one-way ANOVA), with markedly higher use of the right

forepaw in 81% of wall contacts, while the left forepaw and both forepaws accounted for 0.7 and 19% of contacts, respectively (both $P < 0.001$ compared to the right forepaw, Tukey's post hoc test). In line with these results, HPLC-MS/MS analysis of striatal tissue revealed significant diminutions of dopamine, DOPAC and HVA in the lesioned striata when compared to the unlesioned striata, by 97% ($t_{(9.036)} = 6.940$, $P < 0.001$), 70% ($t_{(9.267)} = 6.160$, $P < 0.001$) and 82% ($t_{(9.580)} = 6.416$, $P < 0.001$), respectively as displayed in Fig.1b. In contrast, 5-HT ($t_{(15.45)} = 0.6997$, $P > 0.05$) or 5-HIAA ($t_{(16.35)} = 0.2436$, $P > 0.05$) levels were comparable between both hemispheres. As presented in Table 1, the 6-OHDA lesion induced a significant increase in the turnover rate of dopamine in the ipsilateral striata ($t_{(9.051)} = 4.238$, $P < 0.01$) for DOPAC/dopamine and ($t_{(9.015)} = 3.578$, both $P < 0.01$) for (DOPAC+HVA)/dopamine, respectively, whereas the turnover rate of 5-HT was unaltered ($t_{(16.58)} = 1.096$, $P > 0.05$). Thus, the lesioned striata showed a 14.9-fold-increase in the DOPAC/dopamine ratio and a 10.1-fold-increase in (DOPAC+HVA)/dopamine ratio, respectively.

Effect of granisetron on L-DOPA-induced dyskinesia

Axial, limbs and oro-lingual abnormal involuntary movements AIMs

As shown in Fig. 2a, administration of granisetron in combination with L-DOPA had a significant effect on ALO AIMs duration throughout the 3 h observation period ($F_{time\ (6.52,\ 469.5)} = 0$, $P > 0.05$; $F_{treatment\ (5,\ 72)} = 9.405$, $P < 0.001$; and $F_{interaction\ (45,\ 648)} = 1.520$, $P < 0.05$; two-way RM ANOVA following ranking of data). Thus, as presented in Supplementary Table 3, Supplemental digital content 1, *http://links.lww.com/BPHARM/A56*, at 60 min, granisetron (0.0001, 0.001, 0.01 mg/kg) significantly decreased ALO AIMs duration, by 29%, 40% and 45%, respectively, when compared to vehicle ($P < 0.05$, $P < 0.001$ and $P < 0.001$, Dunn's post hoc test). At 140 min, granisetron

(0.0001, 0.001, 0.01 mg/kg) significantly decreased ALO AIMs duration by 34%, 26% and 34%, respectively, when compared to vehicle ($P < 0.001$, $P < 0.05$ and $P < 0.001$, Dunn's post hoc test). Combining granisetron with L-DOPA also significantly diminished the severity of cumulative ALO AIMs duration (Friedman Statistic [FS] = 15.99, $P < 0.01$) as illustrated in Fig. 2b. Thus, administration of granisetron (0.0001, 0.001 and 0.01 mg/kg) with L-DOPA attenuated ALO AIMs duration by 25%, 29% and 27%, ($P < 0.05$, $P < 0.01$ and $P < 0.05$, Dunn's post hoc test), when compared to L-DOPA/vehicle.

Figure 2c shows that administration of granisetron in combination with L-DOPA also had a significant effect on ALO AIMs amplitude throughout the 3 h observation period ($F_{time\ (4.89,\ 352.3)} = 0$, $P > 0.05$; $F_{treatment\ (5,\ 72)} = 7.250$, $P < 0.001$; and $F_{interaction\ (45,\ 648)} = 1.327$, $P > 0.05$; two-way RM ANOVA following ranking of data). Thus, as presented in Supplementary Table 4, Supplemental digital content 1, *http://links.lww.com/BPHARM/A56*, at 60 min, granisetron (0.001, 0.01, 0.1 and 1 mg/kg) significantly decreased ALO AIMs amplitude, by 30, 27, 26 and 29%, respectively, when compared to vehicle (each, $P < 0.05$, $P < 0.001$, $P < 0.05$ and $P < 0.01$, Dunn's post hoc test). At 140 min, granisetron (0.0001, 0.001, 0.01 and 0.1 mg/kg) significantly decreased ALO AIMs amplitude, by 38, 31, 35 and 26%, respectively, when compared to vehicle ($P < 0.01$, $P < 0.01$, $P < 0.01$ and $P < 0.05$, Dunn's post hoc test). Granisetron significantly diminished the severity of cumulative ALO AIMs amplitude (FS = 20.85, $P < 0.001$). Thus, administration of granisetron (0.0001, 0.001, 0.01, 0.1 and 1 mg/kg) with L-DOPA attenuated ALO AIMs amplitude by 28, 29, 29, 27 and 24%, ($P < 0.01$, $P < 0.001$, $P < 0.001$, $P < 0.05$, and $P < 0.05$, Dunn's post hoc test), when compared to L-DOPA/vehicle, as shown in Fig. 2d.

Abnormal involuntary movement subtypes

As displayed in Fig. 3a, granisetron significantly diminished the severity of cumulative axial AIMs duration (FS = 11.38, $P < 0.05$). Administration of granisetron 0.0001 mg/kg with L-DOPA attenuated axial AIMs duration by 32% ($P < 0.01$, Dunn's post hoc test), when compared to L-DOPA/vehicle. In contrast, granisetron 0.001, 0.01, 0.1 and 1 mg/kg did not significantly reduce the severity of cumulative axial AIMs duration. Granisetron also significantly reduced the severity of cumulative axial AIMs amplitude (FS = 15.76, $P < 0.01$, Fig. 3b). Thus, after administration of granisetron 0.0001, 0.001, 0.01, 0.1 and 1 mg/kg, the severity of axial AIMs amplitude was reduced by 21, 28, 29, 24 and 25%, respectively, when compared to L-DOPA/vehicle ($P < 0.05$, $P < 0.01$, $P < 0.01$, $P < 0.05$ and $P < 0.05$, Dunn's post hoc test).

As shown in Fig. 3c, granisetron significantly diminished the severity of cumulative limbs AIMs duration (FS = 24.95, $P < 0.001$). Administration of granisetron 0.0001, 0.001 and 0.01 mg/kg with L-DOPA attenuated limbs AIMs duration by 30, 45 and 42%, ($P < 0.05$, $P < 0.001$ and $P < 0.001$, Dunn's post hoc test), when compared to L-DOPA/vehicle. As displayed in Fig. 3d, granisetron significantly reduced the severity of cumulative limbs AIMs amplitude (FS = 14.21, $P < 0.05$). Thus, after administration of granisetron 0.0001, 0.001 and 0.01 mg/kg, the severity of limbs AIMs amplitude was reduced by 25, 27 and 24%, respectively, when compared to vehicle ($P < 0.05$, $P < 0.05$ and $P < 0.01$, Dunn's post hoc test).

Finally, as displayed in Fig. 3e, granisetron did not diminish the severity of cumulative oro-lingual AIMs duration (FS = 9.645, $P > 0.05$). In contrast, as shown in Fig. 3f, granisetron significantly reduced the severity of cumulative oro-lingual AIMs amplitude (FS = 13.48, $P < 0.05$). Thus, after administration of granisetron 0.0001, 0.1 and 1 mg/kg, the severity of oro-lingual AIMs amplitude was reduced by 38, 27 and 27%, respectively, when compared to vehicle (all $P < 0.05$, Dunn's post hoc test).

Granisetron does not compromise L-DOPA antiparkinsonian action

We assessed the effect of granisetron on L-DOPA antiparkinsonian action using the cylinder test. As shown in Fig. 4, L-DOPA alone or in combination with granisetron resulted in an improvement in motor performance with less dependence on the right forepaw when making wall contacts ($F_{(4.067,\ 48.80)}$ = 8.384, $P < 0.001$, one-way RM ANOVA). Thus, L-DOPA/vehicle-treated rats displayed a marked decrease in the number of rears using the unlesioned side, by 49% ($P < 0.05$, Tukey's post hoc test), when compared to L-DOPA-untreated 6-OHDA-lesioned animals. The addition of granisetron (0.001, 0.01, 0.1 or 1 mg/kg) did not affect this decrease in rears with the unlesioned forepaw, which was 50, 44, 45and 43%, respectively (each, $P < 0.01$, $P < 0.05$, $P < 0.05$ and $P < 0.05$, Tukey's post hoc test), when compared to L-DOPA-untreated 6-OHDA-lesioned rats. Forepaw use with the unlesioned side was similar between L-DOPA/vehicle and L-DOPA/granisetron, for all doses of granisetron except for the dose of 0.0001 mg/kg ($P < 0.05$, Tukey's post hoc test).

Chapter 3 Discussion

In the present study, we have shown that acute administration of granisetron alleviated established L-DOPA-induced AIMs in the hemi-parkinsonian rat. Moreover, the therapeutic benefit was achieved without worsening L-DOPA antiparkinsonian action. Our results confirm previous findings that blockade of the 5-HT_3 receptor may be a promising therapeutic intervention to reduce dyskinesia in Parkinson's disease.

A limitation of our study is that it was performed in female rats only. We elected to conduct the experiments in female animals, as their weight remains stable over time, in contrast to their male counterpart. Recent clinical trials did not find any difference between individuals from both sexes to the antidyskinetic effect of amantadine (Hauser et al., 2017, Pahwa and Hauser, 2017), which may suggest that the response to an antidyskinetic agent may not be affected by gender variables. However, this potential explanation is speculative with 5-HT_3 antagonists and requires further evaluation.

A recent study in the 6-OHDA-lesioned rat found that administration of the 5-HT_3 antagonist ondansetron commenced concurrently with the first dose of L-DOPA elicited an antidyskinetic effect by decreasing AIMs scores by 54% when compared to vehicle (Aboulghasemi et al., 2018). In line with these findings, in the same model, we reported that 6-OHDA-lesioned rats that were started on ondansetron treatment concurrently with the first dose of L-DOPA during the dyskinesia induction phase exhibited less severe ALO AIMs amplitude, by 64%, compared to rats treated with L-DOPA alone from the beginning (Kwan et al., 2020c). In addition, in that previous study, we found that ondansetron diminished the severity of established ALO AIMs, by 25%, which is in agreement with the magnitude of the ALO AIMs reduction that we obtained here. Our data, therefore, support that 5-HT_3 blockade may alleviate dyskinesia, although the antidyskinetic benefit conferred by this approach appears to be partial.

Prior to our current experiment, it was unclear whether the antidyskinetic efficacy of ondansetron was due to a class effect (blockade of the 5-HT_3 receptor) or solely limited to the action of ondansetron. Granisetron and ondansetron are highly potent 5-HT_3 antagonists with receptor binding affinities (pK_i) of 8.42 and 8.07, respectively (van Wijngaarden et al., 1990a). Ondansetron demonstrates low affinity binding to 5-HT_{1A}, 5-HT_{1B}, α-adrenergic and opioid receptors but its

binding to high-affinity 5-HT_3 receptor sites is about 250- to 1000-fold higher that of other receptors (Kilpatrick et al., 1987, van Wijngaarden et al., 1990a, Gregory and Ettinger, 1998). In contrast, granisetron displays 4000 to 40000 times higher affinity for 5-HT_3 receptors than other receptors, with negligible affinity for other 5-HT and dopamine receptors, which suggests that granisetron mediates its effects through selective 5-HT_3 interaction (Blower, 1990). The duration of action of both compounds appears relatively similar as, following intra-venous (i.v.) administration of granisetron to humans (40-300 µg/kg), the mean plasma half-life ranged from 4.1 to 10.6 h (Carmichael et al., 1989, Allen et al., 1994), whereas intravenous administration of ondansetron in human (109- 115 µg/kg) resulted in mean plasma half-life of 3.2-5.5 h (Blackwell and Harding, 1989, Colthup et al., 1991, Baber et al., 1992). To date, no study has assessed the plasma pharmacokinetics of granisetron following subcutaneous or intraperitoneal administration in rat, but intravenous administration of granisetron (3-6 mg/kg) in rat led to a plasma half-life of 51-52 min, which is similar to the plasma half-life of 39- 43 min with subcutaneous administration of ondansetron (0.1-10 µg/kg) (Kwan et al., 2020a). Interestingly, we found that the slightly longer half-life of granisetron did not translate to a superior reduction in ALO AIMs duration. Other than differences in half-life, pharmacokinetic parameters are comparable between these 5-HT_3 antagonists (Gregory and Ettinger, 1998). Given that granisetron alleviated dyskinesia in the hemi-parkinsonian rat to a similar magnitude and shares the same mechanism of action as ondansetron (5-HT_3 receptor blockade), we may infer that the benefit obtained by both ondansetron and granisetron is mediated by antagonism of the 5-HT_3 receptor.

In contrast to the inverse U-shaped dose-response curve ascribed to the 5-HT_3 receptor antagonist ondansetron (Goudie and Leathley, 1990), studies reported that granisetron exhibits a curvilinear dose-response profile that is linear until subsequent increases in dose do not result in equivalent

increases in response in animal models (Gregory and Ettinger, 1998). Here, the dose-response curve of granisetron does not appear to have a distinct shape, although the therapeutic benefit conferred by granisetron was limited to doses < 0.01 mg/kg and was reminiscent of a U-shaped dose-response curve. Of note, a similar loss of antidyskinetic effect was also encountered in our studies with ondansetron (Kwan et al., 2020c). A recent notion suggests that the efficacy of pharmacological action of neurotransmitter receptor antagonists requires 67-97% occupancy of the target receptor at therapeutically effective doses (Grimwood and Hartig, 2009). Thus, it is possible that, at the dose of granisetron 0.01 mg/kg, receptor occupancy is maximal and receptor occupancy is saturated with higher doses (> 0.01 mg/kg), which does not produce superior antidyskinetic efficacy. To the best of our knowledge, no study has measured plasma or brain levels of granisetron following subcutaneous administration of small doses in rat, so it is difficult to comment on the correlation between the pharmacokinetic profile of granisetron and its effect on dyskinesia. However, a microdialysis experiment in the rat found that following intravenous administration of granisetron (3 and 6 mg/kg), half-life in the brain is significantly longer than in plasma, which suggests appreciable blood-brain barrier penetrance (Huang et al., 1999). In light of these results, additional studies are required to clarify the relationship between the pharmacokinetics of granisetron and its antidyskinetic benefit and to determine whether therapeutically effective doses are clinically relevant.

The link between L-DOPA-induced dyskinesia and fluctuations in dopamine levels in the striatum has been well established and notably, a PET study found that dyskinetic patients show greater changes in striatal dopamine levels than stable responders (de la Fuente-Fernandez et al., 2004). Thus, the ability of 5-HT_3 receptors to modulate dopamine release within the basal ganglia may underlie the antidyskinetic action of antagonists. In fact, in-vivo microdialysis studies found that

5-HT (Benloucif et al., 1993) or the 5-HT_3 agonist phenylbiguanide (Santiago et al., 1995) facilitated dopamine release in the striatum, which is consistent with effects of 5-HT_3 agonists on dopamine levels in rat (Benuck and Reith, 1992) and mouse (Sershen et al., 1995) striatal slides. Although these results are difficult to reconcile with the relatively low density of 5-HT_3 binding in the rat striatum (Kilpatrick et al., 1989), evidence supports a role of the 5-HT_3 receptor in altering dopaminergic transmission-mediated behaviours. Pharmacological studies in the rat found that administration of the 5-HT_3 antagonist MDL 72222 (20 μg/kg) suppressed locomotor and motor activity (Kriem et al., 1995), while low doses (0.001- 0.1 mg/kg) of the antagonist GR38032F attenuated amphetamine-induced hyperactivity to control levels (Costall et al., 1987). In spite of the favourable data that supports a possible role of 5-HT_3 receptor activation in controlling dopamine release in the striatum, additional studies that would determine the neuroanatomical substrate of 5-HT_3 receptor blockade (Barnes et al., 1992) will provide further evidence of the mechanism underlying its antidyskinetic benefit.

Abnormalities in glutamatergic transmission have been reported in animal models of L-DOPA induced dyskinesia, which result in high levels of peri-synaptic and extra-synaptic glutamate receptor stimulation (Sgambato-Faure and Cenci, 2012). To this end, following chronic L-DOPA administration in 6-OHDA rats, dyskinesia expression coincided with a surge of extracellular glutamate levels in the substantia nigra pars reticulata and striatum (Robelet et al., 2004, Mela et al., 2012). Recently, we demonstrated that activation of metabotropic glutamate 2/3 ($mGlu_{2/3}$) receptors with the orthosteric agonist LY-354,740 alleviated dyskinesia in the 6-OHDA rat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned marmoset models (Frouni et al., 2019). Taken together, these findings suggest that reducing glutamatergic hyperactivity may confer an antidyskinetic effect. In the rat, autoradiographic binding studies have consistently

reported moderate 5-HT_3 binding in the cortex (Kilpatrick et al., 1987, Kilpatrick et al., 1988, Laporte et al., 1992), while 5-HT_3 receptor messenger ribonucleic acid (mRNA) has been found in motor, pre-limbic and cingulate cortices (Morales and Bloom, 1997). Moreover, single cell recording studies in the rat have demonstrated that activation of 5-HT_3 receptors modulate glutamatergic synaptic transmission in brain regions such as the hippocampus (Zhang et al., 1994) and area postrema (Funahashi et al., 2004), possibly by suppressing neuronal activity (Ashby et al., 1991, Edwards et al., 1996). Considering the distribution and role of 5-HT_3 receptors in controlling glutamate release, we speculate that blockade of 5-HT_3 receptors, possibly localised on cortico-striatal axons, with granisetron may dampen glutamatergic transmission to achieve its antidyskinetic benefit. Further studies are required to examine whether the antidyskinetic efficacy attributed to 5-HT_3 receptor blockade coincides with reduced glutamatergic neurotransmission.

In general, granisetron did not compromise the antiparkinsonian benefit of L-DOPA, which is consistent with studies that administered ondansetron in the same model (Aboulghasemi et al., 2018, Kwan et al., 2020c). However, the dose of 0.0001 mg/kg did not significantly alter dependence on the unlesioned forepaw compared to drug-naïve animals, which suggests that such a low dose of granisetron may interfere with the therapeutic action of L-DOPA. Importantly, this result was not obtained with ondansetron and further validation is required to clarify this discrepancy. Nevertheless, this apparent reduction of L-DOPA antiparkinsonian action was not encountered with higher doses of granisetron, which may indicate that future studies should administer higher doses of granisetron (> 0.0001 mg/kg) to obtain antidyskinetic efficacy without compromising the therapeutic benefit conferred by L-DOPA.

The mechanism underlying the action of granisetron on cylinder test performance and AIMs severity has not been studied so our explanations remain speculative. One possibility for worsened

performance in the cylinder test with granisetron 0.0001 mg/kg that was associated with improving AIMs severity is the time interval for behavioural analysis. Cylinder test performance is evaluated at 45-55 min post L-DOPA administration, which corresponds to peak L-DOPA plasma levels (Huot et al., 2012a) and correlates with peak AIMs severity. However, when looking at the effect of this dose on AIMs, as shown in Supplementary Tables 3 and 4, Supplemental digital content 1, *http://links.lww.com/BPHARM/A56*, its antidyskinetic effect at 40 or 60 min is milder compared to doses such as 0.001 and 0.01 mg/kg, while at later time points (i.e. 100 or 140 min), the effect is more pronounced. Thus, we may infer that during the cylinder test with granisetron 0.0001 mg/kg, animals may be more dyskinetic, which may inhibit their ability to make wall contacts and as a result, increases dependence on the right (unlesioned) forepaw. On the other hand, looking at cumulative AIMs severity or time course, behaviour was assessed over the 3 h observation period, so we could still capture the antidyskinetic benefit of this dose. These seemingly contradictory results may be due to the different time duration of behavioural tests.

An alternative explanation is based on the fact that blockade of the 5-HT_3 receptor has been shown to inhibit release of dopamine in the striatum (Koulu et al., 1990). The erratic release of striatal dopamine has been linked to the development of AIMs in the 6-OHDA rat (Carta et al., 2007), so we hypothesised that the antidyskientic effect of granisetron may be mediated through dampening abnormal striatal dopamine release. It is possible that the low dose of granisetron (0.0001 mg/kg) might interfere with striatal dopaminergic transmission in such a way that reduces AIMs but at the expense of impairing the therapeutic efficacy of L-DOPA and worsening performance in the cylinder test. It is unclear why higher doses of granisetron do not hinder the antiparkinsonian action of L-DOPA but it may be related to the possibility that granisetron reaches concentrations that target 5-HT_3 receptors in different brain areas or may interact with other receptor subtypes, also

leading to a loss of antidyskinetic efficacy. Future studies should address the mechanisms underlying the antidyskinetic efficacy of granisetron and other 5-HT_3 antagonists, as well as possible interaction(s) with L-DOPA.

Gastro-intestinal dysfunction occurs in the majority of Parkinson's disease patients with constipation (Sung et al., 2014) and gastroparesis (Heetun and Quigley, 2012) as common symptoms. Autoradiographic binding studies have localised 5-HT_3 receptors in the human colon and rectum, where they are predominantly found on myenteric neurons (Sakurai-Yamashita et al., 1999a, Sakurai-Yamashita et al., 1999b), and from the receptor distribution, we may infer that 5-HT_3 antagonists are well situated to modulate gastrointestinal function. Two case reports have suggested that 5-HT_3 antagonists ameliorated constipation and gastrointestinal tract motility in Parkinson's disease patients (Liu et al., 2005, Ogawa et al., 2012). However, these studies recruited a small number of patients, lacked randomisation and placebo controls, and the compounds also exhibited affinity for other receptor subtypes, so we cannot conclude that the prokinetic effect on colorectal motility is solely attributed to blockade of the 5-HT_3 receptor. Contrary to these findings, open-label trials that assessed the antipsychotic efficacy of ondansetron reported that half of patients experienced increased constipation as the major adverse effect (Zoldan et al., 1995). These previous studies suggest that Parkinson's disease patients may be susceptible to constipation following ondansetron administration. Thus, if granisetron and other 5-HT_3 antagonists advance to the clinic, attention should be paid to their effects on the gastrointestinal tract.

The present study adds to the results of previous studies that found that blockade of the 5-HT_3 receptor may represent a new therapeutic strategy to alleviate dyskinesia in Parkinson's disease. Along with other 5-HT_3 antagonists, granisetron is clinically available, which may facilitate repositioning as an adjunct therapy to L-DOPA in the treatment of dyskinesia in Parkinson's

disease. Further studies are required to assess whether tolerance to the antidyskinetic effect of granisetron would develop following prolonged administration and whether the antidyskinetic benefit observed here would also be obtained in the parkinsonian non-human primate.

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Conflicts of Interest

There are no conflicts of interest.

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Chapter 3 Tables and Figures

Table 1 Dopamine and 5-HT turnover rates in the striatum of 6-hydroxydopamine lesioned rats

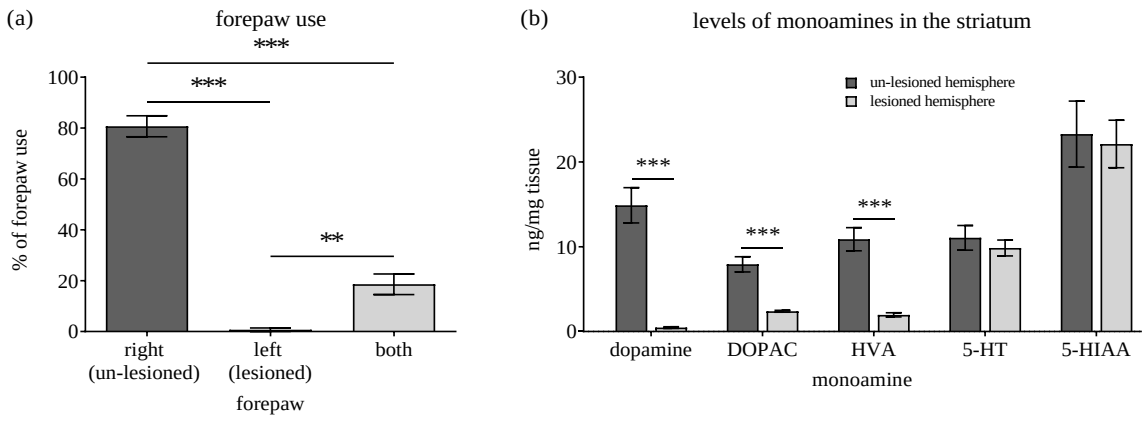
	DOPAC dopamine	$\frac{\text{DOPAC+HVA}}{\text{dopamine}}$	5-HIAA 5-HT
Unlesioned striatum	0.48 (± 0.06)	1.15 (± 0.14)	1.89 (± 0.26)
Lesioned striatum	7.61 (± 1.99) **	12.72 (± 2.73) **	2.24 (± 0.19)

6-OHDA lesion significantly increased the turnover rate of dopamine in the ipsilateral striatum, while the turnover rate of 5-HT remained unchanged compared to the contralateral striatum.

5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin, 6-OHDA, 6-hydroxydopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid.

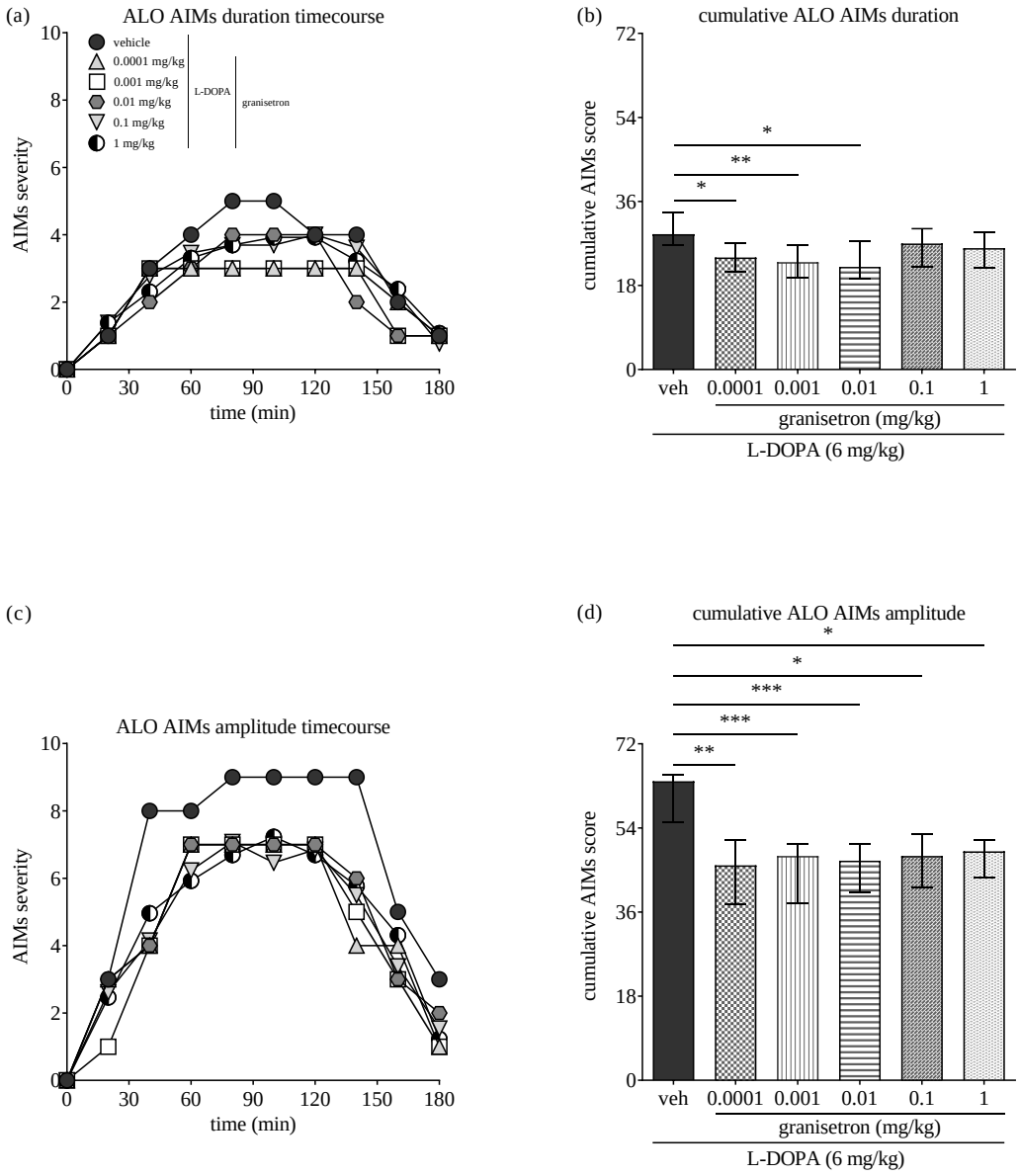
Data are presented as the mean ± SEM ng/mg of wet tissue. **: $P < 0.01$.

Fig. 1



Extent of striatal dopaminergic denervation. (a) Rats selected for behavioural studies displayed preferential use of the un-lesioned (right) forepaw in 81% of rears compared to 0.7% and 19% of rears using the lesioned (left) forepaw and both forepaws, respectively. Data are presented as the mean ± SEM. ***: $P < 0.001$. (b) High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis showed that striatal levels of dopamine and metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) are significantly reduced in the right striata (lesioned) when compared to the left (un-lesioned, by 97%, 70% and 82% respectively). In contrast, levels of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were similar in both striata. N=13. Data are presented as the mean ± SEM. ***: $P < 0.001$.

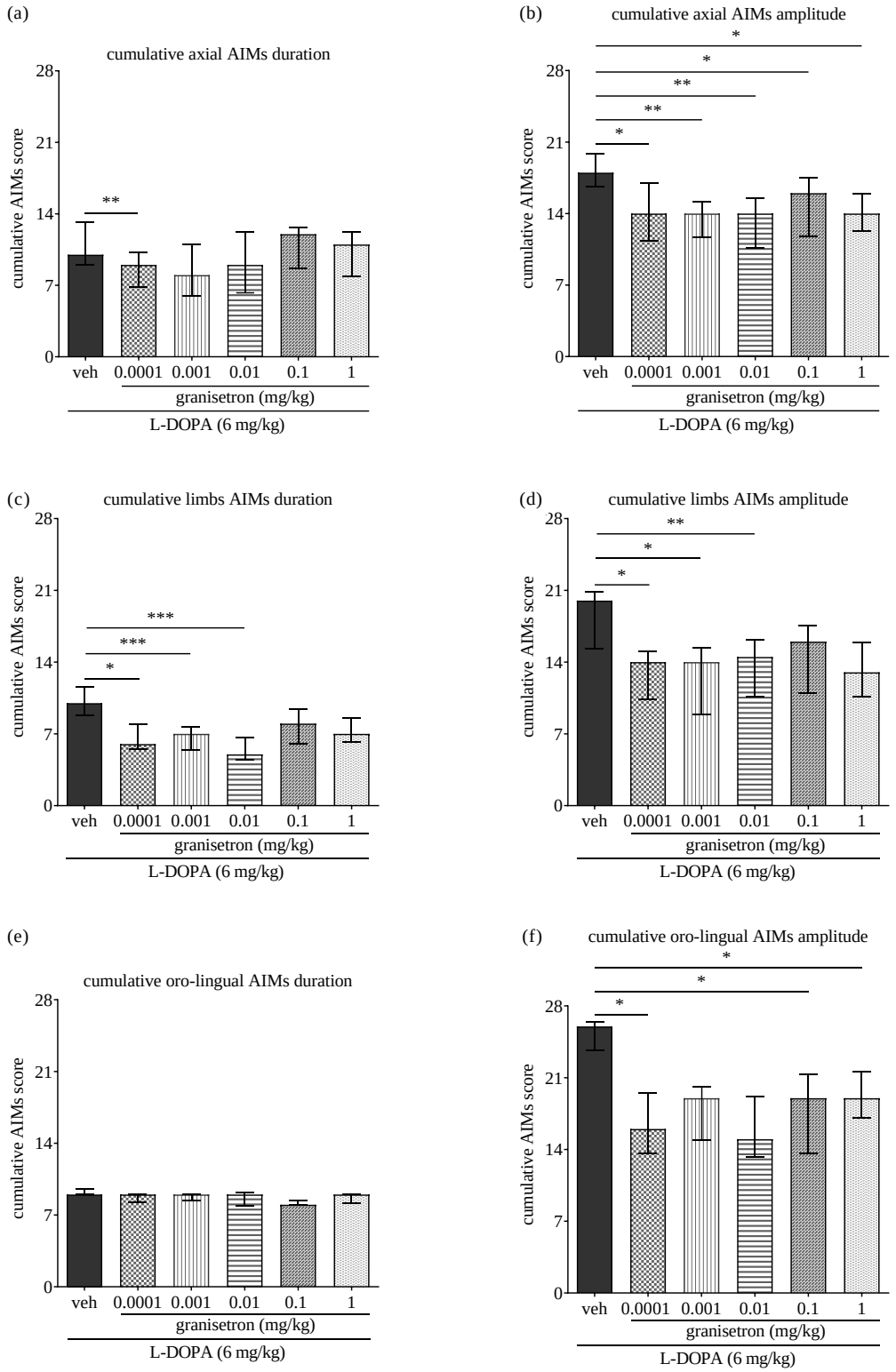
Fig. 2



Effect of acute challenges of granisetron on established L-DOPA induced ALO AIMs. (a) Time course of ALO AIMs duration during the 3 h monitoring period following administration of granisetron and L-DOPA. (b) Treatment with granisetron 0.0001, 0.001 and 0.01 mg/kg in combination with L-DOPA alleviated ALO AIMs duration by 25%, 29% and 27%, respectively.

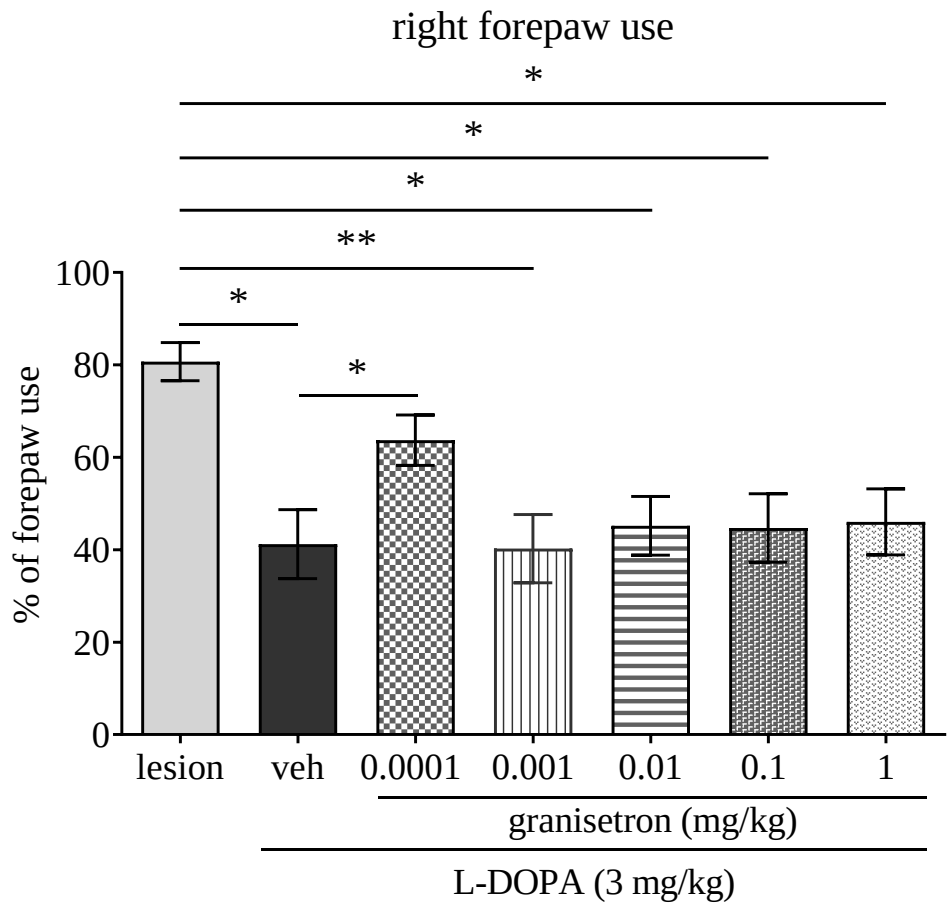
(c) Time course of ALO AIMs amplitude during the 3 h observation period after administration of granisetron and L-DOPA. (d) Adding granisetron 0.0001, 0.001, 0.01, 0.1 and 1 mg/kg to L-DOPA resulted in a marked reduction in ALO AIMs amplitude by 28%, 29%, 29%, 27% and 24%, respectively, compared to vehicle. *N*=13. Data are presented as the median (a, c) and median with semi-interquartile interval (b, d). *: $P < 0.05$; **: $P < 0.01$, ***: $P < 0.001$. AIMs, abnormal involuntary movements; ALO, Axial, limbs and oro-lingual; L-DOPA, L-3,4-dihydroxyphenylalanine.

Fig. 3


(a)
cumulative axial AIMs duration
cumulative AIMs score
28
21
14
7
0
**
veh
0.0001
0.001
0.01
0.1
1
granisetron (mg/kg)
L-DOPA (6 mg/kg)
(b)
cumulative axial AIMs amplitude
*
*
**
**
*
cumulative AIMs score
28
21
14
7
0
veh
0.0001
0.001
0.01
0.1
1
granisetron (mg/kg)
L-DOPA (6 mg/kg)
(c)
cumulative limbs AIMs duration
***
***
*
cumulative AIMs score
28
21
14
7
0
veh
0.0001
0.001
0.01
0.1
1
granisetron (mg/kg)
L-DOPA (6 mg/kg)
(d)
cumulative limbs AIMs amplitude
**
*
*
cumulative AIMs score
28
21
14
7
0
veh
0.0001
0.001
0.01
0.1
1
granisetron (mg/kg)
L-DOPA (6 mg/kg)
(e)
cumulative oro-lingual AIMs duration
cumulative AIMs score
28
21
14
7
0
veh
0.0001
0.001
0.01
0.1
1
granisetron (mg/kg)
L-DOPA (6 mg/kg)
(f)
cumulative oro-lingual AIMs amplitude
*
*
*
cumulative AIMs score
28
21
14
7
0
veh
0.0001
0.001
0.01
0.1
1
granisetron (mg/kg)
L-DOPA (6 mg/kg)

Effect of acute challenges of granisetron on established L-DOPA induced AIMs. (a) Granisetron 0.001 mg/kg significantly diminished axial AIMs duration by 32%. (b) Axial AIMs amplitude was reduced by 21%, 28%, 29%, 24% and 25%, following administration of granisetron 0.0001, 0.001, 0.01, 0.1 and 1 mg/kg, when compared with vehicle. (c) Compared to vehicle, adding granisetron 0.0001, 0.001 and 0.01 mg/kg to L-DOPA attenuated the severity of limbs AIMs duration by 30%, 45% and 42%. (d) Treatment with granisetron 0.0001, 0.001 and 0.01 mg/kg in combination with L-DOPA resulted in reduced limbs AIMs amplitude by 25%, 27% and 24%. (e) Granisetron had no significant effect on oro-lingual AIMs duration. (f) Administration of granisetron 0.0001, 0.1 and 1 mg/kg attenuated oro-lingual AIMs amplitude by 38%, 27% and 27%. N=13. Data are expressed as median with semi-interquartile interval. *: $P < 0.05$; **: $P < 0.01$, ***: $P < 0.001$. AIMs, abnormal involuntary movements; ALO, Axial, limbs and oro-lingual; L-DOPA, L-3,4-dihydroxyphenylalanine.

Fig. 4



Right forepaw rears across treatment conditions. 6-OHDA-lesioned rats used the right (unlesioned) forepaw in 81% of rears. Administration L-DOPA (3/15 mg/kg) decreased the number of rears with the unlesioned forepaw by 49%. This decrease in rears with the unlesioned forepaw was maintained when granisetron 0.001, 0.01, 0.1 or 1 mg/kg was combined with L-DOPA (reductions of 50%, 44%, 45% and 43%, respectively, when compared to the L-DOPA-untreated parkinsonian state). *N*=13. Data are graphed as mean ± SEM. *: $P < 0.05$; **: $P < 0.01$.

Supplementary Table 1 Evaluation of axial, limbs and oro-lingual abnormal involuntary movements (AIMs) duration in the 6-OHDA lesioned rat model (Cenci and Lundblad, 2007)

parameter	score
axial	0: no dyskinesia 1: occasional signs of dyskinesia, present less than 50% of the observation period 2: frequent signs of dyskinesia, present more than 50% of the observation period 3: dyskinesia present during the entire observation period, but suppressed by external stimuli 4: continuous dyskinesia not suppressed by external stimuli
limbs	0: no dyskinesia 1: occasional signs of dyskinesia, present less than 50% of the observation period 2: frequent signs of dyskinesia, present more than 50% of the observation period 3: dyskinesia present during the entire observation period, but suppressed by external stimuli 4: continuous dyskinesia not suppressed by external stimuli
oro-lingual	0: no dyskinesia 1: occasional signs of dyskinesia, present less than 50% of the observation period 2: frequent signs of dyskinesia, present more than 50% of the observation period 3: dyskinesia present during the entire observation period, but suppressed by external stimuli 4: continuous dyskinesia not suppressed by external stimuli

Supplementary Table 2 Evaluation of axial, limbs and oro-lingual abnormal involuntary movements (AIMs) amplitude in the 6-OHDA lesioned rat model (Cenci and Lundblad, 2007)

parameter	score
axial	0: no dyskinesia 1: sustained deviation of the head and neck at approximately 30º angle 2: sustained deviation of the head and neck at less than or equal to 60º angle 3: sustained twisting of the head, neck and upper trunk, angle greater than 60º but less than or equal to 90º 4: sustained twisting of the head, neck and trunk, angle greater than 90º, causing the rat to lose balance from a bipedal position
limbs	0: no dyskinesia 1: tiny movements of the paw around a fixed position 2: movements leading to a visible displacement of the whole limb 3: large displacement of the whole limb with visible contraction of shoulder muscles 4: vigorous limb displacement of maximal amplitude, with concomitant contraction of shoulder and extensor muscles
oro-lingual	0: no dyskinesia 1: twitching of facial muscles accompanied by small masticatory movements without jaw opening 2: twitching of facial muscles accompanied by masticatory movements which occasionally result in jaw opening 3: movements with broad involvement of facial muscles and masticatory muscles, with frequent jaw opening and occasional tongue protrusion 4: involvement of all of the above muscles to the maximal possible degree

Supplementary Table 3 Time course of ALO AIMs duration in 6-OHDA-lesioned rats

		L-DOPA/vehicle vs								
		time (min)								
	granisetron (mg/kg)	20	40	60	80	100	120	140	160	180
L-DOPA	0.0001	ns	*	*	**	**	ns	***	ns	*
	0.001	ns	ns	***	***	**	*	*	*	*
	0.01	ns	***	***	ns	ns	ns	***	*	ns
	0.1	ns	ns	ns	**	ns	ns	ns	ns	***
	1	ns	*	ns	**	ns	ns	ns	ns	**

*: $P < 0.05$ vs L-DOPA/vehicle; **: $P < 0.01$ vs L-DOPA/vehicle; ***: $P < 0.001$ vs L-DOPA/vehicle; ns: not significant

Supplementary Table 4 Time course of ALO AIMs amplitude in 6-OHDA-lesioned rats

		L-DOPA/vehicle vs time (min)								
	granisetron (mg/kg)	20	40	60	80	100	120	140	160	180
L-DOPA	0.0001	ns	ns	ns	*	ns	ns	**	*	**
	0.001	ns	ns	*	**	ns	*	**	**	**
	0.01	ns	*	***	*	ns	ns	**	***	**
	0.1	ns	*	*	ns	*	ns	*	*	***
	1	ns	ns	**	*	ns	ns	ns	*	***

*: $P < 0.05$ vs L-DOPA/vehicle; **: $P < 0.01$ vs L-DOPA/vehicle; ***: $P <$ 0f.001 vs L-DOPA/vehicle; ns: not significant

Transition 2: Assessing the anti-psychotic and anti-dyskinetic efficacy of ondansetron in the gold standard model of PD

In the hemi-parkinsonian rat model of PD, we have previously demonstrated that selective blockade of the 5-HT_3 receptor with the antagonists ondansetron [870] and granisetron (Chapter 3) diminished the severity of dyskinesia. Building on these findings, we sought to examine the anti-dyskinetic efficacy of ondansetron in the MPTP-lesioned marmoset, which exhibits high predictive validity in assessing the therapeutic potential of compounds on parkinsonism, dyskinesia, and psychosis [941, 1015].

A few open-label trials in PD patients found that ondansetron treatment significantly improved psychosis symptoms, particularly visual hallucinations without worsening motor function [1016-1018]. However, these trials did not address the use of concomitant medication and the results of a single placebo-controlled trial assessing the anti-psychotic efficacy of ondansetron have not been disclosed [1019]. Here, we used a randomised controlled paradigm to evaluate the effect of ondansetron on the severity of dyskinesia, psychosis-like behaviours, and parkinsonian disability in the parkinsonian marmoset. We also determined the pharmacokinetic profile of ondansetron in the marmoset to assess the clinical relevance of administered doses.

In Chapter 4, we found that selective blockade of the 5-HT_3 receptor with ondansetron in the MPTP-marmoset alleviated the severity of dyskinesia and psychosis-like behaviours, an effect that was accompanied by significantly reducing the duration of on-time with disabling dyskinesia and psychosis-like behaviours. Moreover, the therapeutic action of ondansetron even potentiated the anti-parkinsonian effect of L-DOPA. Ondansetron demonstrated a safe profile with therapeutic plasma levels similar to well tolerated levels obtained in clinical studies. These

favourable results are timely as there is an ongoing double-blind, randomised controlled Phase II trial that is assessing whether ondansetron treatment (8-24 mg dose range) alleviates PD psychosis (EudraCT T: 2019-003962-41).

Chapter 4 - Selective blockade of the 5-HT_3 receptor acutely alleviates dyskinesia and psychosis in the parkinsonian marmoset

Chapter 4: Selective blockade of the 5-HT_3 receptor acutely alleviates dyskinesia and psychosis in the parkinsonian marmoset

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Short title: Ondansetron alleviates dyskinesia and psychosis in marmoset

Chapter 4 Abstract

In Parkinson's disease (PD), management of L-3,4-dihydroxyphenylalanine (L-DOPA)-related complications, such as L-DOPA induced dyskinesia and psychosis, remains inadequate, which poses a significant burden on the quality of life of patients. We have shown, in the hemi-parkinsonian rat model of PD, that the selective serotonin type 3 (5-HT_3) receptor antagonists ondansetron and granisetron decreased the severity of established dyskinesia, and ondansetron even attenuated the development of dyskinesia. Here, we seek to confirm these favourable data on dyskinesia and to explore the effect of ondansetron on the severity of psychosis-like behaviours (PLBs) in the gold standard model of PD, the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned non-human primate. We first determined the pharmacokinetic profile of ondansetron in the marmoset. Subsequently, six MPTP-lesioned marmosets were administered L-DOPA chronically until they exhibited stable and reproducible dyskinesia and PLBs upon each administration of L-DOPA. On behavioural assessment days, ondansetron (0.01, 0.1 and 1 mg/kg) or vehicle was administered in conjunction with L-DOPA, and the severity of dyskinesia, PLBs and parkinsonism was evaluated. Ondansetron 0.1 mg/kg alleviated global dyskinesia severity by 73% ($P < 0.0001$) and decreased duration of on-time with disabling dyskinesia by 88% ($P = 0.0491$). Ondansetron 0.1 mg/kg reduced the severity of global PLBs by 80% ($P < 0.0001$) and suppressed on-time with disabling PLBs ($P = 0.0213$). Ondansetron enhanced the anti-parkinsonian action of L-DOPA, reducing global parkinsonism by 53% compared to L-DOPA ($P = 0.0004$). These results suggest that selective blockade of the 5-HT_3 receptor with ondansetron may be an effective approach to alleviate L-DOPA-related complications.

Keywords: 5-HT_3 receptor, Ondansetron, Parkinson's disease, Dyskinesia, Psychosis, MPTP-Lesioned marmoset

Chapter 4 Introduction

With a global burden of 6.1 million patients in 2016 (Dorsey et al., 2018a), Parkinson's disease (PD) is the second most common neurodegenerative disease and, amongst the neurological disorders examined, it was the fastest growing in deaths, disability and prevalence (Feigin et al., 2017). Although L-3,4-dihydroxyphenylalanine (L-DOPA) confers benefit for motor symptoms, long-term use is marred by complications including L-DOPA induced dyskinesia and psychosis, which can affect nearly 95% and 75% of patients (Hely et al., 2005, Hely et al., 2008). Management of these complications with currently available drugs remains inadequate. Thus, amantadine treatment for dyskinesia may be limited by side effects and development of tolerance (Thomas et al., 2004), while treatment of psychosis with clozapine raises concerns about long-term safety and monitoring (Zahodne and Fernandez, 2010, Seppi et al., 2019) and pimavanserin appears to only be mildly effective (Cummings et al., 2014, The Lancet, 2018).

Recent studies in the 6-hydroxydopamine (6-OHDA) lesioned rat found that administration of the selective serotonin (5-HT) type 3 (5-HT_3) antagonist ondansetron started concurrently with the initial dose of L-DOPA attenuated the dyskinesia induction process (Aboulghasemi et al., 2018, Kwan et al., 2020d). In the same animal model, ondansetron and another selective 5-HT_3 antagonist, granisetron, both alleviated established dyskinesia by a similar magnitude, ≈ 25% (Kwan et al., 2020d, Kwan et al., 2021a). These results suggest that selectively antagonising 5-HT_3 receptors might represent a novel anti-dyskinetic strategy. Here, following determination of its pharmacokinetic (PK) profile, we sought to confirm the anti-dyskinetic effect of 5-HT_3 receptor

blockade in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned marmoset, a non-human primate with high predictive validity in assessing clinical efficacy for dyskinesia, psychosis and parkinsonism (Veyres et al., 2018, Beaudry and Huot, 2020b). We also explored the effect of ondansetron on psychosis-like behaviours (PLBs) and parkinsonism.

Chapter 4 Materials and Methods

Animals

Twelve common marmosets (*Callithrix jacchus*, N = 6 of each sex) weighing 300-450 g and aged 4-6 years old at the time of the experiments were pair housed under conditions of controlled temperature (24 ± 1°C), humidity (50 ± 5%) and a 12 h light/dark cycle (07:15 a.m. lights on). Animals had *ad libitum* access to water and were provided with food (Mazuri® Marmoset Jelly, boiled eggs, nuts, yoghourt, pasta and fresh fruits) twice daily. Their home cages were enriched with perches and primate toys. Animals were acclimatised to handling, transfer to observational cages for behavioural recordings and sub-cutaneous (s.c.) injections prior to the start of studies. All procedures were approved by the McGill University and the Montreal Neurological Institute Animal Care Committees, in accordance with guidelines established by the Canadian Council on Animal Care.

Pharmacokinetic study

Six marmosets (N = 3 of each sex) were used and, employing a sparse sampling technique, a minimal volume of blood was collected from animals, as we have previously done (Gaudette et al., 2017, Gaudette et al., 2018). Based on a PK study we conducted in the rat (Kwan et al., 2020b),

ondansetron 0.01 mg/kg was administered s.c. at the following time points: baseline, 2 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h and 8 h. Ondansetron 0.1 mg/kg was administered s.c. with three samples collected at 5 min, 30 min and 1 h. Samples were gently inverted and centrifuged to generate plasma aliquots and stored at -80°C until analysis. The analytical method to determine plasma levels of ondansetron using high-performance liquid chromatography and tandem mass spectrometry (HPLC-MS/MS) was developed in rat (Gaudette et al., 2019) and cross validated in marmoset.

Plasma PK parameters were determined from the mean concentration value at each time point by a non-compartmental analysis method using PKSolver (Rowland M and TN., 1995, Zhang et al., 2010). Area under the curve (AUC) was calculated using the linear trapezoidal rule. AUC_{0-t}, $AUC_{0-\infty}$, maximal plasma concentration (C_{max}), time to C_{max} (T_{max}), terminal half-life ($T_{1/2}$), clearance (CL), bioavailability (F), volume of distribution (V_z) and mean residence time (MRT) were all calculated.

Induction of parkinsonism, dyskinesia and psychosis-like behaviours

Six marmosets (N = 3 of each sex) were rendered parkinsonian by daily injections of MPTP hydrochloride (2 mg/kg, s.c., MilliporeSigma, Canada) over 5 days (Hamadjida et al., 2017, Hamadjida et al., 2018c). Following a month recovery period to allow parkinsonian symptoms to stabilise, animals were administered L-DOPA/benserazide (15/3.75 mg/kg, orally, MilliporeSigma) once daily for a minimum of 30 days until the expression of dyskinesia and PLBs was stable and reproducible.

Experimental design

On days of behavioural assessment, marmosets were administered ondansetron (0.01, 0.1, 1 mg/kg free base or vehicle, s.c., MilliporeSigma) in combination with L-DOPA. Ondansetron hydrochloride was dissolved in dimethyl sulfoxide at 100 mg/ml and then diluted to appropriate concentrations in 0.9% NaCl. Drug administration was randomised according to a within-subjects design. Following administration of treatment, each marmoset was placed in individual observation cages (36 × 33 × 22 in) that contained water, food and a wooden perch, and left undisturbed for the 6 h observation period. Treatments were separated by at least 72 h, allowing for complete drug clearance. Behaviours were recorded via webcam for *post hoc* analysis.

Behavioural analysis

The severity of dyskinesia, PLBs and parkinsonism was rated according to previously validated scales (Fox et al., 2010, Kwan et al., 2019, Sid-Otmane et al., 2020). Dyskinesia consisted of chorea and dystonia, which were both rated on a scale from 0 to 4, where 0 = absent, 1 = mild, present less than 70% of the observation period and animal is able to eat and perform normal activity, 2 = moderate, 3 = marked and 4 = severe, present more than 70% of the observation period and animal is unable to perform normal activity. PLBs consisted of hyperkinesia, response to non-apparent stimuli (tracking and staring), repetitive grooming and stereotypies. PLBs were rated on a scale from 0 to 4, where 0 = absent, 1 = mild, present less than 30% of the observation period and animal is able to eat and perform normal activity, 2 = moderate, 3 = marked and 4 = severe, present more than 30% of the observation period and replacing normal activity. Parkinsonian disability comprised range of movement, bradykinesia, posture and attention/alertness. Range of movement was rated on a scale from 0 to 9, where 0 = running, jumping and use of limbs for different activities, whereas 9 = no movement. Bradykinesia was

rated from 0 to 3, where 0 = normal initiation and speed of movement, whereas 3 = prolonged freezing, akinesia and immobile. Postural abnormality was rated 0 or 1, where 0 = normal balance with upright body posture and head is held up, whereas 1 = impaired balance, prone body posture with head down. Attention/alertness was rated 0 or 1, where 0 = normal head checking and movement of neck is smooth in different directions and in small movements, whereas 1 = less or no head checking and head is in one position for more than 50% of the time. Dyskinesia, PLBs and parkinsonian disability were simultaneously scored *post hoc* by a trained evaluator blinded to treatment conditions. Over a 6 h observation period, behaviour was assessed for 5 min every 10 min and respective dyskinesia, PLBs and parkinsonian disability scores were summed for each hour over the entire observation period and during the peak period (90-150 min following L-DOPA administration). The duration of anti-parkinsonian benefit, hereafter referred to as "on-time", corresponded to the number of minutes for which bradykinesia was absent, whereas on-time with disabling dyskinesia and/or PLBs was defined as the number of minutes where dyskinesia and/or PLBs was either marked or severe (scores of 3 or 4).

Statistical Analysis

Dyskinesia, PLBs and parkinsonism scores are presented as the median and were analysed using Friedman test followed by Dunn's *post hoc* test to compare between the four treatment conditions. Time courses of dyskinesia, PLBs and parkinsonism are presented as the median and were analysed by computing the AUC, after which Welch's one-way analysis of variance (ANOVA) followed by Dunnett's T3 *post hoc* test was performed (Dunnett, 1980). On-time data and AUCs are presented as the mean ± standard error of the mean (SEM) and were analysed using one-way repeated measures (RM) ANOVA with Greenhouse-Geisser correction (Greenhouse and

Geisser, 1959) followed by Tukey's *post hoc* test. Statistical significance was assigned when $P < 0.05$ and statistical analyses were performed with GraphPad Prism 8.4.3 (GraphPad Software Inc., USA).

Chapter 4 Results

Pharmacokinetic profile

Plasma PK parameters of ondansetron in the marmoset following s.c. administration are presented in Table 1. Briefly, ondansetron 0.01 mg/kg led to a C_{max} of 1.96 ng/mL that was detected at 30 min, while ondansetron 0.1 mg/kg led to a C_{max} of 18.9 ng/mL. The $T_{1/2}$ following administration of ondansetron 0.01 mg/kg was 49 min.

5-HT_3 blockade alleviates L-DOPA induced dyskinesia

As shown in Fig. 1A, ondansetron significantly reduced the severity of dyskinesia. By computing the AUC of the 6 h dyskinesia time course, we found that ondansetron significantly altered global dyskinesia severity ($F_{(3, 11.08)} = 28.25$, $P < 0.0001$, Welch's one-way ANOVA, Fig. 1B). Ondansetron 0.01, 0.1 and 1 mg/kg decreased the severity of dyskinesia when compared to L-DOPA alone, by 68%, 73% and 71% ($P = 0.0002$, $P < 0.0001$ and $P < 0.0001$, Dunnett's T3 *post hoc* test). In line with these results, ondansetron had a significant effect on peak dyskinesia severity (Friedman Statistic [FS] = 11.40, $P = 0.0040$, Fig. 1C). Ondansetron 0.1 and 1 mg/kg diminished peak dyskinesia severity when compared to L-DOPA, by 64% and 59% ($P = 0.0219$ and $P = 0.0219$, Dunn's *post hoc* test), whereas ondansetron 0.01 mg/kg non-significantly

diminished peak dose dyskinesia severity, by 55% (P = 0.1521, Dunn's *post hoc* test). We also found that ondansetron produced a significant effect on the duration of on-time during which dyskinesia was disabling ($F_{(1.267,6.336)}$ = 12.53, P = 0.0002; one-way RM ANOVA with Greenhouse-Geisser correction, Fig. 1D). Thus, after treatment with L-DOPA alone, duration of on-time with disabling dyskinesia was 42 min, while after administration of L-DOPA in combination with ondansetron 0.01, 0.1 and 1 mg/kg, it was 2 min (96% reduction), 5 min (88%) and 8 min (80% reduction), respectively (P = 0.0519, P = 0.0491, P = 0.0321, Tukey's *post hoc* test).

5-HT$_3$ blockade alleviates psychosis-like behaviours

As presented in Fig. 2A, ondansetron significantly reduced the severity of PLBs. By computing the AUC of the 6 h PLBs course, we found that ondansetron significantly altered global PLBs severity ($F_{(3, 11.04)}$ = 38.37, $P < 0.0001$, Welch's one-way ANOVA, Fig. 2B). Ondansetron 0.01, 0.1 and 1 mg/kg decreased PLBs intensity when compared to L-DOPA alone, by 71%, 80% and 79% (each $P < 0.0001$, Dunnett's T3 *post hoc* test). In line with these results, ondansetron had a significant effect on peak PLBs severity (FS = 13.40, P = 0.0006, Fig. 2C). Ondansetron 0.01, 0.1 and 1 mg/kg diminished peak PLBs severity when compared to L-DOPA, by 59%, 76% and 72% (P = 0.4418, P = 0.0048, P = 0.0219, Dunn's *post hoc* test). Ondansetron also produced a significant effect on the duration of on-time during which PLBs was disabling ($F_{(1.067, 5.337)}$ = 20.53, P = 0.0050; one-way RM ANOVA with Greenhouse-Geisser correction, Fig. 2D). Thus, after treatment with L-DOPA alone, duration of on-time with disabling PLBs was 78 min, while after administration of L-DOPA in combination with ondansetron 0.01 mg/kg, it was 3 min (96%

reduction, P = 0.0223), whereas it was suppressed with ondansetron 0.1 and 1 mg/kg (both P = 0.0213, Tukey's *post hoc* test).

5-HT$_3$ blockade enhances L-DOPA anti-parkinsonian benefit

As illustrated in Fig. 3A, adding ondansetron to L-DOPA resulted in a significant additional anti-parkinsonian effect. By computing the AUC of the 6 h parkinsonian disability time course, we found that ondansetron significantly altered global parkinsonism severity ($F_{(3, 10.99)}$ = 15.49, P = 0.0003, Welch's one-way ANOVA, Fig. 3B). Ondansetron 0.01, 0.1 and 1 mg/kg decreased parkinsonism severity when compared to L-DOPA, by 49%, 46% and 53% (P = 0.0008, P = 0.0048 and P = 0.0004, Dunnett's T3 *post hoc* test). This reduction of parkinsonism was not accompanied by any significant change in the duration of L-DOPA anti-parkinsonian action ($F_{(1.757, 8.786)}$ = 2.634, P = 0.1303; one-way RM ANOVA with Greenhouse-Geisser correction, Fig. 3C).

Chapter 4 Discussion

In the present study, we demonstrated that the selective 5-HT$_3$ antagonist ondansetron significantly alleviated both the severity of dyskinesia and PLBs acutely in the MPTP-lesioned marmoset. Ondansetron was well tolerated by animals and sedation was not observed. In addition, ondansetron enhanced L-DOPA anti-parkinsonian action. Our results suggest that selective blockade of the 5-HT$_3$ receptor may be an effective therapeutic approach to treat L-DOPA related complications and amenable to the testing of clinically-available 5-HT$_3$ antagonists in Phase II clinical trials.

Acute administration of ondansetron 0.01 mg/kg in marmosets produced a C_{max} of 1.96 ng/mL, which is comparable to the C_{max} of 2.99 ng/mL obtained with the same dose in rats (Kwan et al., 2020b). The C_{max} associated with the higher dose of 0.1 mg/kg, 18.9 ng/mL, is close to the lower range of C_{max} values reported in clinical studies (26.4 to 42.0 ng/mL) where healthy volunteers received a single 8 mg oral dose of ondansetron (Colthup et al., 1991, Baber et al., 1992). Although we did not determine the PK of ondansetron 1 mg/kg in the marmoset, based on data from the other two doses, we may infer that the C_{max} would approximate the higher range reported in clinical trials, similar to the C_{max} of 94.6-194.4 ng/mL obtained following a single 24 mg oral dose of ondansetron in healthy subjects (Novartis Pharmaceuticals Corporation, 1999, VanDenBerg et al., 2000). Thus, characterisation of ondansetron PK parameters in marmosets suggests that doses of ondansetron associated with a therapeutic effect would be safe and well tolerated by patients.

Here, in the MPTP-lesioned marmoset, the addition of ondansetron to L-DOPA led to a ≈ 73% reduction in global dyskinesia severity, whereas in the same animal model, the clinically efficacious anti-dyskinetic agent amantadine led to a ≈ 45% to ≈ 63% reduction (Hill et al., 2004, Kobylecki et al., 2011). However, it is unclear whether these doses would be well tolerated in the clinic, as the PK profile of amantadine has not been described in the marmoset. In PD patients, significant reduction of dyskinesia was achieved with amantadine plasma concentrations of ≈ 1,500 ng/mL (Pahwa et al., 2015), which corresponds to an effective plasma concentrations of ≈ 1,400 ng/mL in macaque (Brigham et al., 2018). In the MPTP-lesioned macaque, clinically relevant plasma levels of amantadine are achieved with doses of 10 to 20 mg/kg (Brigham et al., 2018) and generally lead to a reduction of dyskinesia severity by ≈ 23% to ≈ 29% (Grégoire et al., 2013, Ko et al., 2014), although one study found an absence of anti-dyskinetic efficacy (Bezard et

al., 2013). From our results, we may therefore infer that ondansetron compares favourably to a clinically efficacious treatment of dyskinesia (Fox et al., 2018).

In the current experiments, ondansetron 0.01-1 mg/kg conferred similar levels of anti-dyskinetic efficacy in the MPTP-lesioned marmoset, suggesting that maximal anti-dyskinetic efficacy is achieved with ondansetron 0.01 mg/kg and plasma concentrations of ≈ 2 ng/mL. To the best of our knowledge, the effects of selectively antagonising 5-HT_3 receptors on dyskinesia and PLBs in PD have only been assessed in studies employing behavioural methodology. The paucity of reports on the possible mechanism(s) of action(s) that govern 5-HT_3 antagonists' effects limits our understanding of these drugs. Nevertheless, there is support in the literature to speculate on the benefit of 5-HT_3 blockade in L-DOPA induced dyskinesia. A predominant hypothesis suggests that the dysregulated release of striatal dopamine triggers dyskinesia (Iravani et al., 2006, Carta et al., 2007), as evidenced by the large fluctuations in dopamine levels in dyskinetic PD patients compared to those who do not exhibit dyskinesia (de la Fuente-Fernandez et al., 2004). In rat striatal slices, application of 5-HT_3 agonists enhanced dopamine release (Zazpe et al., 1994), an effect that was blocked by 5-HT_3 antagonists (Blandina et al., 1989), which is in line with findings in *in vivo* microdialysis studies that reported increased dopamine levels following administration of 5-HT (Benloucif et al., 1993) or of the 5-HT_3 agonist phenylbiguanide (Santiago et al., 1995). Furthermore, at the behavioural level, 5-HT_3 antagonists diminished dopaminergic transmission-mediated motor behaviours such as amphetamine-induced hyperactivity (Costall et al., 1987), rotations (Bachy et al., 1993) and stereotypies (Shankar et al., 2000). Collectively, these studies suggest that 5-HT_3 receptors modulate striatal dopamine release and we may infer that ondansetron exerted its effect by regulating the erratic dopamine release that occurs in the dyskinetic state, but further investigation is warranted.

Modulation of the 5-HT system has been implicated in PD psychosis (Chang and Fox, 2016). An autoradiographic binding study using *post-mortem* tissue showed increased 5-HT_{2A} receptor binding in the infero-lateral temporal cortex in PD patients with visual hallucinations (Huot et al., 2010), which suggests that alterations in 5-HT transmission in visual processing pathways may be involved in PD hallucinations (Chang and Fox, 2016). Autoradiographic binding studies have detected moderate levels of 5-HT_3 receptor binding in the human limbic system (Waeber et al., 1989), while moderate densities of 5-HT_3 receptors in mesolimbic structures also receive input from the ventral tegmental area (Kilpatrick et al., 1996), which lend support to the role of the 5-HT_3 receptor in regulating mesolimbic dopaminergic activity. Ondansetron and other 5-HT_3 antagonists have been shown to attenuate dopamine-induced motor hyperactivity and reduce adverse behaviour associated with a hyperactive dopaminergic state in rodent and non-human primate models (Costall et al., 1987, Hagan et al., 1990). Moreover, ondansetron reduced neuropeptide-induced hyperactivity in the rat, an effect that was accompanied by a decrease in dopamine metabolism in the nucleus accumbens (Hagan et al., 1987). Thus, 5-HT_3 receptor antagonists such as ondansetron may modulate the facilitatory role of 5-HT on dopaminergic transmission, which would dampen dopaminergic hyperactivity and alleviate psychotic symptoms.

In line with these findings, three open-label clinical trials conducted by the same group with PD patients found that ondansetron (mean 17- 20 mg/day) reduced the severity of psychosis by ≈ 19-24% (Zoldan et al., 1993, Zoldan et al., 1995, Friedberg et al., 1998). Ondansetron was particularly effective against visual hallucinations, paranoid delusions and confusion, and was well tolerated by patients, without worsening motor symptoms. In addition, an improvement in visual hallucinations in PD patients was achieved with daily doses of ondansetron of 4-8 mg/day (Kazunori et al., 1999), while another trial reported a lack of anti-psychotic efficacy and tolerance

with daily doses that ranged from 8 to 24 mg/day (Eichhorn et al., 1996). Based on the doses administered in these trials, ondansetron plasma levels associated with anti-psychotic efficacy may be within the range of 26.4-42.0 ng/mL, according to the published, albeit partial, PK profile of ondansetron in human (Colthup et al., 1991, Baber et al., 1992, Novartis Pharmaceuticals Corporation, 1999). Importantly, an issue that these studies did not address was the concomitant use of medication (*e.g.* L-DOPA, dopamine agonists and amantadine), which may have confounded results given their potential role in psychosis (Carey et al., 1995, Diederich et al., 2009, Ecker et al., 2009). In the present study, we addressed this limitation by assessing the effect of ondansetron (0.01. 0.1 and 1 mg/kg) in a randomised controlled paradigm in the parkinsonian primate and found a similar reduction in PLBs severity, without compromising L-DOPA therapeutic action. To the best of our knowledge, the outcome of the single randomised-controlled trial assessing the anti-psychotic efficacy of ondansetron in PD patients (Melamed et al., 1999) has not been disclosed. Nevertheless, these favourable data provide support for testing the anti-psychotic efficacy of ondansetron in PD patients in larger randomised-controlled studies. The expense of ondansetron was previously regarded as cost prohibitive (Eichhorn et al., 1996) and deterred such studies, but the availability of generic forms of ondansetron has quelled price-related issues (Kwan and Huot, 2019).

In the current study, we found that all doses of ondansetron potentiated the anti-parkinsonian action conferred by L-DOPA to a similar extent, with a ≈ 50% improvement, without extending the duration of on-time. In contrast to these results, in the 6-OHDA-lesioned rat, ondansetron did not alter L-DOPA anti-parkinsonian action (Aboulghasemi et al., 2018, Kwan et al., 2020d, Kwan et al., 2021a), an effect that was also reported in clinical trials in PD patients that had found ondansetron conferred anti-psychotic efficacy, without worsening motor symptoms

(Zoldan et al., 1993, Zoldan et al., 1995, Friedberg et al., 1998). The lack of antagonistic action of ondansetron at dopamine D_2 receptors (van Wijngaarden et al., 1990a) may explain why the anti-dyskinetic and anti-psychotic action of ondansetron is not accompanied by a deterioration in motor function. Understanding of the additional L-DOPA anti-parkinsonian benefit encountered with ondansetron administration could be improved upon with studies that would determine whether it is mediated through changes in metabolite concentrations, neuronal activity or a yet to be explored mechanism of action.

In summary, we demonstrated the acute anti-dyskinetic and anti-psychotic effects of the 5-HT_3 antagonist ondansetron in the MPTP-lesioned marmoset. Subsequent studies are required to determine whether these effects are maintained in a chronic administration paradigm. Ondansetron conferred these benefits while also enhancing the anti-parkinsonian action of L-DOPA. The doses of ondansetron administered were well tolerated by animals and reached plasma levels comparable to those encountered in clinical practice. Potent and selective 5-HT_3 antagonists with diverse pharmacological profiles are clinically available and these promising data support their transition to testing in Phase II clinical trials for PD-related endpoints.

Authors roles

1) Research project: A. Conception, B. Organisation, C. Execution;

2) Manuscript: A. Writing of the first draft, B. Review and Critique.

Kwan: 1 B, 1C, 2 A, 2 B; Nuara: 1 B, 1C, 2 B; Bédard: 1C, 2 B; Gaudette: 1C, 2 B; Gourdon: 1 A, 1 B, 2 B; Beaudry: 2 B; Huot: 1 A,1 B,1C,2A,2 B.

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Chapter 4 Tables and Figures

Table 1

Derived PK parameters in the plasma following s.c. administration of ondansetron in the marmoset

parameters	0.01 mg/kg	0.1 mg/kg
	mean (± SD)	mean (± SD)
C_{max} (ng•mL^{-1})	1.96 (± 0.47)	18.9 (± 2.05)
T_{max} (min)	30 (± 8.66)	
$T_{1/2}$ (min)	49 (± 7.96)	
AUC_{0-t} (ng•h•mL^{-1})	3.25 (± 0.19)	
$AUC_{0-\infty}$ (ng•h•mL^{-1})	3.27 (± 0.18)	
CL/F (L•h^{-1}• kg^{-1})	3.06 (± 0.17)	
V_z/F (L•h^{-1}• kg^{-1})	3.58 (± 0.70)	
MRT (h)	1.39 (± 0.08)	

AUC: area under the curve; CL: clearance; C_{max}: maximal plasma concentration; F: bioavailability; MRT: mean residence time; $T_{1/2}$: terminal half-life; T_{max}: time to maximal plasma concentration; V_z: volume of distribution.

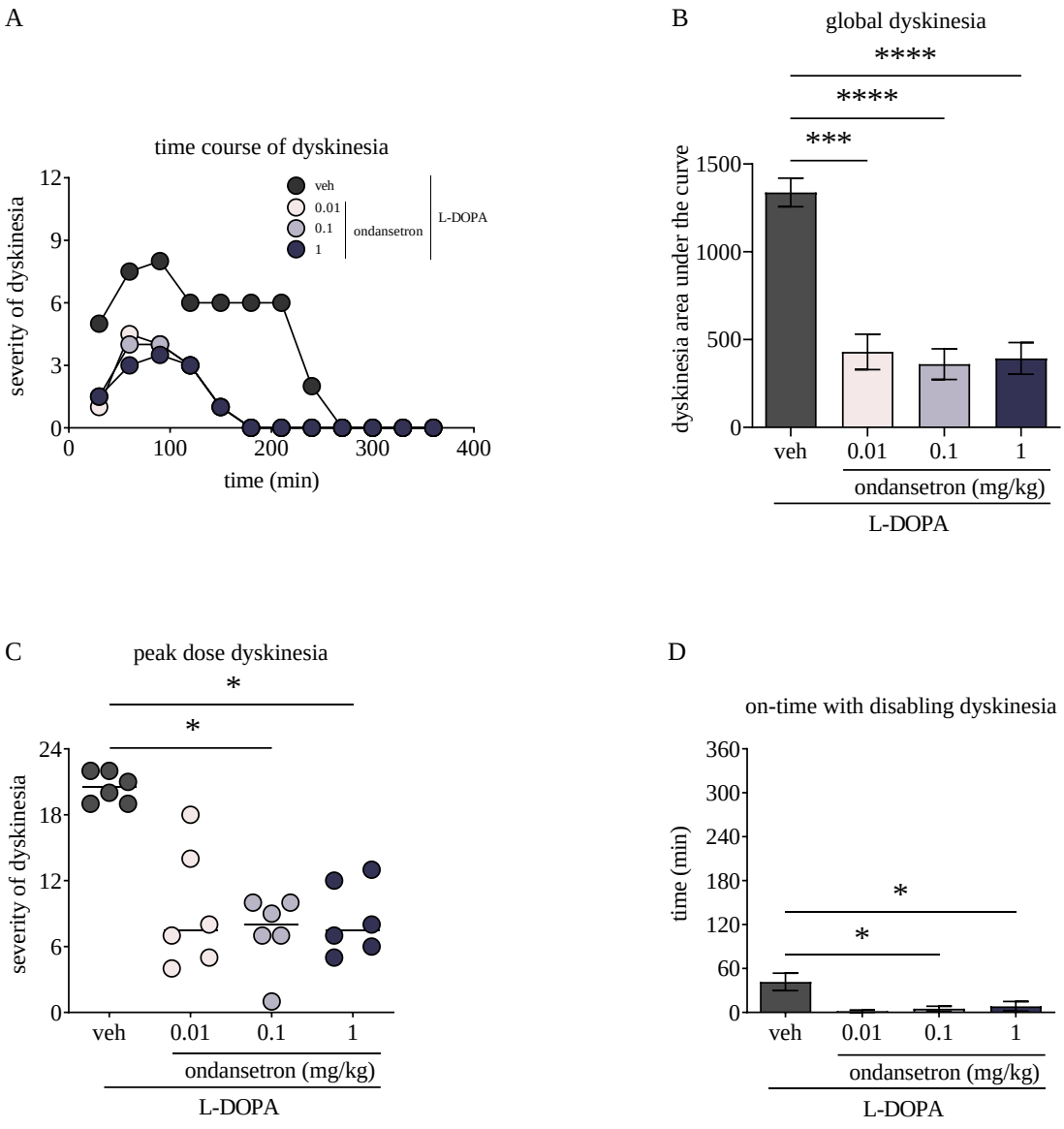

Fig. 1. Ondansetron alleviates L-DOPA induced dyskinesia.

In MPTP-lesioned marmosets, administration of ondansetron (0.01, 0.1 and 1 mg/kg) in combination with L-DOPA significantly alleviated global dyskinesia severity as evidenced by the time course of dyskinesia (**A**) and AUC of dyskinesia time course, by 68%, 73% and 71%, respectively, compared to L-DOPA treatment (**B**). Ondansetron 0.1 and 1 mg/kg also decreased peak dose dyskinesia severity, by 64% and 59%, compared to L-DOPA/vehicle (**C**). The anti-

dyskinetic effect of ondansetron (0.01, 0.1 and 1 mg/kg) was accompanied by a decrease in the duration of on-time with disabling dyskinesia, by 96%, 88% and 80%, compared to LDOPA/vehicle (**D**). Data are expressed as the median (**A**), the mean ± SEM (**B**), median with individual values (**C**) and the mean ± SEM (**D**). *: $P < 0.05$, ****: $P < 0.0001$.

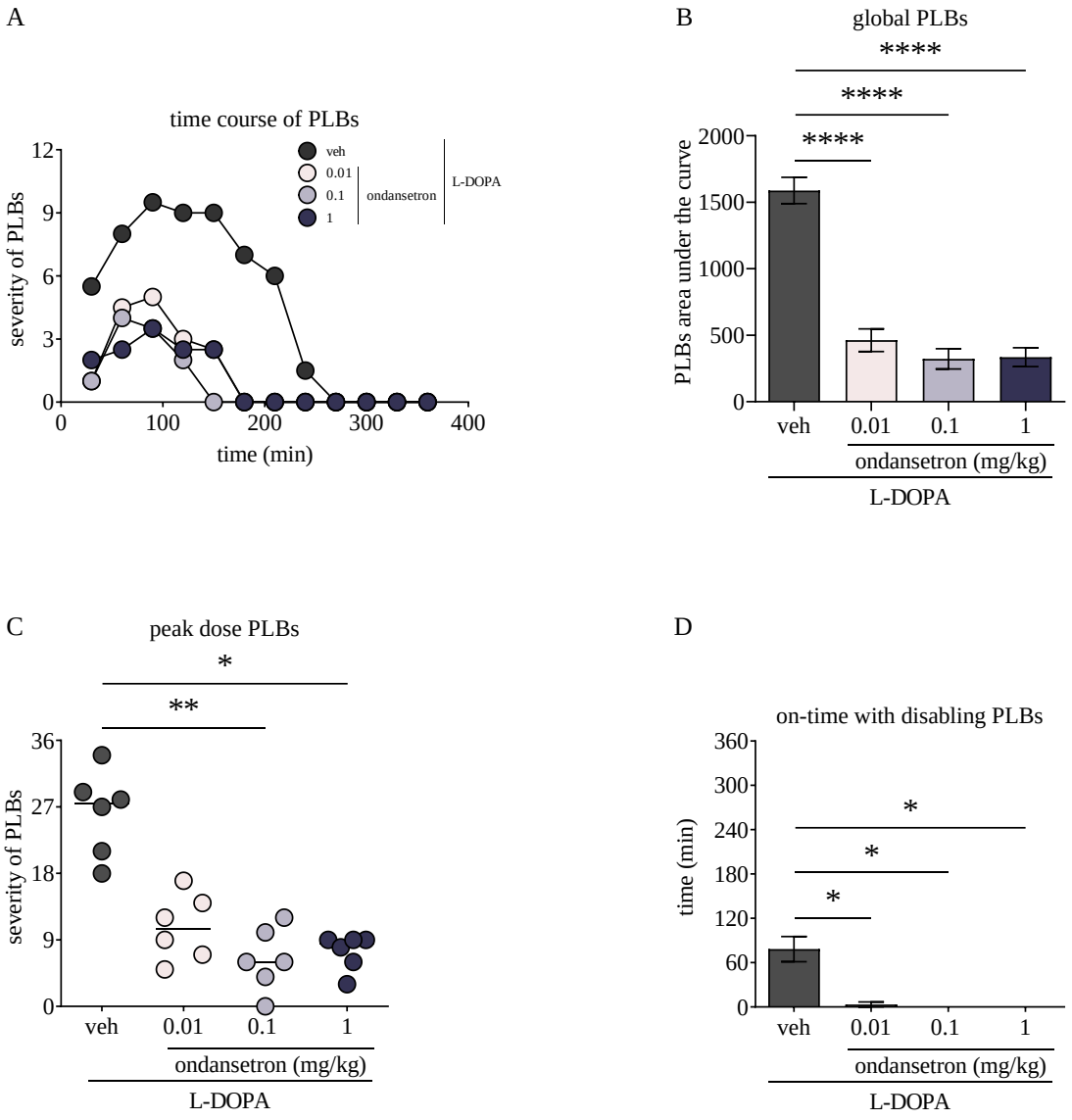

Fig. 2. Ondansetron alleviates psychosis-like behaviours.

In MPTP-lesioned marmosets, administration of ondansetron (0.01, 0.1 and 1 mg/kg) in combination with L-DOPA significantly alleviated global PLBs severity as evidenced by the time course of PLBs (**A**) and AUC of PLBs time course, by 71%, 80% and 79%, respectively, compared to L-DOPA treatment (**B**). Ondansetron 0.1 and 1 mg/kg also decreased peak dose PLBs severity, by 76% and 72%, compared to L-DOPA/vehicle (**C**). The anti-dyskinetic effect of ondansetron

0.01 mg/kg was accompanied by a decrease in the duration of on-time with disabling PLBs, by 96%, whereas it was completely suppressed with doses of 0.1 and 1 mg/kg, compared to L-DOPA/vehicle (**D**). Data are expressed as the median (**A**), the mean ± SEM (**B**), median with individual values (**C**) and the mean ± SEM (**D**). *: $P < 0.05$, **: $P < 0.01$, ****: $P < 0.0001$.

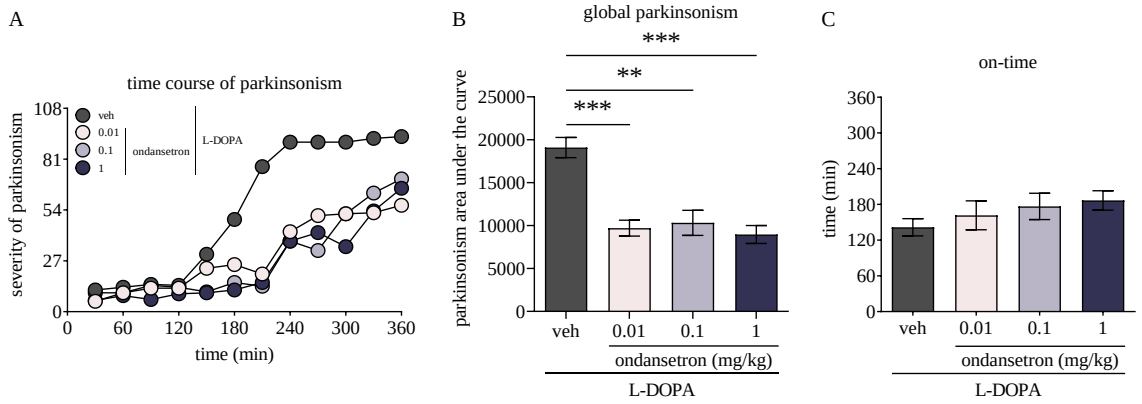

Fig. 3. Ondansetron enhances the anti-parkinsonian benefit of L-DOPA. In MPTP-lesioned marmosets, administration of ondansetron (0.01, 0.1 and 1 mg/kg) in combination with L-DOPA significantly alleviated global parkinsonism severity as displayed by the time course of parkinsonism (**A**) and the AUC of parkinsonism time course, by 49%, 46% and 53%, respectively, compared to L-DOPA/vehicle (**B**). This reduction of parkinsonism was not accompanied by any significant alteration to duration of on-time (**C**). Data are expressed as the median (**A**), the mean ± SEM (**B**) and the mean ± SEM (**C**). **: $P < 0.01$, ***: $P < 0.001$.

Transition 3: Characterising novel behaviours in the parkinsonian marmoset

PD psychosis is one of the most frequent and debilitating nonmotor symptoms that afflicts as many as 60% of PD subjects [718, 753] and is associated with increased nursing home placement [1020] and mortality risk [715]. The MPTP-lesioned marmoset model of PD exhibits high positive predictive value in assessing the clinical efficacy of drug candidates to treat PD psychosis [941, 1015]. In this model, psychosis-like behaviours span four categories including hallucinations, stereotypies, hyperkinesia, and grooming, which are idiosyncratic but are reproducible with each administration of L-DOPA [977]. Here, we sought to expand on the existing repertoire of psychosis-like behaviours in the MPTP-lesioned marmoset, to enrich the testing paradigm for antipsychotic candidates in PD (Chapter 5).

In the MPTP-lesioned marmoset, we found stereotypical behaviours that were not previously described. For instance, whereas initial findings limited circling behaviours to the cage floor, we report and provide visual support that circling behaviours occurred in different environments (e.g., floor, wall, perch, and ceiling) that were specific to each animal. The time course and severity of these novel behaviours is similar to other psychosis-like behaviours evaluated using the original rating scale, whereas the profile of psychosis-like behaviours was variable depending on the animal. We proposed that these stereotypical behaviours should be amended to the original scale. We also found that each animal had its own distinctive repeated patterns of behaviours. Moreover, we discovered that the stereotypical behaviours described in the MPTP-lesioned marmoset are reminiscent of punding in PD patients, which are stereotyped, purposeless and repetitive behaviours, and are often derived from occupations and hobbies [1021]. Although the aetiology of punding is unclear, there is evidence to support an association with

dopaminergic neurotransmission and the transition from voluntary control to habitual routines [1022, 1023]. Thus, further characterisation of psychosis-like behaviours in the MPTP-lesioned marmoset may improve our understanding of the mechanisms underlying punding and PD psychosis, and in turn, the clinical development of new therapeutic targets for these conditions.

Chapter 5 - Further characterisation of psychosis-like behaviours induced by L-DOPA in the MPTP-lesioned marmoset

Chapter 5: Further characterisation of psychosis-like behaviours induced by L-DOPA in the MPTP-lesioned marmoset

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Short title: Novel psychosis behaviours in marmoset

Chapter 5 Abstract

Parkinson's disease (PD) psychosis afflicts over half of patients and poses a significant burden on quality of life. The aetiology of PD psychosis is multifactorial and likely arises from the complex interaction between dopamine replacement therapy and disease state. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned common marmoset is a validated model to predict the efficacy of therapeutic compounds for treatment-related complications, including PD psychosis. In this model, psychosis-like behaviours (PLBs) encompass stereotypies that are idiosyncratic in nature and reproducible with each L-3,4-dihydroxyphenylanaline (L-DOPA) administration. In the present study, we sought to expand upon the existing repertoire of PLBs through the characterisation of novel stereotypical behaviours that appear dependent on the environment. We then discuss our findings in the context of clinical reports on stereotypical behaviours termed "punding" in subjects with PD, which consists of stereotypical repetitive and senseless behaviours. The poor understanding of the pathophysiology governing punding and consequent lack of effective therapies stand to benefit from enhanced characterisation of these stereotypical behaviours in a validated pre-clinical model. We hope that further characterisation of PLBs in the MPTP-lesioned marmoset will be helpful in the evaluation of interventions that seek to alleviate PD psychosis symptoms.

Keywords Psychosis · Parkinson's disease · MPTP-lesioned marmoset · Punding · L-DOPA

Chapter 5 Introduction

Parkinson's disease (PD) is a neurodegenerative disease that afflicts approximately 6 million individuals worldwide and the global burden of PD is expected to double in the next few decades (Dorsey et al., 2018a). The main treatment, L-3,4-dihydroxyphenylanaline (L-DOPA), provides relief for motor symptoms during early stages of disease. However, with disease progression and long-term therapy, its utility is marred by complications such as L-DOPA-induced dyskinesia and psychosis (Hely et al., 2005, Hely et al., 2008). In addition to the dopaminergic system, there is evidence that serotonergic, acetylcholinergic and glutamatergic systems are implicated in PD psychosis (Powell et al., 2020). Psychotic symptoms are a frequent occurrence in PD, with over 50% of subjects eventually affected (Fénelon et al., 2010), and management is limited (Seppi et al., 2019), which underscores that PD psychosis is a clinical unmet need. PD psychosis predominantly present as hallucinations, with visual hallucinations being the most common (Fénelon and Alves, 2010), but also encompasses delusions, illusions and false sense of presence (Fernandez et al., 2008, Zahodne and Fernandez, 2010).

Upon administration of dopaminergic agents, the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned marmoset exhibits behaviours that are reminiscent of PD psychosis (Fox et al., 2006, Visanji et al., 2006, Fox et al., 2010) and can reliably predict the anti-psychotic efficacy of compounds in the clinic (Veyres et al., 2018). Psychosis-like behaviours (PLBs), the equivalent of PD psychosis in the MPTP-lesioned marmoset, span four general categories including response to non-apparent stimuli (visual hallucinations and tracking), stereotypies, hyperkinesia and repetitive grooming (Fox et al., 2010). In particular, stereotypical behaviours (e.g. repetitive side-to side jumping, fiddling with the cage and running in circles) are

idiosyncratic and reproducible with each administration of L-DOPA (Fox et al., 2010). These features may be reminiscent of punding behaviours described in PD patients, which is defined as complex stereotypical behaviours that consist of excessive, repetitive and non-goal-oriented behaviours and may occur following L-DOPA treatment (Evans et al., 2004). The animal specific stereotypies encountered in MPTP-lesioned marmosets are therefore reminiscent of the heterogeneous manifestations of psychotic symptoms in PD patients.

Here, we sought to expand on the existing behavioural repertoire of PLBs in the MPTP-lesioned marmoset, to help with the evaluation of future potential anti-psychotic agents in the treatment of PD psychosis.

Chapter 5 Methods

Animals

Six common marmosets (*Callithrix jacchus*, *N*=3 of each sex), weighing 300-450 g were pair housed under conditions of controlled temperature (24 ± 1°C), humidity (50 ± 5%) and a 12 h light/dark cycle (lights on at 07:15 a.m.). Animals had unrestricted access to water and were fed twice daily (Mazuri® Marmoset Jelly, fresh fruits, boiled eggs, pasta, nuts, etc.) and were provided with enrichment in home cages (perches and primate toys). Marmosets were acclimatised to handling, transfer to observational cages for behavioural recordings and sub-cutaneous (s.c.) injections prior to the start of studies. All procedures were approved by the McGill University and the Montreal Neurological Institute Animal Care and Use Committees, in accordance with guidelines established by the Canadian Council on Animal Care.

Induction of parkinsonism and psychosis-like behaviours

Animals were rendered parkinsonian by administration of MPTP hydrochloride (2 mg/kg, s.c., MilliporeSigma, Canada) once daily or every other day for a total of 3 to 5 doses, tailored to the animals' reaction (Hamadjida et al., 2017, Hamadjida et al., 2018b, Frouni et al., 2019, Kwan et al., 2019). Following recovery, animals were administered L-DOPA/benserazide (hereafter L-DOPA, 15/3.75 mg/kg, orally, MilliporeSigma) once daily for a minimum of 30 days until the severity of PLBs was stable and reproducible.

Behavioural analysis

On experimental days, L-DOPA (15/3.75 mg/kg, s.c., MilliporeSigma) was administered to animals. The severity of PLBs and parkinsonism was rated according to previously published scales (Fox et al., 2010, Huot et al., 2011, Hamadjida et al., 2017, Hamadjida et al., 2018a, Frouni et al., 2019, Kwan et al., 2019). The PLB rating scale is detailed in Table 1. Briefly, PLBs consisted of hyperkinesia, response to non-apparent stimuli (tracking and staring), repetitive grooming and stereotypies. PLBs were rated on a scale from 0 to 4, where 0 = absent, 1 = mild, present less than 30% of the observation period and animal is able to eat and perform normal activity, 2 = moderate, 3 = marked and 4 = severe, present more than 30% of the observation period and replacing normal activity. The parkinsonian disability rating scale is presented in Table 2. Parkinsonian disability comprised of range of movement, bradykinesia, posture and attention/alertness. Range of movement was rated on a scale from 0 to 9, where 0 = running, jumping and use of limbs for different activities, 6 = on the wall of cage, perch or ceiling, whereas 9 = no movement. Bradykinesia was rated from 0 to 3, where 0 = normal initiation and speed of movement, whereas

3 = prolonged freezing, akinesia and immobile. Postural abnormality was rated 0 or 1, where 0 = normal balance with upright body posture and head is held up, whereas 1 = impaired balance, prone body posture with head down. Attention/alertness was rated 0 or 1, where 0 = normal head checking and movement of neck is smooth in different directions and in small movements, whereas 1 = less or no head checking and head is in one position for more than 50% of the time. Over a 6-h observation period, PLBs and parkinsonian disability were simultaneously assessed post hoc for 5 min every 10 min. PLBs and parkinsonian disability scores were summed for each half hour over the entire observation period.

Statistical Analysis

This article expands the repertoire of previously described behaviours that occur following the administration of L-DOPA to marmosets. The analysis was performed in a qualitative fashion.

Chapter 5 Results

In MPTP-lesioned marmosets, following administration of L-DOPA, animals exhibited PLBs over a 6-h observation period (Fig. 1). The PLBs displayed by animals encompassed hyperkinesia, hallucinatory-like behaviours, scratching and stereotypies, with the majority of the PLB severity score attributed to hallucinatory-like behaviours and stereotypies (Fig. 2A). Moreover, the profile of PLBs varied between the six animals (Fig. 2B). From the analysis of the PLBs, we also observed that animals displayed stereotypical behaviours that had not previously been described (Table 1). Thus, whereas circling behaviour was initially reported, it was incorporated in the scale only when

it occurred on the cage floor. Here, we report, along with visual support, that the circling behaviour is not restricted to the cage floor. Different marmosets circled in environments that were specific to each animal, e.g. on the floor for animal 1, on the cage wall for animal 2, on the perch for animal 3, and on the ceiling for animal 4 (see Video, Online Resource 1, which demonstrates circling behaviours in different environments). Moreover, circling behaviours were expanded to encompass 360° rotations around a fixed point or object. Consistent with other PLBs, there was considerable variation in the amplitude of these stereotypies that ranged from mild to disabling and the severity was scored according to the original scale. Stereotypies displayed by animals were idiosyncratic in nature, such as repetitive and distinct patterns of movements (see Video, Online Resource 2, which demonstrates unique patterns of repetitive behaviour).

The time course of parkinsonism in MPTP-lesioned marmosets is presented in Fig. 3. In terms of range of movement that is evaluated under parkinsonism severity, we observed that some animals hung from the ceiling without any locomotion, which could not be scored using the traditional rating scale. We propose that this behaviour is reminiscent of hanging on the cage wall or perch without movement. As shown in Table 2, we have amended the scale for range of movement to include hanging from the ceiling, wall or perch as equivalent behaviours.

Chapter 5 Discussion

In the present study, we presented novel PLBs, namely stereotypies, in the MPTP-lesioned marmoset, to expand the behavioural repertoire encountered in the model and hopefully increase the translational potential of studies conducted in this small primate. These additions to the existing

scale appear dependent on the environment and may be analogous to punding behaviours presented by PD patients, which reinforces the importance of further characterising pre-clinical models.

Amongst the spectrum of psychotic symptoms in PD, visual hallucinations predominate in the clinic, afflicting as many as 60% of patients (Fénelon et al., 2010). Consistent with these findings, we found that in MPTP-lesioned marmosets administered L-DOPA, the most common PLBs were response to non-apparent stimuli, the marmoset equivalent to visual hallucinations (Fox et al., 2010), followed by stereotypies. As iterated by the authors of the original PLB rating scale, stereotypies are idiosyncratic and can be reproduced with administration of L-DOPA (Fox et al., 2006, Visanji et al., 2006, Fox et al., 2010). Here, stereotypies represented the second most severe subtype amongst the four PLBs subtypes, which further supports the importance of accurately detecting and quantifying these behaviours. Importantly, although animals may also exhibit chorea and/or dystonia, stereotypies are clearly distinct from them. Indeed, dystonia consists of abnormal posture, while chorea consists of random and non-stereotypical movements (Pearce et al., 1995, Henry et al., 1999). The novel behaviours described here, i.e. circling in 360° rotations on the ceiling, cage wall and wooden perch, as well as sequential patterns of behaviour, are also specific to each animal and occur with each L-DOPA administration. Based on the description of the original scale, we believe that these behaviours correspond to stereotypies. We propose that they should be rated according to the previously published scale, where 0 corresponds to the absence of such behaviours and 4 is the most disabling and occurs > 30% of the assessment period.

In addition to the wide phenotypical repertoire of these stereotypical behaviours, an intriguing feature of these is that they appear to manifest differently depending on the environment. The circling behaviour, for example, is not limited to the cage floor but for some animals, may occur on the cage wall, perch or ceiling. Of note, such an environmental-dependence of behaviours

induced by L-DOPA was previously reported in the 6-hydroxydopamine-lesioned rat (Lane et al., 2011) but, to the best of our knowledge, not in the MPTP-lesioned non-human primate.

We previously suggested that these repetitive, exaggerated and driven gross motor behaviours, may represent behavioural correlates of neuropsychiatric symptoms in PD (Fox et al., 2010). Indeed, the complex stereotypies exhibited by these animals are reminiscent of punding, which may occur in PD patients (Fasano and Petrovic, 2010) and subjects with psychostimulant addiction (Rylander, 1972, Brady et al., 1991, Fasano et al., 2008). Punding is described as a stereotyped behaviour characterised by senseless and repetitive activity that often arises from prepotent idiosyncratic habits and hobbies (Evans et al., 2004). The similarities between stereotypical behaviours in the MPTP-lesioned marmoset and clinical features enhances the value of the model and would benefit from pharmacological validation, although there is currently no efficacious treatment to address punding in PD (Seppi et al., 2019).

First described in PD patients in 1994 (Friedman, 1994), the prevalence of punding behaviours ranges from 1.4 to 14% of PD population (Evans et al., 2004, Miyasaki et al., 2007), but due to its poor characterisation, punding is likely underreported in clinical practice (Lawrence et al., 2007). Punding in the PD population correlates with poorer disease-related quality of life (Lawrence et al., 2007) and devastating psychosocial consequences (Voon, 2004). Evidence supports a strong association between dopaminergic transmission and punding, particularly with high doses of L-DOPA (Evans et al., 2004), as well as a few reports with dopamine agonists (Miyasaki et al., 2007, Fasano and Petrovic, 2010, Vargas et al., 2019), and symptoms improve after reduction or cessation of L-DOPA or dopamine agonists (Fernandez and Friedman, 1999, Miwa and Kondo, 2008, Fasano and Evans, 2013). Although some studies found cases of punding in PD patients with exceedingly high doses of L-DOPA (1,350 to 7,500 mg) (Fernandez and

Friedman, 1999, Serrano-Dueñas, 2002, Kurlan, 2004, Kumar, 2005, Kummer et al., 2007), there were also cases of punding in PD patients associated with relatively lower doses of L-DOPA (300 to 500 mg) (Fernandez and Friedman, 1999, Miwa et al., 2004, Miwa and Kondo, 2005, Fasano et al., 2006, Miwa and Kondo, 2008, Wingo et al., 2009, Kulisevsky and Pagonabarraga, 2010, Spencer et al., 2011). It is noteworthy that the doses of L-DOPA discussed represent the total daily dose, not the dose administered during a single intake, which rarely exceeds 250-300 mg. In addition, another study did not report a significant difference in the Levodopa Equivalent Daily Dose (LEDD) between PD patients that exhibit punding and those who do not, average of 464 mg and 431 mg, respectively (Pettorruso et al., 2016). Moreover, similar behaviours are exhibited in users of cocaine and amphetamine (Rylander, 1972), both of which enhance dopaminergic transmission (dela Peña et al., 2015). In contrast to these findings, an isolated case report found that two PD patients exhibited punding behaviours following an improvement in psychosis with the anti-psychotic quetiapine, which adds complexity and could suggest an interaction between serotonin and stereotypies (Miwa et al., 2004). However, as only a subset of PD patients develop punding, it is possible that it arises from a complex interaction between pharmacological and non-pharmacological clinical features (Fasano et al., 2011).

The pathophysiology of punding remains unclear but high co-morbidity rates with L-DOPA-induced dyskinesia (Miwa et al., 2004, Silveira-Moriyama et al., 2006), behavioural addictions and psychosis lend support to a shared neural substrate. Thus, both punding and dyskinesia require chronic drug administration to induce behavioural sensitisation, such that once established, a single dose of drug can trigger the behaviour (Fasano et al., 2011). Moreover, punding behaviours share phenotypical similarities with stereotypical behaviours in animals after administration of L-DOPA or amphetamine (Robbins et al., 1990). However, while dyskinesia

(Cotzias et al., 1967), impulse control disorders (Garcia-Ruiz et al., 2014), behavioural addictions (Rusyniak, 2011) and punding (Fasano et al., 2011) are all associated with dopaminergic medication, future studies are required to discern the exact contribution of dopaminergic medication to these conditions.

The predominant hypothesis for the pathophysiology underlying punding in PD is based on plastic changes in the dorsal and ventral striatum (Ikemoto and Panksepp, 1999), whereby dopaminergic projections to the dorsal striatum are progressively lost while those to the ventral striatum are relatively spared (Kish et al., 1988). It has been proposed that dopamine replacement therapy results in the overstimulation of the dorsal striatum and facilitates the transition from goal-oriented actions to automated behaviour with loss of voluntary control (Beaulieu-Boire and Lang, 2015). This is consistent with reports that individuals cannot control automatic response (Evans et al., 2004) and the dissociation between knowledge and behaviour (Toates, 1998). Furthermore, variation in individual susceptibility to develop punding may be linked to frontal cortex projections that inhibit dopamine-induced stereotypical behaviours (Ridley, 1994). Dysfunction of this process may underlie conditions that involve abnormal expression of stereotypical behaviours (Mink, 1996), as evidenced by lesions to the frontal cortex (Volle et al., 2002) and basal ganglia (Laplane et al., 1984, Laplane et al., 1989) sometimes leading to stereotypical behaviours reminiscent of punding. However, this theory is difficult to reconcile with relatively sparing of cognitive function in PD patients who exhibit punding behaviours (Fasano et al., 2011), which underscores the need to study cognitive profile in these patients.

The MPTP-lesioned marmoset model has previously demonstrated its value in evaluating the therapeutic potential of L-DOPA related side effects such as impulse control disorders and dopamine dysregulation syndrome (Johnston et al., 2011). Here, we have expanded the PLB

repertoire in the MPTP-lesioned marmoset by describing novel behaviours that are idiosyncratic and dependent on the environment. Characterisation of these behaviours will be beneficial in identifying mechanisms to target pharmacologically PD psychosis. Lastly, in light of similarities with punding behaviours in PD patients, moving towards more comprehensive understanding of PLBs may aid drug development of therapies for punding.

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Author contribution CK and PH conceived and designed research. CK, SGN and PH conducted experiments. CK analysed data. CK wrote the manuscript. SGN, JCG and PH revised the manuscript. All authors read and approved the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Data availability Not applicable.

Declarations

Ethics approval Experiments were approved by McGill University and the Montreal Neurological Institute-Hospital (The Neuro) Animal Care Committees, which are in accordance with the regulations defined by the Canadian Council on Animal Care.

Consent to participate Not applicable.

Consent to publish All authors have read and approved the manuscript for publication.

Competing interests The authors declare no competing interests.

Chapter 5 References

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Chapter 5 Tables and Figures

Table 1 Proposed new psychosis-like behaviour rating scale in the MPTP-lesioned common marmoset

Parameter	Score
Hyperkinesia	Locomotor activity: running, jumping or climbing, that is faster than normal and/ or inability of animal to remain in one position for > 5 seconds without exhibiting locomotion 0: activity absent 1: present for < 30% of assessment time and not disabling (animal can walk, run, and eat) 2: present for > 30% of assessment time and not disabling 3: present for < 30% and disabling (interferes with walking, running, eating – takes over normal activity) 4: present for > 30% and disabling
Hallucinatory-like response to apparent non-stimuli	Tracking: head movements following non-apparent stimuli (> 10 seconds/min) and/ or Staring: head still, looking in one direction at non-apparent stimulus for extended period (> 10 seconds/min) 0: activity absent. 1: present for < 30% of assessment time and not disabling (animal can walk, run, and eat). 2: present for > 30% of assessment time and not disabling. 3: present for < 30% and disabling (interferes with walking, running, eating – takes over normal activity). 4: present for > 30% and disabling
Obsessive grooming	Grooming or scratching repetitively (> 5 times/min) 0: activity absent. 1: present for < 30% of assessment time and not disabling (animal can walk, run, and eat). 2: present for > 30% of assessment time and not disabling. 3: present for < 30% and disabling (interferes with walking, running, eating – takes over normal activity). 4: present for > 30% and disabling

Stereotypies	a) Side-to-side repetitive whole body jumping movements on floor of cage (> 2 times/ min) b) Head checking movements that are repetitive, quick, side-to-side, exaggerated large amplitude, often with associated body movements (> 3 times/ min) c) Circling behaviour – whole body turning in circles (**or 360° rotations)** on floor, **cage wall, perch or ceiling** (>2 times/ min) d) Fiddling with and/ or repetitively grasping at cage bars with forearms (> 2 times/ min) 0: activity absent 1: present for < 30% of assessment time and not disabling (animal can walk, run, and eat). 2: present for > 30% of assessment time and not disabling. 3: present for < 30% and disabling (interferes with walking, running, eating – takes over normal activity). 4: present for > 30% and disabling

For each of the behavioural parameters tested, the score assigned is the most representative of psychosis-like behaviour over a 5-min period. The psychosis-like behaviour score attributed was the most disabling of any of the four behavioural parameters observed during the 5-min period (Fox et al., 2010). Adapted from S. H. Fox, N. Visanji, G. Reyes, P. Huot, J. Gomez-Ramirez, T. Johnston and J. M. Brotchie. Neuropsychiatric behaviors in the MPTP marmoset model of Parkinson's disease. *Can J Neurol Sci*, 2010, 37(1): 86-95. Changes to the original scale are emboldened.

Table 2 Proposed new parkinsonian disability rating scale in the MPTP-lesioned common marmoset

Parameter	Score
Range of movement	0: running, jumping between roof, walls, perch, using limbs through a wide range of activity 1: climbing up and down the walls of the cage or along perch 2: climbing onto wall of cage or perch 3: hopping on floor of cage 4: walking around floor 5: on **ceiling**, wall of cage or perch, movement of limbs, but no locomotion 6: on **ceiling**, wall of cage or perch, movement of head or trunk 7: on the floor of the cage, movement of limb, but no locomotion 8: on the floor of the cage, movement of head 9: no movement
Bradykinesia	0: normal initiation and speed of movement 1: slight slowing of movement 2: moderate slowing of movement, marked freezing, difficulty initiating and maintaining movement 3: prolonged freezing, akinesia, inability to move
Postural abnormality	0: normal balance, upright posture, head held up 1: impaired balance, crouched posture, head down
Attention/alertness	0: normal head checking movements, movement of neck in variable directions, smooth, small movements 1: reduced or absent head checking, head in one position for more than 50% of observation period

For each behavioural parameter tested, the score attributed is the most representative over a 5-min period. A global parkinsonism score is calculated according to the following formula: [(bradykinesia × 3) + (posture × 9) + (range of movement × 1) + (alertness × 9)]. The maximal disability score for any given observation period is 36, while normal animals score under 6 (Huot et al., 2011). Reprinted from

Experimental Neurology. 188(1). M. A. Silverdale, S. L. Nicholson, P. Ravenscroft, A. R. Crossman, M. J. Millan and J. M. Brotchie. Selective blockade of D3 dopamine receptors enhances the anti-parkinsonian properties of ropinirole and levodopa in the MPTP-lesioned primate. 128-138. Copyright (2020) with permission from Elsevier. Changes to the original scale are emboldened.

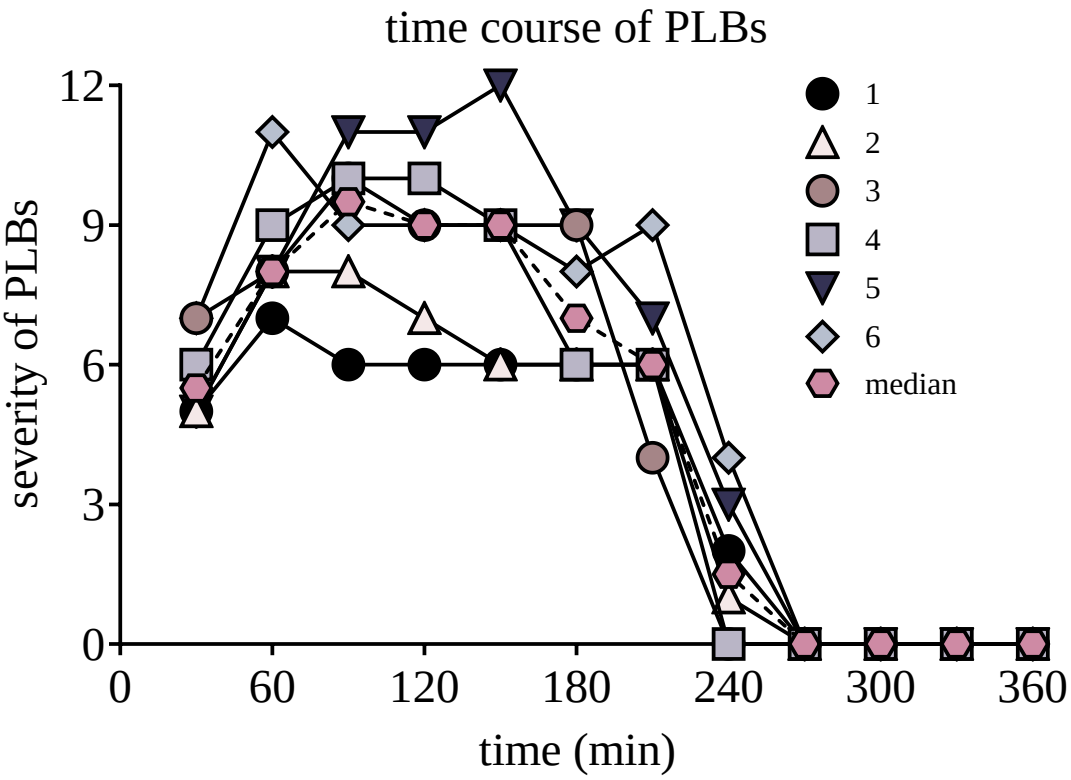

Fig. 1 Time course of PLBs in MPTP-lesioned marmosets. L-DOPA administration in MPTP-lesioned marmosets elicited PLBs. Median and individual time course of PLBs of the six animals over the 6-h observation are presented. Data are expressed as the median. The maximal PLB score at any time point is 12

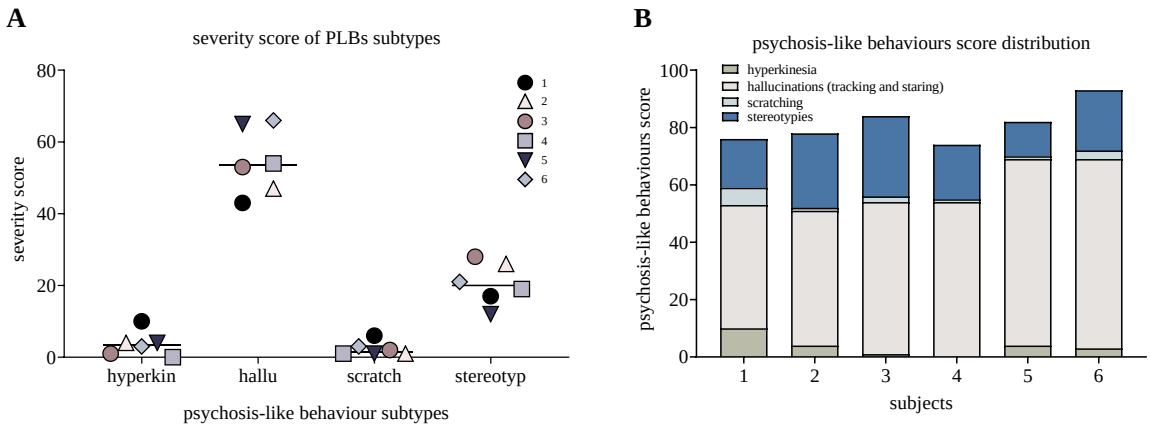

Fig. 2 Distribution of PLB scores in MPTP-lesioned marmosets. The severity score of PLB subtypes (hyperkinesia, hallucinatory-like behaviours, scratching and stereotypies) of the six MPTP-lesioned marmosets are presented in a. The distribution of PLB scores for each individual animal is presented in b. Data are expressed as the median. hyper, hyperkinesia; hallu, response to non-apparent stimuli (tracking and staring, i.e. visual hallucinations); scratch, scratching; stereoty, stereotypies

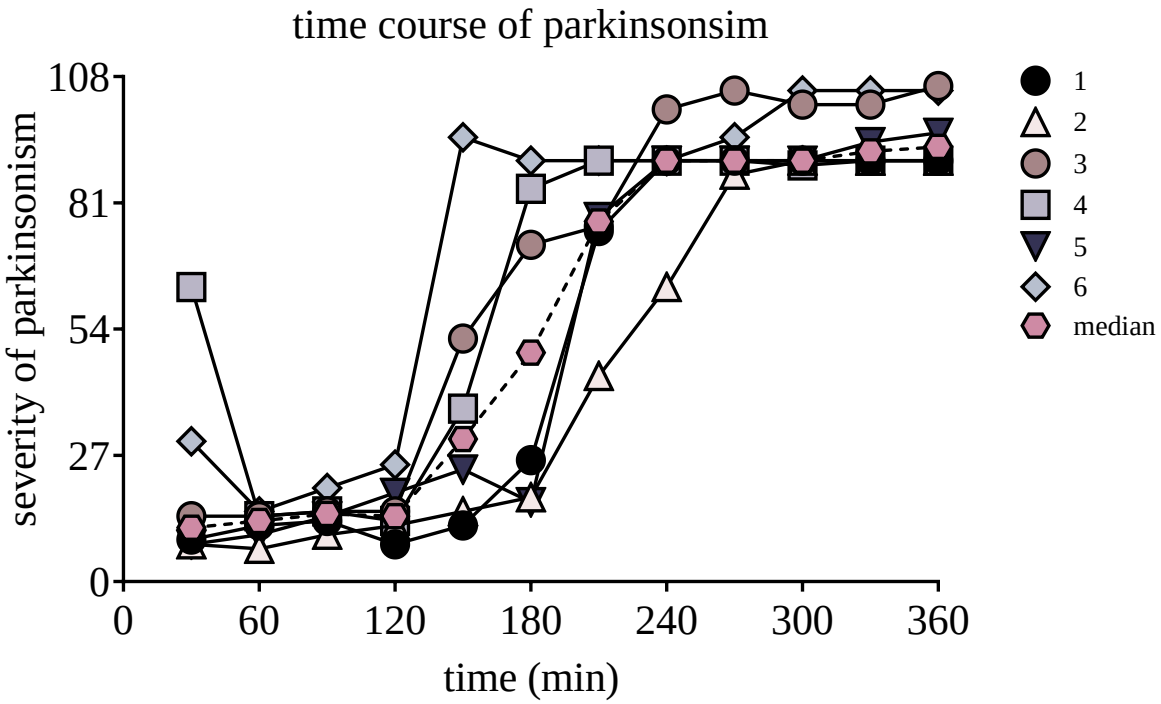

Fig. 3 Time course of parkinsonism in MPTP-lesioned marmosets. Median and individual time course of parkinsonism of six MPTP-lesioned marmosets over the 6-h observation is presented. All animals were administered L-DOPA. Data are expressed as the median. The maximal parkinsonian disability score at any time point is 108

Supplementary Video 1

https://doi.org/10.1007/s00210-021-02090-6

Supplementary Video 2

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Transition 4: Altered 5-HT_3 receptor levels underlie L-DOPA induced dyskinesia in the hemi-parkinsonian rat

Investigation of blockade of the 5-HT_3 receptor as an approach to alleviate dyskinesia in PD is a very recent development and, to date, studies have been limited to behavioural pharmacological experiments. In the hemi-parkinsonian rat, the 5-HT_3 receptor antagonists ondansetron [559, 870] and granisetron (Chapter 3) both alleviated the severity of established dyskinesia, and ondansetron even prevented the development of dyskinesia [870]. Building upon these findings, ondansetron treatment in the parkinsonian marmoset also reduced the severity of established dyskinesia (Chapter 4). Altogether, these behavioural studies suggest that the anti-dyskinetic efficacy of these selective antagonists may be attributed to blockade of the 5-HT_3 receptor. However, insight into possible mechanism(s) of action that govern the anti-dyskinetic efficacy of 5-HT_3 antagonists is lacking, which limits the translational potential of these well tolerated and clinically available compounds. Thus, a better understanding of brain areas where 5-HT_3 receptor levels are altered may shed light onto the possible mechanism of action governing these drugs.

Using autoradiographic binding, we examined the relationship between L-DOPA induced dyskinesia and 5-HT_3 receptor levels in the hemi-parkinsonian rat by comparing [^{3}H]GR65630 binding levels between control and experimental subjects (Chapter 6). The distribution of 5-HT_3 receptors was examined in brain areas implicated in L-DOPA-induced dyskinesia, i.e., the motor loop of the basal ganglia, as well as the primary motor cortex and ventral anterior/ventral lateral (VA/VL) nuclei of the thalamus. We found a regionally selective upregulation of [^{3}H]GR65630 binding in 6-OHDA-lesioned rats that were either L-DOPA naïve or dyskinetic due to chronic L-DOPA administration. While this upregulation was predominantly observed in the bilateral

subthalamic nucleus, it was also observed, to a lesser extent, in the ipsilateral entopeduncular nucleus and VA/VL thalamus. [^{3}H]GR65630 binding remained unchanged in the other brain regions studied, including the primary motor cortex, striatum, and substantia nigra pars reticulata. Lastly, dyskinesia severity scores negatively correlated with binding levels in the ipsilateral striatum and contralateral subthalamic nucleus. Taken together, our findings suggest that the 5-HT_3 receptor may contribute to the pathophysiology of L-DOPA-induced dyskinesia and provide some insight into the brain regions worth further study. Further studies are warranted to unravel the role that 5-HT_3 receptors in the subthalamic nucleus, entopeduncular nucleus and VA/VL thalamus may play in the development or expression of L-DOPA induced dyskinesia.

Chapter 6 - Autoradiographic labelling of 5-HT_3 receptors in the hemi-parkinsonian rat brain

Chapter 6: Autoradiographic labelling of 5-HT_3 receptors in the hemi-parkinsonian rat brain

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Short title: 5-HT_3 binding in hemi-parkinsonian rat

Chapter 6 Abstract

L-3,4-dihydroxyphenylalanine (L-DOPA) is the mainstay treatment for Parkinson's disease, but its effectiveness during early disease is marred by the eventual development of L-DOPA induced dyskinesia. In hemi-parkinsonian rats, the serotonin type 3 (5-HT_3) antagonists ondansetron and granisetron alleviated dyskinesia induced by L-DOPA without impeding its anti-parkinsonian action; in parkinsonian marmosets, ondansetron alleviated dyskinesia and enhanced L-DOPA anti-parkinsonian action. Here, we sought to gain insight into the mechanisms governing the anti-dyskinetic action of 5-HT_3 antagonists and measured 5-HT_3 receptor levels across different brain, using [^{3}H]GR65630 autoradiographic binding. Brain sections were chosen from 6-hydroxydopamine (6-OHDA)-lesioned rats exhibiting abnormal involuntary movements (AIMs), as well as L-DOPA-naïve 6-OHDA and sham-lesioned animals. [^{3}H]GR65630 binding increased in the ipsilateral subthalamic nucleus of 6-OHDA-lesioned rats with mild and severe AIMs, (3-fold changes, $P < 0.001$). [^{3}H]GR65630 binding also increased in the ipsilateral entopeduncular nucleus and thalamus of 6-OHDA-lesioned rats with severe AIMs (75% and 88%, $P < 0.05$). AIMs scores negatively correlated with [^{3}H]GR65630 binding in the ipsilateral dorsolateral striatum and contralateral subthalamic nucleus ($P < 0.05$). These results suggest that alterations in 5-HT_3 mediated neurotransmission may contribute to the pathophysiology of L-DOPA induced dyskinesia.

Keywords:

5-HT_3 receptor

Parkinson's disease

Dyskinesia

Granisetron

[^{3}H]GR65630

Autoradiography

Chapter 6 Introduction

By 2040, Parkinson's disease (PD) is projected to affect over 17 million people worldwide (Dorsey et al., 2018b). Since its introduction (Cotzias et al., 1967), L-3,4-dihydroxyphenylalanine (L-DOPA) has remained the most effective symptomatic treatment for PD. However, with chronic L-DOPA treatment and disease progression (Fox and Lang, 2008), patients develop debilitating complications, such as L-DOPA-induced dyskinesia, which affects nearly 95 % of patients after 15 years of treatment (Hely et al., 2005). To date, the non-selective *N*-methyl-D-aspartate (NMDA) antagonist amantadine is the sole pharmacological agent approved by the United States Food and Drug Administration to alleviate dyskinesia, but its use is hampered by the development of hallucinations and loss of efficacy with repeated administration (Hauser et al., 2017, Pahwa and Hauser, 2017).

Preclinical studies have implicated the raphe-striatal serotonin (5-HT) system as a modulator of L-DOPA-induced dyskinesia (Carta and Tronci, 2014). Notably, anti-dyskinetic efficacy has been demonstrated both for 5-HT type 1A (5-HT_{1A}) receptor agonists and 5-HT type 2A (5-HT_{2A}) receptor antagonists in rodent and non-human primate models of PD (Bibbiani et al., 2001, Hamadjida et al., 2018d). However, in clinical trials, 5-HT_{1A} receptor agonists failed to alleviate dyskinesia and/or compromised L-DOPA therapeutic efficacy (Ludwig et al., 1986, Kannari et al., 2002b, Goetz et al., 2007, Goetz et al., 2008b). Furthermore, the efficacy of 5-HT_{2A} receptor antagonists in dyskinetic patients has been mixed and limited to small trials (Maertens de Noordhout and Delwaide, 1986, Meco et al., 1988) and requires further study in placebo-controlled trials.

Modulation of the 5-HT type 3 (5-HT_3) receptor provides another potential approach to alleviate L-DOPA induced dyskinesia. In particular, the 5-HT_3 receptor antagonists ondansetron and granisetron were observed to reduce the severity of dyskinesia in the 6-hydroxydopamine (6-OHDA) lesioned rat (Aboulghasemi et al., 2018, Kwan et al., 2020c, Kwan et al., 2021a). An anti-dyskinetic efficacy of ondansetron was also demonstrated in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned marmoset (Kwan et al., 2021b); the latter is a model with high positive predictive value in forecasting clinical efficacy for dyskinesia, psychosis and parkinsonism (Veyres et al., 2018, Beaudry and Huot, 2020a).

To date, the anti-dyskinetic effects of 5-HT_3 receptor blockade have been investigated only at the behavioural level; mechanistic studies are lacking. Thus, a better understanding of brain areas where 5-HT_3 receptor levels are altered may shed light onto the possible mechanism(s) of action governing these drugs. Autoradiographic binding studies in the intact rat have reported high levels of 5-HT_3 receptor binding in the brainstem and limbic areas (Kilpatrick et al., 1988), with lower levels in the striatum and thalamus (Kilpatrick et al., 1987). However, to the best of our knowledge, 5-HT_3 receptor expression has not been studied in L-DOPA-induced dyskinesia. Therefore, in the present study, we determined the distribution of the 5-HT_3 receptor in brain areas implicated in L-DOPA-induced dyskinesia, including the motor loop of the basal ganglia, by performing autoradiographic binding with the highly selective antagonist [^{3}H]GR65630 in 6-OHDA- and sham-lesioned rats. We also tested for correlations between the severity of L-DOPA induced dyskinesia and specific [^{3}H]GR65630 binding levels in different brain areas.

Chapter 6 Material and methods

Animals

Adult female Sprague-Dawley rats (225–250 g, Charles River, Saint-Constant, Canada) were used. Rats were housed in groups of three under controlled temperature (21± 1°C), humidity (55%), light (12-h light/dark cycle, lights on at 07:00) conditions, with unlimited access to food and water. Upon arrival, rats remained undisturbed to acclimatise to housing conditions for at least 5 days. All procedures were approved by the Montreal Neurological Institute-Hospital (The Neuro) Animal Care Committee, in accordance with the guidelines established by the Canadian Council on Animal Care.

Induction of hemi-parkinsonism

Rats were rendered hemi-parkinsonian by unilateral injection of 6-OHDA, as previously described (Kwan et al., 2020c). Briefly, animals were administered desipramine (10 mg/kg s.c., MilliporeSigma, Canada) and pargyline (5 mg/kg s.c., MilliporeSigma) and, once anaesthetised with isoflurane (2-4 %; MilliporeSigma) in 100 % oxygen (1 L/min), they were placed into a stereotaxic frame (David Kopf Instruments, USA). Thirty minutes later, they were injected with 2.5 µL of 6-OHDA hydrobromide (7 µg/µL, MilliporeSigma) in the right medial forebrain bundle at the following coordinates: antero-posterior: − 2.8 mm, medio-lateral: − 2.0 mm, dorso-ventral: − 9.0 mm) relative to Bregma, with the incisor bar set 3.3 mm below ear bars (Paxinos and Watson, 2007). Sham-lesioned animals were injected with 2.5 µL of 6-OHDA vehicle (0.9 % saline with 0.02 % ascorbate) using the same stereotaxic coordinates. Following the lesion, animals entered a 21-day recovery period.

Assessment of hemi-parkinsonism

After the recovery period, animals underwent the cylinder test to determine the extent of hemi-parkinsonism (Schallert et al., 2000, Frouni et al., 2018). Rats were placed in a transparent cylinder (14 cm diameter × 28 cm height) and recorded for 10 min, following which video recordings were viewed in order to count the number of wall touches that each rat made during a rearing movement, *i.e.* using the forelimb contralateral and ipsilateral to the lesion side, as well as simultaneous placement of both forelimbs. Only animals that displayed use of the forelimb ipsilateral to the lesion in ≥ 70 % of rears were selected for further study, a score that is indicative of approximately 90 % striatal dopamine tissue content depletion (Schallert et al., 2000).

Experimental design

Animals were randomly allocated into four groups. Group A (sham-vehicle, $N = 8$) animals were intracranially injected with 6-OHDA vehicle and then primed with a daily s.c. injection of L-DOPA/benserazide vehicle (0.9 % saline with 0.1 % ascorbate) for 14 days. The remaining rats were unilaterally injected with 6-OHDA and were allocated to groups B, C and D, as follows. Animals in group B (lesion-vehicle, $N=7$) received the L-DOPA/benserazide vehicle daily for 14 days The remaining lesioned animals instead underwent priming with L-DOPA/benserazide (10/15 mg/kg s.c.) daily for 14 days. Following the 14-day treatment period, the severity of AIMs was assessed, with each animal receiving a cumulative abnormal involuntary movements (AIMs) score, based on axial, limbs and oro-lingual (ALO) subtypes of dyskinesia. Based on AIMs scores, the lesioned rats that had received L-DOPA were subdivided into Groups C (lesion-L-DOPA-mild, $N=5$) and D (lesion-L-DOPA-severe, $N=6$). Animals with cumulative ALO AIMs scores (sum of

duration and amplitude components) of 50 and above were assigned to Group D, while those that scored below the cut-off score were assigned to Group C.

Assessment of AIMs severity

Following the 14-day priming period, ALO AIMs severity was assessed by an observer blinded to treatment, according to a scale previously described, which encompasses both duration and amplitude (Cenci and Lundblad, 2007). On the day of behavioural scoring, following administration of L-DOPA/benserazide (10/15 mg/kg, s.c.), ALO AIMs were rated for 2 min every 20 min over a 3-hour observation period. Both ALO AIMs duration and amplitude were rated on a scale from 0 to 4 in each monitoring interval. Each AIMs subtype (*i.e.* axial, limbs and oro-lingual) provided a maximum possible score per session of 36; hence, the maximum possible cumulative ALO AIMs duration and amplitude score was 108 per session.

Tissue preparation

Animals were administered their usual treatment based on group assignment and were anaesthetised with 4% isoflurane in 100 % oxygen (1 L/min) 45 min later, to allow trans-cardial perfusion with 0.9 % saline. Brains were collected and flash frozen in 2-methyl-butane at -56°C. Brains were set in optical cutting temperature (OCT) compound and cut into 12-µm thick coronal sections using a cryostat (Leica CM3050 S, Canada). Sections were thaw-mounted on SuperFrost Plus slides (ThermoFisher, Canada) and then dried at room temperature before being stored at -80°C until use.

[^{3}H]GR65630 autoradiographic binding

Regions of interest selected for processing were based on their role in the motor loop of the basal ganglia, which is implicated in the pathophysiology of dyskinesia (Wichmann and Dostrovsky, 2011, Lanciego et al., 2012). These regions were identified according to a rat brain atlas (Paxinos and Watson, 2007) and comprised the primary motor cortex, dorsolateral striatum, globus pallidus, entopeduncular nucleus, subthalamic nucleus, ventral anterior/ventral lateral (VA/VL) nuclei of the thalamus, and substantia nigra pars compacta and pars reticulata. 5-HT_3 receptor levels were determined by autoradiographic binding adapted from a previously-published protocol (Kilpatrick et al., 1988). Sections were removed from storage at -80°C, and then thawed to dry at room temperature overnight. Sections were pre-incubated by washing twice in HEPES buffer (50 mM, pH 7.4) for 15 min at room temperature. Sections were then incubated in HEPES buffer (50 mM, pH 7.4) containing 2.0 nM [^{3}H]GR65630 (American Radiolabeled Chemicals, USA; specific activity: 60 Ci/mmol) for 30 min at room temperature to define total binding. Non-specific binding was defined by the addition of 10 µM of the 5-HT_3 antagonist granisetron (Cedarlane Laboratories, Canada). Sections were then washed twice in HEPES buffer at 4 ºC for 30 s. Subsequently, sections were dipped in 4 ºC distilled water for 2 s and air dried at room temperature. Sections were apposed to [^{3}H]-sensitive Biomax MR films (MilliporeSigma, Canada) for 6 weeks at 4 ºC with [^{3}H]-microscale standards (ART0123B and ART0123C, American Radiolabeled Chemicals, 5 mm × 7 mm), after which they were developed for densitometric analysis.

Autoradiograms were analysed using optical densitometry with ImageJ software (NIH, version 1.52p). The [^{3}H]-microscale standards were used to calculate a reference curve of radioactivity *versus* grey-level values of autoradiograms (Zilles et al., 2002) and used to quantify signal intensity as nCi per mg of tissue. For each brain area, four consecutive sections were

processed to determine total binding, and four to evaluate non-specific binding. To calculate specific binding, non-specific binding was subtracted from total binding. Signal intensity (nCi/mg) was divided by the specific activity of the radioligand (Ci/mmol) to obtain fmol of receptor per mg of tissue (fmol/mg) (Huot et al., 2012b).

The autoradiographic signal generated by tritiated ligands is quenched to variable degrees, depending on the brain region (Herkenham and Sokoloff, 1984, Geary et al., 1985). Binding values were therefore corrected by applying empirically derived regional quenching coefficients (Geary and Wooten, 1985, Happe and Murrin, 1990). Exceptionally, no such correction was applied to the subthalamic nucleus, as its quenching coefficient has not been published. In Tables S1 and S2, quenching-corrected [^{3}H]GR65630 binding levels are presented for brain regions ipsilateral and contralateral to the lesion; these values are for information only, and no statistical analysis was performed.

Immunohistochemistry

Immunohistochemical staining was performed on striatal brain sections that were thaw-mounted on slides. Slides were dried overnight at room temperature and post-fixed by immersion in pre-cooled (-20°C) acetone for 10 min and left to air dry for 20 min (Matsumoto, 1985). Sections were then subjected to the following incubations, with 3 × 5 min rinses in Tris buffered saline (TBS; 100 mM Tris-Cl, pH 7.40, containing 240 mM NaCl) between each incubation: (1) quenching in 0.5 % H_2O_2 for 10 min, (2) block for 1 h in 10 % normal goat serum (NGS) and 5% bovine serum albumin (BSA) in TBS containing 0.3 % Triton X-100, (3) mouse monoclonal antibody raised against tyrosine hydroxylase (TH) (1:1000, MilliporeSigma, MAB318) in 5% NGS and 2% BSA in TBS containing 0.1 % Triton X-100 (TBS-T) overnight at 4 °C, (4) goat

anti-mouse biotinylated secondary antibody (1:200, Invitrogen, USA, 31800) in 5% NGS and 2% BSA in TBS-T for 1 h, (5) avidin-biotin complex detection kit (ABC; Vector Laboratories, USA, PK-6100) for 2 h. Sections were developed in TBS-T containing 1.25 mg/mL nickel ammonium sulphate hexahydrate (MilliporeSigma, 574988), 0.25 mg/mL 3,3'-diaminobenzidine (MilliporeSigma, D5637) and 0.015% H_2O_2 (Patterson et al., 2019). After washing in TBS, sections were dried, rehydrated in water and dehydrated in graded alcohols, cleared with xylene and coverslipped using Permount mounting medium (Fisher Scientific, USA, SP15-100).

TH-immunoreactivity was quantified by densitometry in sections containing the dorsolateral striatum (Bregma ~ +1.20 mm). Images were captured by a Nikon Eclipse E800 microscope (The Neuro Microscope Core Facility) using Stereo Investigator software (MBF Bioscience, USA, version 11). Optical density was measured in the ipsilateral and contralateral dorsolateral striatum of four adjacent brain sections in ImageJ software (NIH, version 1.53c) (Linkert et al., 2010, Schindelin et al., 2012, Schneider et al., 2012). Relative TH-immunoreactivity of each section was calculated as a percentage of the contralateral (non-lesioned) hemisphere. Mean relative TH optical density was calculated for each subject.

Statistical Analysis

Assessment of hemi-parkinsonism

Relative striatal TH optical density values are presented as a percentage of the contralateral hemisphere. Combining TH data of 6-OHDA-lesioned animals did not violate the Brown-Forsythe test of homogeneity of variance assumption. In addition, a 1-way Welch's ANOVA ($W_{(2, 9.554)}$ = 1.369, $P > 0.05$) found no significant differences in mean dopaminergic denervation across 6-OHDA-lesioned groups, *i.e.*, between group B (lesion-vehicle), group C (lesion-L-DOPA-mild),

and group D (lesion-L-DOPA-severe). Based on these analyses, TH data were pooled and then compared with sham-lesioned animals by Student's *t* test.

Assessment of AIMs severity

AIMs data are discrete and discontinuous values that do not follow a normal distribution, therefore, non-parametric statistical analyses were performed because they do not rely on the assumption of normality (Corder and Foreman, 2014). ALO AIMs scores of mild and severe AIMs 6-OHDA-lesioned animals are presented as median ± semi-interquartile range and were analysed by Mann-Whitney *U* test.

Autoradiographic binding levels

In contrast to AIMs data, autoradiographic binding levels data follow a normal distribution, so parametric statistical analyses were performed (Sheskin, 2011). For each region of interest, specific binding levels are presented as the mean ± standard error of the mean (SEM). Multiple Student's *t* tests (Hsu, 1999, Ruxton and Beauchamp, 2008) were performed to compare each 6-OHDA-lesioned group with the sham-lesioned one, as well as between the mild and severe AIMs groups, and *P* values were corrected using the Holm-Sidak multiple comparisons test. Correlations between ALO AIMs scores and specific [^{3}H]GR65630 binding were analysed using Pearson correlation coefficient.

Statistical analyses were performed with GraphPad Prism 8.4.3 (GraphPad Software Inc., USA). Statistical significance was assigned when $P < 0.05$.

Chapter 6 Results

Parkinsonism and lesion severity

Animals subject to 6-OHDA displayed use of the forelimb ipsilateral to the lesion in 72.5 ± 3.3 % of rears, which is indicative of extensive deficit of striatal dopamine. In agreement with preferential forelimb use, densitometric analysis revealed reduced TH immunoreactivity in the dorsolateral striatum of 6-OHDA lesioned animals (Fig. 1A-1C). When TH data of L-DOPA naïve, mild and severe AIMs 6-OHDA animals was combined, the Brown-Forsythe test of homogeneity of variance was valid ($F_{(2, 13.03)} = 1.957$, $P > 0.05$), and consequently, data were pooled. As shown in Fig. 1A and 1C, the extent of striatal TH immunoreactivity in sham-lesioned rats was comparable between hemispheres. In rats administered 6-OHDA, TH striatal immunoreactivity was significantly reduced, by 90 %, compared to sham-lesioned rats ($t_{(24)} = 22.52$, $P < 0.0001$; Fig. 1B and 1C). As shown in Fig. S1, amongst 6-OHDA-lesioned animals, TH striatal immunoreactivity was comparable between those administered L-DOPA vehicle and those that received L-DOPA and expressed mild or severe AIMs ($W_{(2, 9.554)} = 1.369$, $P > 0.05$, 1-way Welch's ANOVA).

ALO AIMs severity

As shown in Fig. S2, the severely dyskinetic group of lesioned animals had approximately 3-fold higher ALO AIMs scores than the mildly dyskinetic group (median ± semi-interquartile range ALO AIMs scores for Groups C and D, respectively: 32.0 ± 6.25 and 82.5 ± 18.0; $U = 0$, $P < 0.01$). In contrast to the literature (Cenci and Lundblad, 2007), no 6-OHDA-lesioned animal treated with L-DOPA was completely devoid of ALO AIMs, as some animals exhibited mild oro-

lingual AIMs, although axial and limbs AIMs were not present. Importantly, the expression of oro-lingual AIMs was not evocative of stereotypies, so 6-OHDA-lesioned animals treated with L-DOPA that exhibited mild AIMs were included in Group C.

Specific [^{3}H]GR 65630 binding is altered in the brains of 6-OHDA-lesioned rats

Fig. 2 shows examples of autoradiograms of [^{3}H]GR65630 binding in the rat subthalamic nucleus, substantia nigra, striatum and primary motor cortex. Specific binding comprised 25 % of total binding across all regions of interest.

Specific [^{3}H]GR 65630 binding in the ipsilateral hemisphere

In Table 1 and Fig. 3A-3H, [^{3}H]GR65630 binding in the ipsilateral hemisphere of normal and 6-OHDA-lesioned rats is presented. Detailed statistical results of multiple Student *t* tests are presented for each region of interest in Table S3. In the absence of L-DOPA treatment, the 6-OHDA lesion exerted little or no effect, except possibly in the subthalamic nucleus where binding was higher in the L-DOPA naïve *versus* sham group ($t_{(33)} = 2.275$, uncorrected $P = 0.0295$). The combination of 6-OHDA lesion and L-DOPA treatment significantly increased binding in three brain regions (Fig. 3A-3C). This increase was most marked in the subthalamic nucleus, with a large effect (≈3-fold increase) seen in both the mild and severe AIMs groups (Fig. 3A). In the other two regions, VA/VL thalamus and entopeduncular nucleus (Fig. 3B and 3C), only the severe AIMs group showed a significant increase. In the five other brain regions, [^{3}H]GR65630 binding appeared unaltered by the combination of 6-OHDA/L-DOPA treatments.

Specific [^{3}H]GR 65630 binding in the contralateral hemisphere

In Table 2 and Fig. 4A-4H, [^{3}H]GR65630 binding in the contralateral hemisphere of brains of normal and 6-OHDA-lesioned rats is presented. Detailed statistical results of multiple Student *t* tests are presented for each region of interest in Table S4. In general, 6-OHDA lesion alone did not significantly alter [^{3}H]GR65630 binding, except for in the subthalamic nucleus, where there was an ≈ 2-fold increase in the L-DOPA naïve group compared to sham (Fig. 4A). Moreover, 6-OHDA lesion in combination with L-DOPA treatment also resulted in increased (≈ 3- to 5-fold) binding in the subthalamic nucleus in both mild and severe AIMs groups (Fig. 4A). In the substantia nigra pars compacta, binding significantly decreased by ≈ 58% in the severe AIMs group compared to the mild AIMs group (Fig. 4E). Contrary to these findings, [^{3}H]GR65630 binding remained unaffected by the combination of 6-OHDA/L-DOPA treatments in the other brain regions assessed (Fig. 4B-4D, 4F-4H).

Correlation between ALO AIMs scores and [^{3}H]GR 65630 binding in 6-OHDA-lesioned

As shown in Fig. 5A, [^{3}H]GR65630 binding in the ipsilateral dorsolateral striatum and ALO AIMs scores of 6-OHDA-lesioned animals were negatively correlated ($r = -0.6028$, $P < 0.05$). Similarly, [^{3}H]GR65630 binding in the contralateral subthalamic nucleus was negatively correlated with ALO AIMs scores of 6-OHDA-lesioned rats ($r = -0.6133$, $P < 0.05$) (Fig. 5B). As displayed in Fig. S3, [^{3}H]GR65630 binding in the ipsilateral subthalamic nucleus, entopeduncular nucleus, and thalamus was not correlated with ALO AIMs scores of 6-OHDA-lesioned animals ($r = 0.1368$, $r = 0.2061$, $r = 0.2685$, all $P > 0.05$). In the contralateral entopeduncular nucleus and thalamus of 6-OHDA-lesioned animals, [^{3}H]GR65630 binding did not correlate with ALO AIMs scores ($r = -0.08538$, $r = 0.1111$, both $P > 0.05$). Similarly, [^{3}H]GR65630 binding in other brain regions was not correlated with ALO AIMs scores.

Chapter 6 Discussion

This is the first study to assess the distribution of the 5-HT_3 receptor in animals displaying L-DOPA induced dyskinesia. The main novel finding was a regionally-selective upregulation of [^{3}H]GR65630 binding in 6-OHDA-lesioned rats that were either L-DOPA naïve or exhibiting AIMs due to chronic L-DOPA administration. This upregulation was largely confined to the subthalamic nucleus, where it manifested bilaterally; a smaller degree of ipsilateral-only upregulation was also seen in the VA/VL thalamus and entopeduncular nucleus. In the contralateral hemisphere, binding was downregulated in the substantia nigra pars compacta of 6-OHDA-lesioned rats exhibiting severe AIMs compared to those with mild AIMs. In contrast, [^{3}H]GR65630 binding appeared unaltered in the five other brain regions examined: *i.e.* primary motor cortex, dorsolateral striatum, globus pallidus, and substantia nigra pars reticulata. Finally, ALO AIMs score were negatively correlated with binding levels in the ipsilateral dorsolateral striatum and contralateral subthalamic nucleus. These results suggest that increased 5-HT_3 receptor levels may contribute to the pathophysiology of L-DOPA-induced dyskinesia and potentially provide an anatomical basis for the anti-dyskinetic efficacy of 5-HT_3 antagonists in PD.

Brain 5-HT_3 receptors are generally expressed at low levels (De Deurwaerdère and Di Giovanni, 2017), and distinguishing specific receptor binding can thus be challenging, with low signal-to-noise ratios reported across a variety of radioligands and unlabelled displacing ligands, in rodents (Kilpatrick et al., 1987, Ge et al., 1997) and primates (Barnes et al., 1990a, Jones et al., 1992). In line with previous reports, specific binding in our study represented only 25 % of total

binding, but by using a minimal number of animals to conduct valid statistical analysis while abiding by the 3R principles for ethical use of animals in research results, we were able to measure a reliable autoradiographic signal.

Our results reveal similarities with, and differences from, previously published work. For example, in sham-lesioned animals, we found high specific binding in the cerebral cortex, concurring with previous studies using the same radioligand (Kilpatrick et al., 1987, Kilpatrick et al., 1988). We also observed relatively high levels of radioligand binding in the basal ganglia and thalamus, which was consistent with earlier studies using either the same radioligand (Chen and Lawrence, 2000) or radiolabelled (*R*)-zacopride (Ge et al., 1997), although a few studies failed to detect binding in these structures (Kilpatrick et al., 1987, Barnes et al., 1990a). The stronger binding signal that we observed possibly reflects our decision to use a higher concentration of [^{3}H]GR65630 (*i.e.* 2 *versus* 0.2 nM in the earlier studies by Kilpatrick et al.). Another possible explanation would be that the ligand chosen for the excess cold condition, granisetron, shared off-target binding sites with [^{3}H]GR65630, such that our calculated displaceable binding overestimated binding to 5-HT$_3$ receptors. However, this explanation seems very unlikely: first, granisetron has negligible known affinity for other receptors (Blower, 1990) and, second, any additional binding sites would probably not be shared with the radioligand given that the two drugs are structurally unrelated (van Wijngaarden et al., 1993). In contrast, several published autoradiographic 5-HT$_3$ binding studies have employed cold ligands such as metoclopramide that exhibit considerable affinity for non-target receptors (Kilpatrick et al., 1988, Kilpatrick et al., 1989, Jones et al., 1992).

In the present study, the most pervasive upregulation of [^{3}H]GR65630 binding occurred in the subthalamic nucleus. Upregulation in this structure was large, bilateral, and detectable in

lesioned animals even without subsequent L-DOPA treatment. Although 6-OHDA-lesion/L-DOPA treatment resulted in both [^{3}H]GR65630 binding upregulation and AIMs, AIMs severity was not significantly correlated with [^{3}H]GR65630 binding in the ipsilateral subthalamic nucleus. Instead, a negative correlation was observed in the subthalamic nucleus contralateral to the lesion. It is unclear whether this contralateral elevation in [^{3}H]GR65630 binding is functionally related to dyskinesia, either as a contributor or as a compensatory mechanism. More generally, our findings add to previous evidence implicating the subthalamic nucleus in L-DOPA induced dyskinesia. Notably, injection of the 5-HT_{1A} agonist sarizotan into the subthalamic nucleus abolished dyskinesia induced by L-DOPA in the same animal model (Marin et al., 2009), and bilateral deep brain stimulation of this nucleus in PD patients is a clinically efficacious therapy for dyskinesia (Fox et al., 2018). These finding point towards key roles of the subthalamic nucleus and of 5-HT in the pathophysiology of dyskinesia in PD. Thus, it can be suggested that blockade of 5-HT_3 receptors in the subthalamic nucleus may contribute to the anti-dyskinetic efficacy of 5-HT_3 antagonists. In addition to the subthalamic nucleus, [^{3}H]GR65630 binding was also upregulated in the ipsilateral entopeduncular nucleus and VA/VL thalamus of severely dyskinetic rats. These brain regions form part of the cortico-basal ganglia-thalamo-cortical loop (Parent and Hazrati, 1995, Wallace et al., 2017), and hence may be well positioned to mediate dyskinesia in PD (Lanciego et al., 2012). Further studies are required to elucidate the role that 5-HT_3 receptors in these three brain areas may play in the development or expression of L-DOPA induced dyskinesia.

Behavioural studies in the parkinsonian rat and non-human primate provide support for the role of 5-HT_3 receptor antagonists in alleviating L-DOPA-induced dyskinesia (Aboulghasemi et al., 2018, Kwan et al., 2020c, Kwan et al., 2021a, Kwan et al., 2021b), but insight into possible mechanism(s) of action remains largely lacking. Prior to the current experiments, only two

autoradiographic binding studies had determined 5-HT_3 receptor levels in PD but they were limited in relation to dyskinesia as a complication experienced by patients: in one, medication and co-morbidities (*i.e.* dyskinesia) were not disclosed (Steward et al., 1993), and in the other, the cortico-basal ganglia network was not studied (Cicin-Sain and Jenner, 1993). Whereas [^{3}H]GR65630 binding significantly decreased in the pre-frontal and entorhinal cortices of 6-OHDA-lesioned animals compared to control animals (Cicin-Sain and Jenner, 1993), we did not observe any significant differences in binding in the primary motor cortex between 6-OHDA L-DOPA naïve and L-DOPA treated rats with sham-lesioned ones. The discrepancy in findings may be explained by differences in cortical areas studied as an immunohistochemical study of the mouse brain reported variations in 5-HT_3 receptor expression across cortical areas, with weak to moderately high signal intensity in the pre-frontal and entorhinal cortices but strong signal intensity in the primary motor cortex (Koyama et al., 2017). Moreover, there were notable methodological differences between the studies, including the dose of 6-OHDA administered, strain of rats, as well as the affinity of the cold ligand used (metoclopramide versus granisetron). Future studies examining the cortical distribution of 5-HT_3 receptors in the 6-OHDA-lesioned rat model are warranted.

Considerable evidence *in vitro* and *in vivo* has implicated the role of the 5-HT_3 receptor in mediating dopamine release in the striatum (Costall et al., 1987, Blandina et al., 1989, Santiago et al., 1995, Yoo et al., 2008). Given that dysregulated dopamine release is thought to play an aetiological role in L-DOPA-induced dyskinesia (Carta et al., 2007, Muñoz et al., 2009), modulation of the 5-HT_3 receptor may alleviate dyskinesia by dampening the erratic release of striatal dopamine that occurs in the dyskinetic state (Carta et al., 2006). Contrary to expectations, [^{3}H]GR65630 binding was comparable between 6-OHDA-lesioned animals and sham-lesioned

animals, irrespective of treatment or dyskinesia severity. These findings are consistent with a membrane binding study in rats that found 5-HT_3 receptor levels in the striatum were not significantly affected by 6-OHDA lesion (Kidd et al., 1993). Moreover, a post-mortem study in humans demonstrated that 5-HT_3 receptor levels were comparable in PD patients to controls (Steward et al., 1993). These results collectively suggest that 5-HT_3 receptors may not be located on dopaminergic terminals of the striatum, but may point to a localisation of 5-HT_3 receptors on striatal interneurons (Steward et al., 1993) or on non-dopaminergic afferents to the striatum.

The rating scale used to assess AIMs in the 6-OHDA-lesioned rat is well established and validated (Monville et al., 2005, Dekundy et al., 2007). Compared to the 6-OHDA rat model, the MPTP-lesioned non-human primate model of PD bears greater similarities with the human condition (Philippens et al., 2010) and could be the next step in assessing levels of 5-HT_3 receptor binding in dyskinesia. Recent efforts in the development of positron emission tomography (Mu et al., 2016) and fluorescent probes (Jack et al., 2015) that target the 5-HT_3 receptor may further characterise alterations in 5-HT_3 receptor levels in L-DOPA induced dyskinesia. Specific ligands with nanomolar affinity demonstrated a high signal to noise ratio (Jack et al., 2015, Mu et al., 2016), unlike the high non-specific binding reported in autoradiographic binding studies with the 5-HT_3 receptor, may enable studying the distribution of the receptor *in vivo* (Lochner and Thompson, 2015). Lastly, receptor binding levels were corrected by regional brain quenching coefficients derived from a different paradigm (Herkenham and Sokoloff, 1984, Geary et al., 1985). To our knowledge, no study has directly studied the effects of 6-OHDA lesion or L-DOPA treatment on lipid distribution – a known factor of quenching (Zilles et al., 1990); further studies are warranted to shed light on their effects on tritium quenching.

In summary, the present study suggests that alterations in 5-HT_3 receptor levels in the subthalamic nucleus, as well as the ipsilateral entopeduncular nucleus and VA/VL thalamus may modulate L-DOPA induced dyskinesia in the 6-OHDA rat at the post-mortem level. Taken together, these findings provide insight into the brain regions worth further study, in an attempt to better understand the role of 5-HT_3 receptors in L-DOPA induced dyskinesia and how 5-HT_3 antagonists alleviate dyskinesia in PD.

Data availability

No data was used for the research described in the article.

Data will be made available on request.

Authors' contributions

PH conceived the project. CK, AH and PH designed the project. CK, DB and IF performed and collected data. CK, CL, JMY, DL and PBSC contributed to data analysis. CK wrote the first draft of the manuscript. All the authors contributed to the final manuscript. All the authors read and approved the final manuscript.

Declaration of Competing Interest

Authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.neures.2021.12.004.

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Chapter 6 Tables and Figures

Table 1

[^{3}H]GR65630 binding levels in the rat brain ipsilateral to lesion

	[^{3}H]GR65630 binding levels (fmol/mg)			
	sham	6-OHDA L-DOPA naïve	6-OHDA mild AIMs	6-OHDA severe AIMs
primary motor cortex	2.083 ± 0.096	2.110 ± 0.130	2.142 ± 0.149	1.902 ± 0.111
basal ganglia				
dorsolateral striatum	17.943 ± 5.025	14.628 ± 1.547	28.086 ± 5.716	16.055 ± 2.947
globus pallidus	23.390 ± 4.03	25.648 ± 4.883	15.679 ± 4.193	13.41 ± 2.064
entopeduncular nucleus	12.027 ± 1.981	13.636 ± 2.544	15.784 ± 0.932	21.017 ± 2.953 *
subthalamic nucleus	7.478 ± 2.863	19.465 ± 2.607	31.159 ± 1.965 ***	31.368 ± 6.163 ***
substantia nigra				
pars compacta	27.741 ± 1.994	22.065 ± 5.454	19.684 ± 6.594	20.967 ± 2.507
pars reticulata	18.326 ± 5.005	11.076 ± 1.996	19.377 ± 2.906	19.718 ± 5.552
VA/VL thalamus	14.726 ± 3.088	17.44 ± 2.594	19.462 ± 3.418	27.643 ± 3.997 *

Data are presented as mean ± standard error of the mean (SEM) specific binding (fmol/mg of tissue). 6-OHDA, 6-hydroxydopamine; AIMs, abnormal involuntary movements; L-DOPA, L-3,4-dihydroxyphenylalanine. *N*=5-8 per group. *: $P < 0.05$ compared to sham-lesioned group. ***: $P < 0.001$ compared to sham-lesioned group.

Table 2

[^{3}H]GR65630 binding levels in the rat brain contralateral to lesion

	[^{3}H]GR65630 binding levels (fmol/mg)			
	sham	6-OHDA L-DOPA naïve	6-OHDA mild AIMs	6-OHDA severe AIMs
primary motor cortex	2.108 ± 0.129	2.128 ± 0.139	2.154 ± 0.144	1.912 ± 0.140
basal ganglia				
dorsolateral striatum	24.352 ± 3.693	22.843 ± 3.437	21.827 ± 2.827	23.036 ± 3.129
globus pallidus	25.571 ± 3.146	31.587 ± 5.909	14.183 ± 4.988	17.907 ± 3.343
entopeduncular nucleus	18.012 ± 2.332	16.71 ± 2.647	16.693 ± 1.579	17.836 ± 3.175
subthalamic nucleus	5.922 ± 2.573	20.377 ± 2.544 ***	34.376 ± 1.799 ****	25.243 ± 2.541 **** †
substantia nigra				
pars compacta	27.025 ± 2.503	23.467 ± 4.587	34.498 ± 7.93	14.62 ±5.298 †
pars reticulata	15.719 ± 4.306	19.691 ± 3.678	19.667 ± 3.907	13.623 ± 3.139
VA/VL thalamus	18.739 ± 3.831	15.04 ± 3.651	21.926 ± 3.251	23.11 ± 2.246

Data are presented as the mean ± SEM specific binding (fmol/mg of tissue). 6-OHDA, 6-hydroxydopamine; AIMs, abnormal involuntary movements; L-DOPA, L-3,4-dihydroxyphenylalanine. *N*=5-8 per group. *: $P < 0.05$ compared to sham-lesioned group. ****: $P < 0.0001$ compared to sham-lesioned group. †: $P < 0.05$ compared to mild AIMs 6-OHDA-lesioned group.

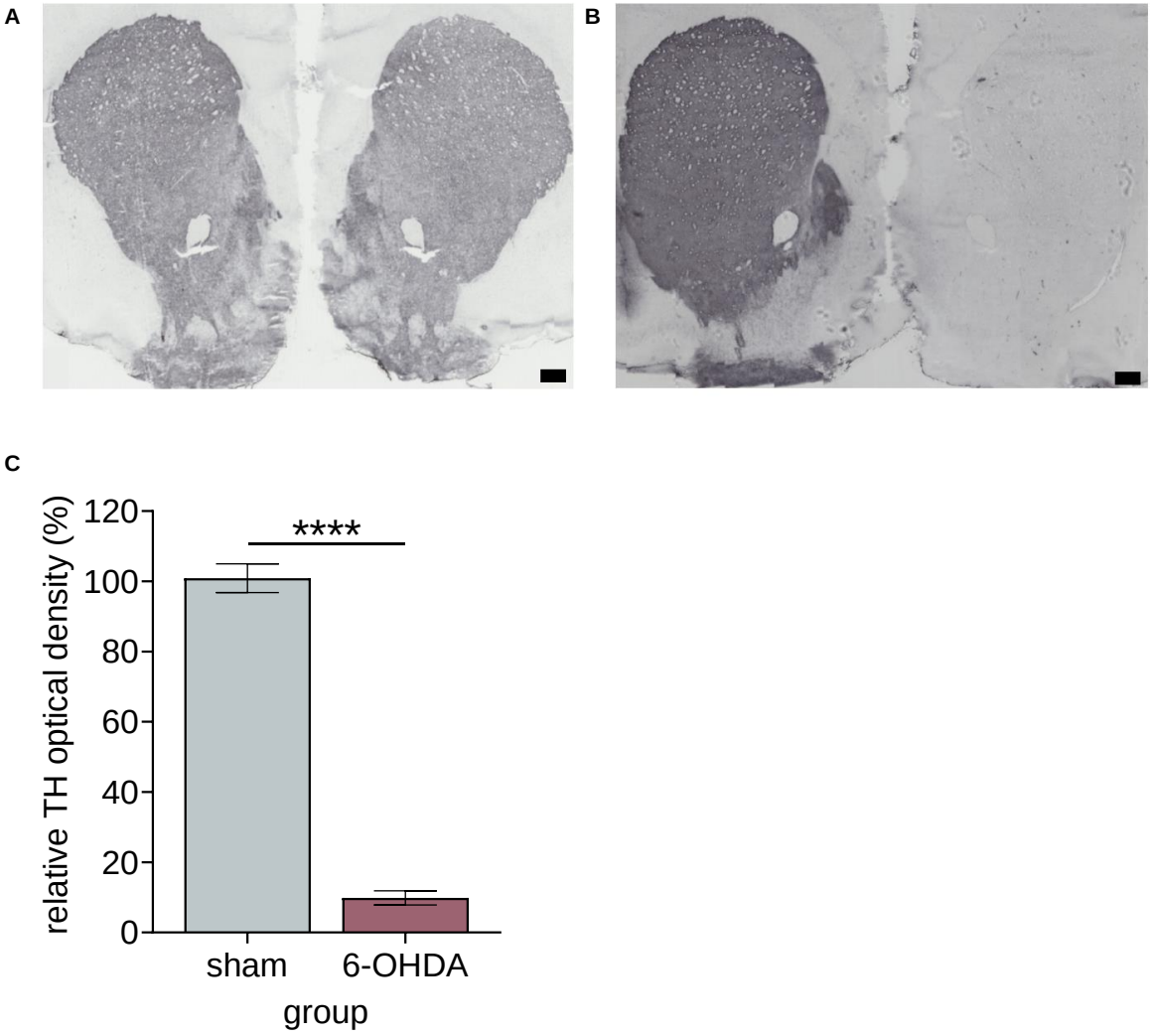


Fig. 1. Relative TH optical density in the rat striatum.

Representative photomicrographs of TH immunoreactive fibres in the striatum of sham-lesioned (**A**) and 6-OHDA-lesioned (**B**) animals. Relative TH densitometry was significantly reduced by 90% in the dorsolateral striatum of animals administered 6-OHDA compared to sham-lesioned animals (**C**). Data are presented as the mean ± SEM. Relative optical density measurements are presented as a percentage of the contralateral (non-lesioned) hemisphere. 6-OHDA, 6-

hydroxydopamine; TH, tyrosine hydroxylase. $N = 8$ in the sham-lesioned vehicle group; $N = 18$ in the pooled 6-OHDA-lesioned group. ****: $P < 0.0001$. Scale bar: 400 μm.

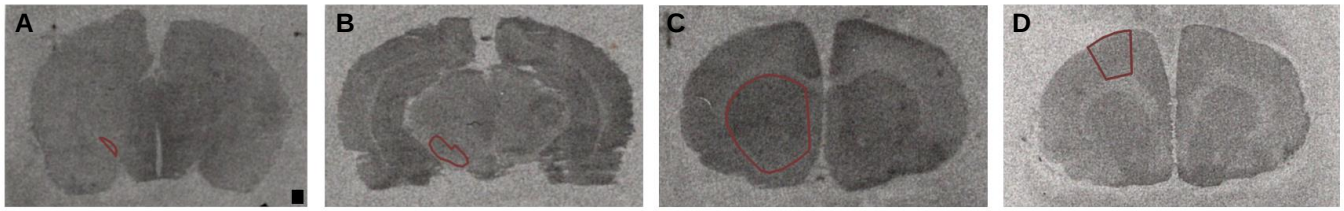


Fig. 2. Autoradiograms of [^{3}H]GR65630 binding across different brain regions of interests in the rat.

Representative autoradiograms of total [^{3}H]GR65630 binding in the rat subthalamic nucleus (**A**), substantia nigra (**B**), striatum (**C**) and primary motor cortex (**D**) are presented. All autoradiograms were selected from sham-lesioned animals. The region of interest contralateral to lesion is outlined in red. Scale bar: 400 μm.

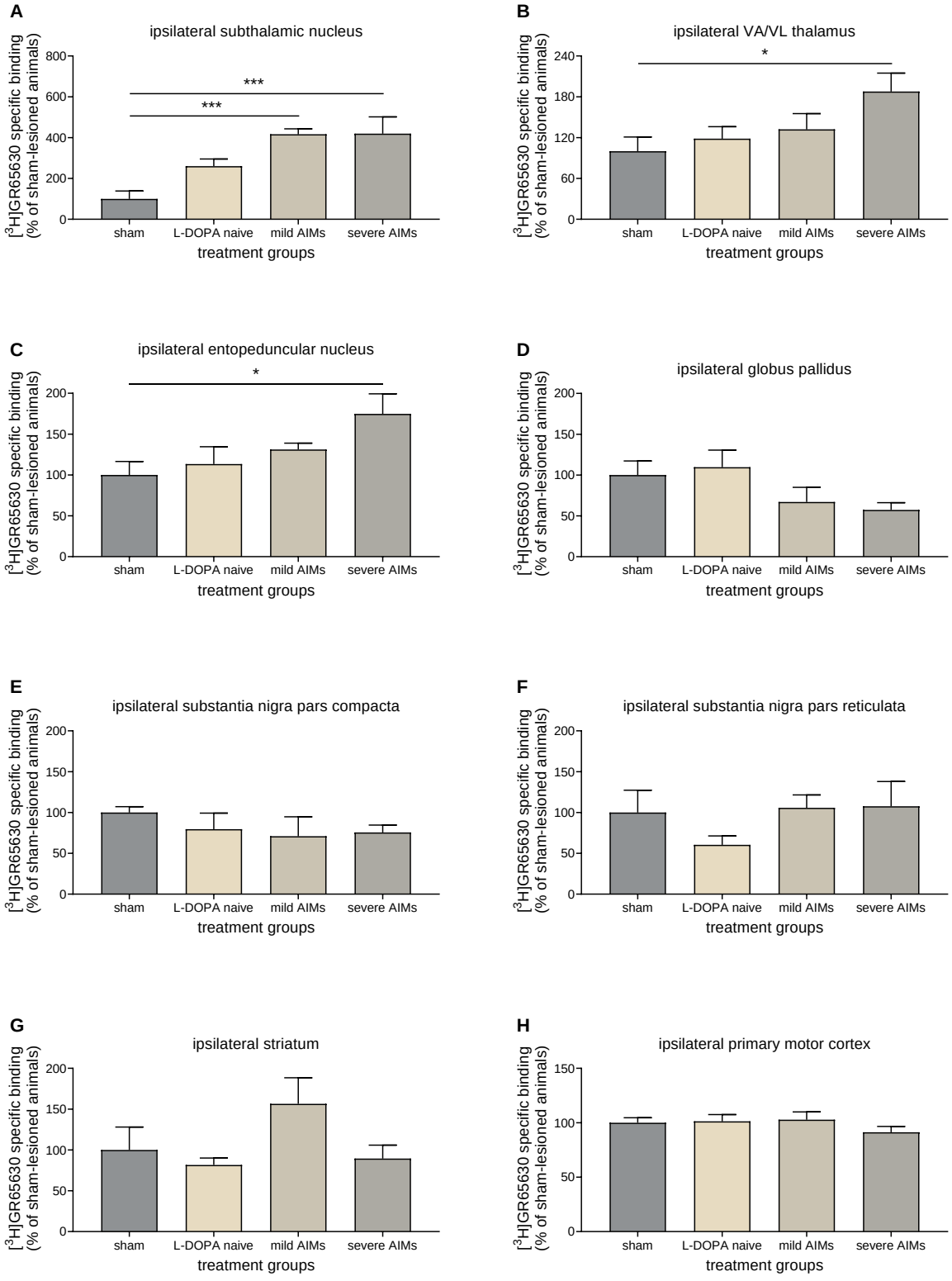

A
ipsilateral subthalamic nucleus
[3H]GR65630 specific binding (% of sham-lesioned animals)
800
600
400
200
0
***
***
sham
L-DOPA naive
mild AIMs
severe AIMs
treatment groups
B
ipsilateral VA/VL thalamus
[3H]GR65630 specific binding (% of sham-lesioned animals)
240
180
120
60
0
*
sham
L-DOPA naive
mild AIMs
severe AIMs
treatment groups
C
ipsilateral entopeduncular nucleus
[3H]GR65630 specific binding (% of sham-lesioned animals)
200
150
100
50
0
*
sham
L-DOPA naive
mild AIMs
severe AIMs
treatment groups
D
ipsilateral globus pallidus
[3H]GR65630 specific binding (% of sham-lesioned animals)
200
150
100
50
0
sham
L-DOPA naive
mild AIMs
severe AIMs
treatment groups
E
ipsilateral substantia nigra pars compacta
[3H]GR65630 specific binding (% of sham-lesioned animals)
200
150
100
50
0
sham
L-DOPA naive
mild AIMs
severe AIMs
treatment groups
F
ipsilateral substantia nigra pars reticulata
[3H]GR65630 specific binding (% of sham-lesioned animals)
200
150
100
50
0
sham
L-DOPA naive
mild AIMs
severe AIMs
treatment groups
G
ipsilateral striatum
[3H]GR65630 specific binding (% of sham-lesioned animals)
200
150
100
50
0
sham
L-DOPA naive
mild AIMs
severe AIMs
treatment groups
H
ipsilateral primary motor cortex
[3H]GR65630 specific binding (% of sham-lesioned animals)
150
100
50
0
sham
L-DOPA naive
mild AIMs
severe AIMs
treatment groups

Fig. 3. Autoradiography with [^{3}H]GR65630 binding shows an increase in 5-HT$_3$ receptor levels in the ipsilateral hemisphere of 6-OHDA-lesioned rats. There was a 3-fold increase in [^{3}H]GR65630 binding in the ipsilateral subthalamic nucleus of both mild and severe AIMs animals, when compared to sham-lesioned ones (**A**). There was also a 27% decrease in binding in severe AIMs animals compared to mild AIMs ones. [^{3}H]GR65630 binding in the ipsilateral VA/VL thalamus in severe AIMs 6-OHDA-lesioned rats increased by 88% compared to sham-lesioned ones (**B**). Whereas [^{3}H]GR65630 binding increased by 75% in the ipsilateral entopeduncular nucleus of severe AIMs 6-OHDA-lesioned animals, compared to sham-lesioned ones (**C**), it was similar across treatment groups in the ipsilateral globus pallidus (**D**). [^{3}H]GR65630 binding in the ipsilateral substantia nigra pars compacta (**E**) and pars reticulata (**F**) was comparable between 6-OHDA-lesioned animals and sham-lesioned ones. There were no significant differences in [^{3}H]GR65630 binding in the ipsilateral dorsolateral striatum (**G**) and primary motor cortex (**H**) between 6-OHDA-lesioned and sham-lesioned animals. Data are presented as the mean ± SEM. [^{3}H]GR65630 binding of L-DOPA naïve, mild and severe AIMs animals was calculated as a percentage of sham-lesioned animals. 6-OHDA, 6-hydroxydopamine; AIMs, abnormal involuntary movements; L-DOPA, L-3,4-dihydroxyphenylalanine. N = 5-8 per group. *: $P < 0.05$ compared to the sham-vehicle group. ***: $P < 0.001$ compared to sham-vehicle group.

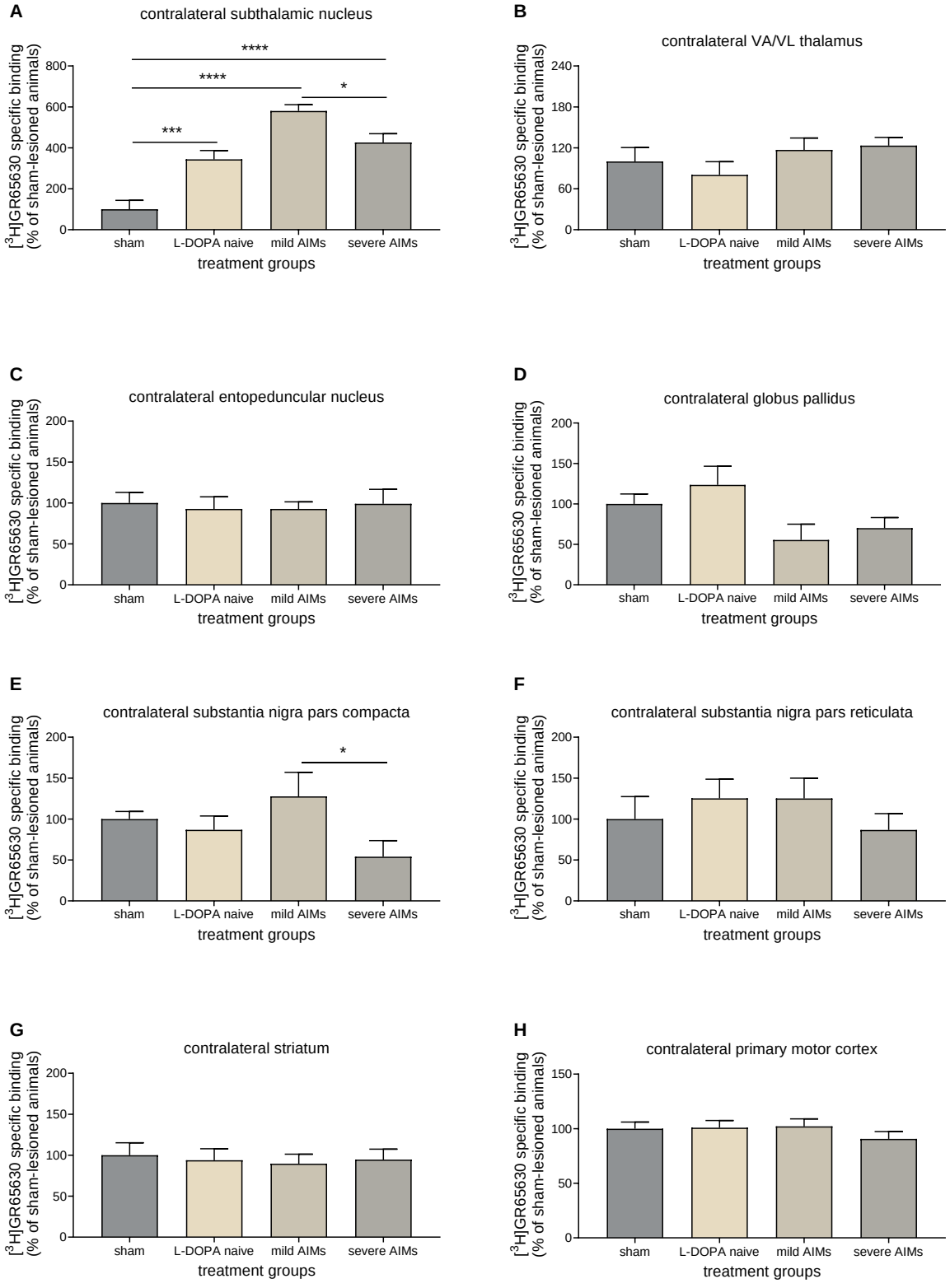

A
contralateral subthalamic nucleus
[3H]GR65630 specific binding (% of sham-lesioned animals)
****
****
*
***
sham
L-DOPA naive
mild AIMs
severe AIMs
treatment groups
B
contralateral VA/VL thalamus
C
contralateral entopeduncular nucleus
D
contralateral globus pallidus
E
contralateral substantia nigra pars compacta
F
contralateral substantia nigra pars reticulata
G
contralateral striatum
H
contralateral primary motor cortex

Fig. 4. Autoradiography with [^{3}H]GR65630 binding shows an increase in 5-HT$_3$ receptor levels in the contralateral subthalamic nucleus of 6-OHDA-lesioned rats. There were 2-fold, 5-fold and 3-fold increases in [^{3}H]GR65630 binding in the contralateral subthalamic nucleus of L-DOPA naïve, mild and severe AIMs 6-OHDA-lesioned rats, compared to sham-lesioned controls (**A**). [^{3}H]GR65630 binding in the contralateral VA/VL thalamus was similar across treatments groups (**B**). In contrast, [^{3}H]GR65630 binding in the contralateral entopeduncular nucleus and (**C**) and globus pallidus (**D**) was comparable across treatment groups. [^{3}H]GR65630 binding in the contralateral substantia nigra pars compacta of severe AIMs 6-OHDA-lesioned rats decreased by 58% compared to mild AIMs animals (**E**). Neither 6-OHDA lesion or AIMs severity had a significant effect on [^{3}H]GR65630 binding in the contralateral substantia nigra pars reticulata (**F**). There were no significant differences in [^{3}H]GR65630 binding in the contralateral dorsolateral striatum (**G**) and primary motor cortex (**H**) between 6-OHDA-lesioned rats and sham-lesioned ones. Data are presented as the mean ± SEM. [^{3}H]GR65630 binding of L-DOPA naïve, mild and severe AIMs animals was calculated as a percentage of sham-lesioned animals. 6-OHDA, 6-hydroxydopamine; AIMs, abnormal involuntary movements; L-DOPA, L-3,4-dihydroxyphenylalanine. N = 5–8 per group. *: $P < 0.05$ compared to sham-vehicle group. ***: $P < 0.001$ compared to sham-vehicle group. ****: $P < 0.0001$ compared to sham-vehicle group. †: $P < 0.05$ compared to lesion-L-DOPA-mild AIMs group.

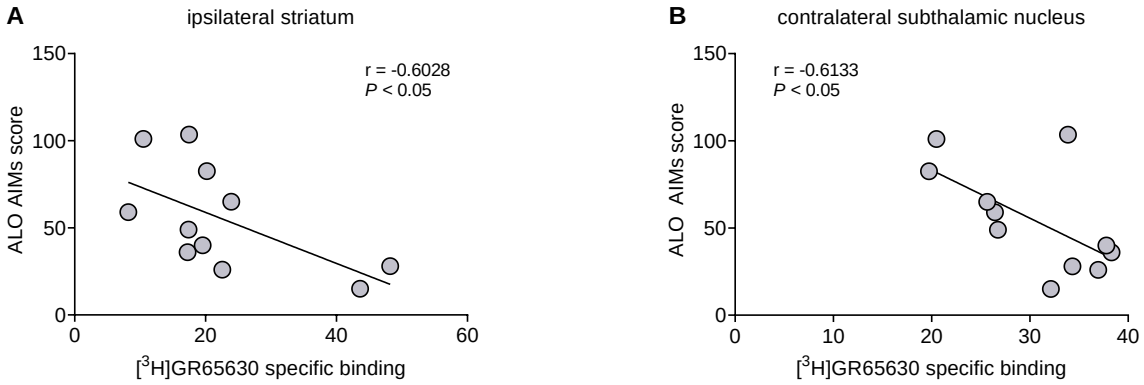


Fig. 5. Correlation between mean ALO AIMs scores and [^{3}H]GR65630 binding levels in 6-OHDA-lesioned rats. [^{3}H]GR65630 binding in the ipsilateral dorsolateral striatum (**A**) and contralateral subthalamic nucleus (**B**) were negatively correlated with mean ALO AIMs scores of 6-OHDA-lesioned animals. 6-OHDA, 6-hydroxydopamine; AIMs, abnormal involuntary movements; ALO, axial, limbs and oro-lingual. N = 11.

Table S1.

[^{3}H]GR65630 binding levels corrected by regional tritium coefficients in the rat brain ipsilateral to lesion

	[^{3}H]GR65630 binding levels (fmol/mg)			
	sham	6-OHDA L-DOPA naïve	6-OHDA mild AIMs	6-OHDA severe AIMs
primary motor cortex	2.75 ± 0.126	2.785 ± 0.171	2.827 ± 0.196	2.511 ± 0.147
basal ganglia				
dorsolateral striatum	24.42 ± 6.839	19.909 ± 2.106	38.225 ± 7.78	21.851 ± 4.011
globus pallidus	37.565 ± 6.472	41.19 ± 7.841	25.181 ± 6.734	21.536 ± 3.314
entopeduncular nucleus	19.182 ± 3.159	21.749 ± 4.058	25.175 ± 1.487	33.522 ± 4.71
substantia nigra				
pars compacta	37.451 ± 2.331	29.788 ± 7.363	26.573 ± 8.902	28.306 ± 3.385
pars reticulata	28.717 ± 7.842	17.356 ± 3.128	30.364 ± 4.553	30.898 ± 8.70
VA/VL thalamus	19.718 ± 4.135	23.352 ± 3.474	26.059 ± 4.577	37.014 ± 5.352

Data are presented as the mean ± SEM specific binding (fmol/mg of tissue). Regional tritium coefficients for all regions of interest except for the subthalamic nucleus were obtained from (Geary and Wooten, 1985), while the coefficient for the entopeduncular nucleus was obtained from (Happe and Murrin, 1990). Corrected binding levels in the subthalamic nucleus were omitted because the corresponding regional tritium coefficient has not been published. 6-OHDA, 6-hydroxydopamine; AIMs, abnormal involuntary movements; L-DOPA, L-3,4-dihydroxyphenylalanine. *N*=5-8 per group.

Table S2.

[^{3}H]GR65630 binding levels corrected by regional tritium coefficients in the rat brain contralateral to lesion

	[^{3}H]GR65630 binding levels (fmol/mg)			
	sham	6-OHDA L-DOPA naïve	6-OHDA mild AIMs	6-OHDA severe AIMs
primary motor cortex	2.782 ± 0.171	2.809 ± 0.183	2.843 ± 0.19	2.524 ± 0.184
basal ganglia				
dorsolateral striatum	33.143 ± 5.026	31.089 ± 4.677	29.707 ± 3.847	31.353 ± 4.259
globus pallidus	41.068 ± 5.052	50.729 ± 9.489	22.778 ± 8.011	28.759 ± 5.368
entopeduncular nucleus	28.729 ± 3.72	26.652 ± 4.223	26.625 ± 2.518	28.449 ± 5.064
substantia nigra				
pars compacta	36.483 ± 2.927	31.68 ± 6.192	46.572 ± 10.706	19.737 ± 7.152
pars reticulata	24.632 ± 6.748	30.856 ± 5.763	30.818 ± 6.122	21.347 ± 4.918
VA/VL thalamus	25.091 ± 5.129	20.138 ± 4.888	29.359 ± 4.354	30.944 ± 3.008

Data are presented as the mean ± SEM specific binding (fmol/mg of tissue). Regional tritium coefficients for all regions of interest except for the subthalamic nucleus were obtained from (Geary and Wooten, 1985), while the coefficient for the entopeduncular nucleus was obtained from (Happe and Murrin, 1990). Corrected binding levels in the subthalamic nucleus were omitted because the corresponding regional tritium coefficient has not been published. 6-OHDA, 6-hydroxydopamine; AIMs, abnormal involuntary movements; L-DOPA, L-3,4-dihydroxyphenylalanine. *N*=5-8 per group.

Table S3.

Summary of Student *t* tests for [^{3}H]GR65630 binding levels ipsilateral to lesion

	group comparisons	degrees of freedom	t ratio	P value
primary motor cortex	sham – L-DOPA naïve	41	0.1573	0.9299
	sham – mild AIMs	41	0.3406	0.9299
	sham – severe AIMs	41	0.9734	0.7073
	mild AIMs – severe AIMs	41	1.330	0.5712
basal ganglia				
dorsolateral striatum	sham – L-DOPA naïve	40	0.4811	0.8654
	sham – mild AIMs	40	1.550	0.3411
	sham – severe AIMs	40	0.2740	0.8654
	mild AIMs – severe AIMs	40	1.689	0.3411
globus pallidus	sham – L-DOPA naïve	38	0.3734	0.9164
	sham – mild AIMs	38	1.397	0.5240
	sham – severe AIMs	38	1.401	0.5240
	mild AIMs – severe AIMs	38	0.3185	0.9164
entopeduncular nucleus	sham – L-DOPA naïve	42	0.5710	0.5710
	sham – mild AIMs	42	1.281	0.3715
	sham – severe AIMs	42	2.912	0.0227 *
	mild AIMs – severe AIMs	42	1.639	0.2918
subthalamic nucleus	sham – L-DOPA naïve	33	2.275	0.0582
	sham – mild AIMs	33	4.365	0.0005 ***
	sham – severe AIMs	33	4.237	0.0005 ***

	group comparisons	degrees of freedom	t ratio	P value
subthalamic nucleus	mild AIMs – severe AIMs	33	0.04124	0.9674
substantia nigra				
pars compacta	sham – L-DOPA naïve	29	0.9302	0.5903
	sham – mild AIMs	29	1.689	0.3494
	sham – severe AIMs	29	1.290	0.5018
	mild AIMs – severe AIMs	29	0.2208	0.8268
pars reticulata	sham – L-DOPA naïve	30	1.080	0.7441
	sham – mild AIMs	30	0.1566	0.9957
	sham – severe AIMs	30	0.2074	0.9957
	mild AIMs – severe AIMs	30	0.04638	0.9957
VA/VL thalamus	sham – L-DOPA naïve	41	0.5965	0.5541
	sham – mild AIMs	41	1.041	0.5157
	sham – severe AIMs	41	2.697	0.0398 *
	mild AIMs – severe AIMs	41	1.652	0.2860

Paired *t* tests were performed for each region of interest. To correct for multiple Student *t* tests, the Holm-Sidak correction was applied to adjust *P* values. AIMs, abnormal involuntary movements; L-DOPA, L-3,4-dihydroxyphenylalanine. *N*=5-8 per group. Sham: sham-vehicle (Group A); L-DOPA naïve: lesion-vehicle (Group B); mild AIMs: lesion-L-DOPA-mild (Group C); severe AIMs: lesion-L-DOPA-severe (Group D). *: $P < 0.05$ compared to sham-lesioned group. ***: $P < 0.001$ compared to sham-lesioned group.

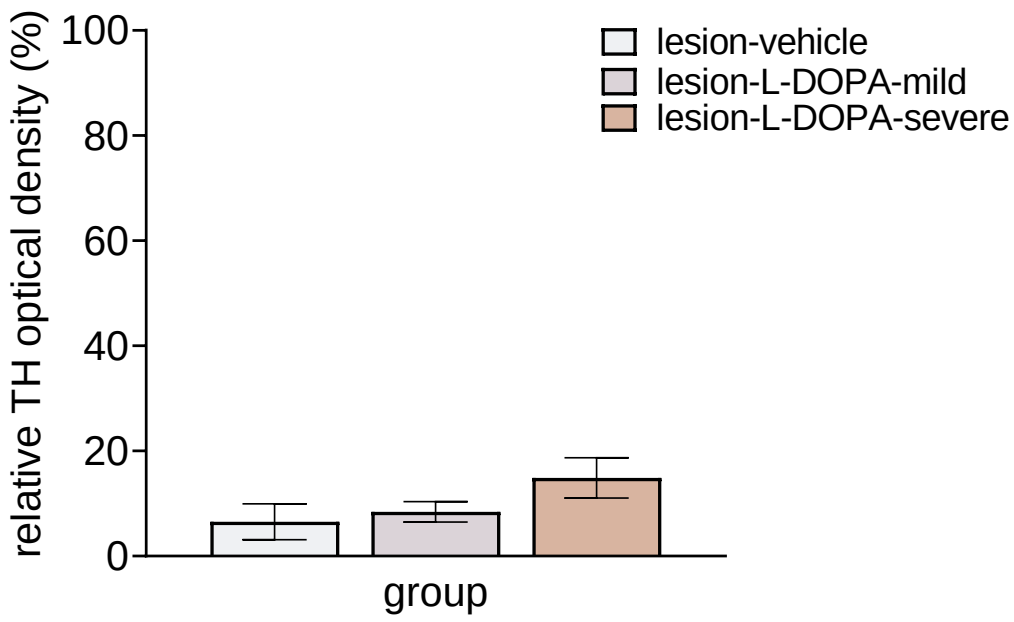
Table S4.

Summary of multiple Student *t* tests for [^{3}H]GR65630 binding levels contralateral to lesion

	group comparisons	degrees of freedom	t ratio	P value
primary motor cortex	sham – L-DOPA naïve	41	0.1048	0.9643
	sham – mild AIMs	41	0.2407	0.9643
	sham – severe AIMs	41	0.9425	0.7272
	mild AIMs – severe AIMs	41	1.203	0.6588
basal ganglia				
dorsolateral striatum	sham – L-DOPA naïve	38	0.3253	0.9838
	sham – mild AIMs	38	0.5443	0.9716
	sham – severe AIMs	38	0.2704	0.9838
	mild AIMs – severe AIMs	38	0.2486	0.9838
globus pallidus	sham – L-DOPA naïve	35	1.015	0.5489
	sham – mild AIMs	35	2.016	0.1907
	sham – severe AIMs	35	1.214	0.5489
	mild AIMs – severe AIMs	35	0.5897	0.5592
entopeduncular nucleus	sham – L-DOPA naïve	41	0.3938	0.9910
	sham – mild AIMs	41	0.3990	0.9910
	sham – severe AIMs	41	0.05054	0.9910
	mild AIMs – severe AIMs	41	0.3177	0.9910
subthalamic nucleus	sham – L-DOPA naïve	30	3.951	0.0009 ***
	sham – mild AIMs	30	7.589	<0.0001 ****
	sham – severe AIMs	30	4.990	0.0001 ****

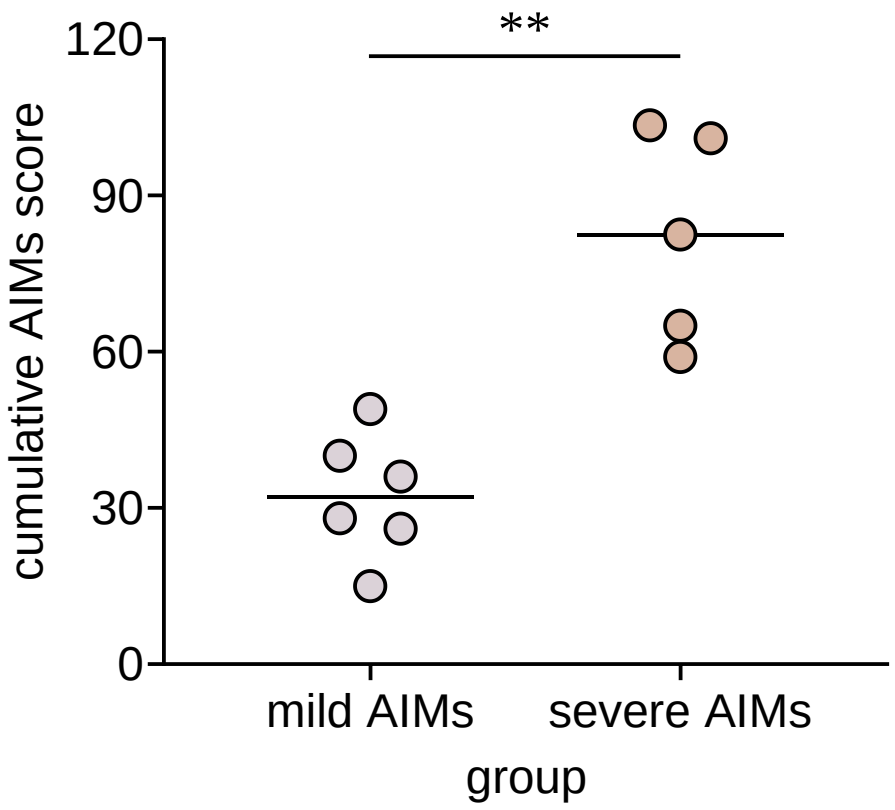
	group comparisons	degrees of freedom	t ratio	P value
subthalamic nucleus	mild AIMs – severe AIMs	30	2.845	0.0079 †
substantia nigra				
pars compacta	sham – L-DOPA naïve	31	0.5881	0.5607
	sham – mild AIMs	31	1.235	0.4009
	sham – severe AIMs	31	1.862	0.2009
	mild AIMs – severe AIMs	31	2.696	0.0442 †
pars reticulata	sham – L-DOPA naïve	29	0.6236	0.8769
	sham – mild AIMs	29	0.6790	0.8769
	sham – severe AIMs	29	0.3606	0.8769
	mild AIMs – severe AIMs	29	0.9490	0.8220
thalamus	sham – L-DOPA naïve	42	0.7874	0.8709
	sham – mild AIMs	42	0.6517	0.8709
	sham – severe AIMs	42	0.8493	0.8709
	mild AIMs – severe AIMs	42	0.2225	0.8709

Paired *t* tests were performed for each region of interest. To correct for multiple Student *t* tests, the Holm-Sidak correction was applied to adjust *P* values. AIMs, abnormal involuntary movements; L-DOPA, L-3,4-dihydroxyphenylalanine. *N*=5-8 per group. Sham: sham-vehicle (Group A); L-DOPA naïve: lesion-vehicle (Group B); mild AIMs: lesion-L-DOPA-mild (Group C); severe AIMs: lesion-L-DOPA-severe (Group D). ***: $P < 0.001$ compared to sham-lesioned group. ****: $P < 0.0001$ compared to sham-lesioned group. †: $P < 0.05$ compared to mild AIMs 6-OHDA-lesioned group.



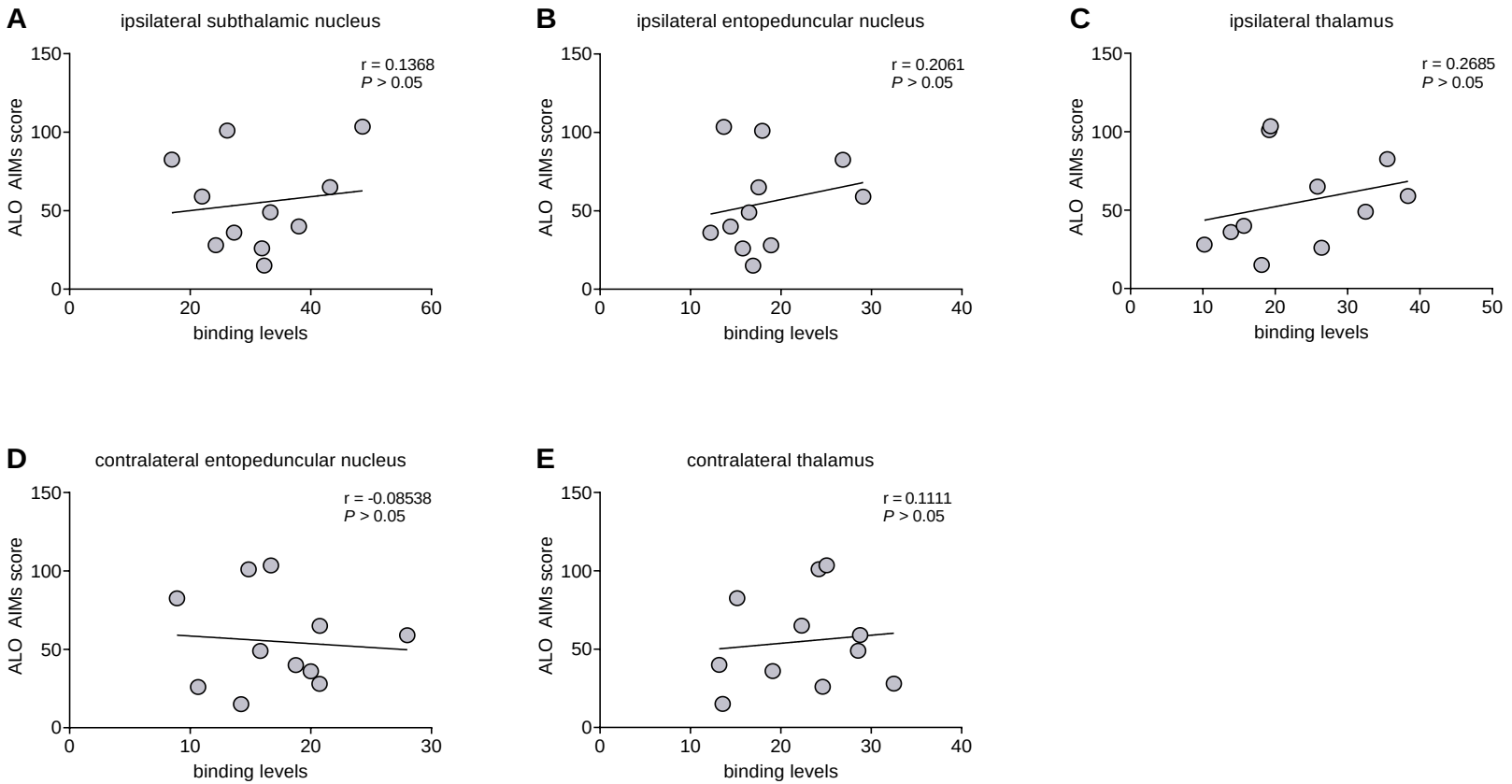
Supplementary Fig. 1. Comparable relative TH optical density in the striatum of 6-OHDA-lesioned rats

There were no significant differences in relative TH densitometry between 6-OHDA-lesioned animals treated with L-DOPA vehicle and those treated with L-DOPA and exhibiting mild or severe AIMs. TH, tyrosine hydroxylase. *N*=7 in the lesion-vehicle group; *N*=5 in the lesion-L-DOPA-mild group; *N*=6 in the lesion-L-DOPA-severe group.



Supplementary Fig. 2. Scatter diagram of cumulative AIMs scores of 6-OHDA-lesioned animals administered L-DOPA.

Animals in the severe AIMs group (median = 82.5) exhibited significantly higher AIMs scores than those in the mild AIMs group (median = 32.0). AIMs: abnormal involuntary movements; L-DOPA: L-3,4-dihydroxyphenylalanine. *N*=5 in the lesion-L-DOPA-mild group; *N*=6 in the lesion-L-DOPA-severe group. **: $P < 0.01$.



Supplementary Fig. 3. No correlation between mean ALO AIMs scores and [^{3}H]GR65630 binding levels in the ipsilateral subthalamic nucleus, entopeduncular nucleus, and thalamus.

[^{3}H]GR65630 binding in the ipsilateral subthalamic nucleus (**A**), entopeduncular nucleus (**B**), and thalamus (**C**) was not correlated with mean ALO AIMs scores of 6-OHDA-lesioned animals. Similarly, binding in the contralateral entopeduncular nucleus (**D**) and thalamus (**E**) of 6-OHDA-lesioned animals did not correlate with ALO AIMs scores. 6-OHDA, 6-hydroxydopamine; AIMs, abnormal involuntary movements; ALO, axial, limbs and oro-lingual. *N*=11.

Transition 5: Coupling co-registration of imaging data with frameless stereotaxic navigation to localise surgical targets in the common marmoset

The previous chapters have focussed on the evaluation of symptomatic treatments in PD using neurotoxin models that induced the rapid degeneration of nigrostriatal dopaminergic neurons to reproduce the sporadic form of the disease [1024]. Whereas these models have enhanced our understanding of disease processes (e.g., oxidative stress, inflammatory responses, cell death), they fail to recapitulate the processes that are involved in the neurodegeneration of the disease. For instance, the 6-OHDA-lesioned rat [1025] and MPTP-lesioned non-human primate [1026] models do not produce the formation of Lewy-like inclusions that are observed in PD [1027, 1028]. As a result, neurotoxin-based models provide little utility to assess disease-modifying treatments. Genetic models are also inadequate as they induce little to no loss of dopaminergic neurons and Lewy pathology [1029].

As iterated above, the development of animal models that can faithfully reproduce key features of PD will aid the development of therapies that can mitigate PD. Selection of the animal species is paramount in this endeavour. Rodent models may not be good predictors of clinical success, considering the failure of clinical development of drug candidates, such as BIIB054 (NCT03318523) and nilotinib [1030]. In contrast, non-human primates share greater genetic, neuroanatomical, and neurophysiological similarity to humans over rodents [1031], and these characteristics lend to the superior translational potential of these species as relevant models of PD. Therefore, we have elected to use the common marmoset, a non-human primate species, to establish a new animal model of PD that recapitulates the degeneration that occurs in patients.

The common marmoset is an increasingly popular model in neuroscience research [1032]. Whereas the success of stereotaxic procedures depends upon accurate localisation of surgical targets, traditional two-dimensional stereotaxic brain atlases of marmosets compromise accuracy due to the small sample size employed [1033-1035]. To address the heterogeneity in skull and brain size between marmosets, co-registration of imaging data of individual subjects allows for more precise surgical targeting. Coupled with frameless stereotaxic navigation, this paradigm shift improves upon limitations of traditional frame-based approaches [1036], while demonstrating equivalent accuracy and improving upon safety and patient outcomes [1037, 1038].

In Chapter 7, subject-specific registration of imaging modalities, *i.e.*, from computed topography, magnetic resonance imaging and positron emission topography, was coupled with frameless stereotaxic navigation to localise the putamen in two common marmosets for injection of alpha-synuclein pre-formed fibrils. Importantly, this was the first time that the methodology of frameless stereotaxic navigation, particularly generation of the laser point cloud and unique coordinate system, was described in non-human primates. Subsequent registration of the point cloud to corresponding CT images generated a three-dimensional reconstruction of the marmoset skull surface, which helped to determine the optimal path to the putamen. Previous studies reported nigro-striatal denervation following intra-striatal injection of alpha-synuclein pre-formed fibrils in rodents and non-human primates [995, 998, 1010, 1011]. In lines with these results, four months following surgery, there was evidence of degeneration of the nigro-striatal system, which suggests that the marmoset putamen was accurately targeted during surgery. In contrast to other methods, our approach considers the unique characteristics of each marmoset's skull and brain; the detailed descriptions here, may be beneficial in enhancing the accuracy of stereotaxic procedures and to

assess endpoints for longitudinal studies. Our results are timely given the increasing popularity of marmosets to model neurological and neurodegenerative diseases.

Chapter 7 - Co-registration of Imaging Modalities (MRI, CT and PET) to Perform Frameless Stereotaxic Robotic Injections in the Common Marmoset

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Short title: Stereotaxy in the marmoset

Chapter 7 Abstract

The common marmoset has emerged as a popular model in neuroscience research, in part due to its reproductive efficiency, genetic and neuroanatomical similarities to humans and the successful generation of transgenic lines. Stereotaxic procedures in marmosets are guided by 2D stereotaxic atlases, which are constructed with a limited number of animals and fail to account for inter-individual variability in skull and brain size. Here, we developed a frameless imaging-guided stereotaxic system that improves upon traditional approaches by using subject-specific registration of computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET) data to identify a surgical target, namely the putamen, in two marmosets. The skull surface was laser-scanned to create a point cloud that was registered to the 3D reconstruction of the skull from CT. Reconstruction of the skull, as well as of the brain from MR images, was crucial for surgical planning. Localisation and injection into the putamen was done using a 6-axis robotic arm controlled by a surgical navigation software (Brainsight™). Integration of subject-specific registration and frameless stereotaxic navigation allowed target localisation specific to each animal. Injection of alpha-synuclein fibrils into the putamen triggered progressive neurodegeneration of the nigro-striatal system, a key feature of Parkinson's disease. Four months post-surgery, a PET scan found evidence of nigro-striatal denervation, supporting accurate targeting of the putamen during co-registration and subsequent surgery. Our results suggest that this approach, coupled with frameless stereotaxic neuronavigation, is accurate in localising surgical targets and can be used to assess endpoints for longitudinal studies.

Key words: marmoset, stereotaxy, robotic surgery, magnetic resonance imaging, computed tomography, positron emission tomography.

Chapter 7 Introduction

In recent years, the New World primate common marmoset (*Callithrix jacchus*) has emerged as a popular model in neuroscience research over Old World monkeys (Hashikawa et al., 2015, Mitchell and Leopold, 2015). This interest can be attributed to its smaller size, a lissencephalic cortex that permits a wider variety of manipulations, and advances in genetic engineering technologies (Silva, 2017, Sasaki, 2019). Marmosets also have a rapid developmental cycle with a gestation period of 5 months and sexual maturity reached by 18 months (Abbott and Hearn, 1978, Tardif et al., 2003). These features offer key advantages over Old World monkeys in studies on development and ageing (Ross and Salmon, 2019) and have led to insights into the development of the primate neocortex (Homman-Ludiye and Bourne, 2017, Homman-Ludiye and Bourne, 2021). Moreover, the marmoset is an excellent model for studies on the visual cortex (Solomon and Rosa, 2014, Mitchell and Leopold, 2015), motor cortex (Bakola et al., 2015, Walker et al., 2017), auditory cortex (Philibert et al., 2005), as well as somatosensory cortex (Kaas, 2021). Owing to its highly developed prefrontal cortex, the marmoset is also a suitable model for neuropsychiatric disorders (Kaasinen et al., 2000, Miller et al., 2016, Oikonomidis et al., 2017). Recent efforts towards characterising cortico-cortical (Majka et al., 2020) and cortico-subcortical anatomical connections (Hori et al., 2020b) of the marmoset brain, as well as network and functional connectivity (Liu et al., 2019a, Hori et al., 2020a, Theodoni et al., 2021), implicate their utility for neuroinformatics.

Stereotaxic procedures in the marmoset, including electrophysiological recordings (Debnath et al., 2018, Pomberger and Hage, 2019), lesion, and tracer mapping studies (Liebetanz et al., 2002, Abe et al., 2017) have been guided by two-dimensional (2D) brain atlases. However, the majority of atlases have collected data from a restricted number of animals

(Stephan et al., 1980, Tokuno et al., 2009, Paxinos et al., 2012) or a single sex (Stephan et al., 1980), which fail to account for the variable skull and brain size between animals. Whilst recent atlases of the marmoset brain have employed relatively larger sample sizes (N = 5-20) to address individual variability (Liu et al., 2018, Risser et al., 2019, Majka et al., 2021), some templates only collected data from a single subject (Liu et al., 2018, Risser et al., 2019), a common practice of stereotaxic atlases. Moreover, most of these marmoset brain atlases have only localised cortical areas (Majka et al., 2016, Risser et al., 2019, Majka et al., 2020, Majka et al., 2021), which offers limited use for targeting subcortical structures. Therefore, the success of procedures that depend upon accurate localisation of targets and replication in different animals may be compromised by the use of traditional stereotaxic atlases and requires a more reliable and robust approach to identify target structures.

The paradigm shift from frame-based stereotaxic approaches to frameless stereotaxic neuronavigation in research and clinical settings (Moorthy et al., 2016) has improved upon limitations of traditional approaches such as difficulty to access large areas of the brain, particularly, subcortical targets, scanning with a frame in place, and the lack of flexibility to change target coordinates without modifying the target approach (Frey et al., 2004). Moreover, frameless stereotaxic neuronavigation, as seen in humans, has demonstrated equivalent accuracy to frame-based stereotaxy (Mascott, 2006) and allowed for real-time feedback during procedures (Gempt et al., 2012), which has resulted in improved safety and patient outcome by reducing surgical time (Dorward et al., 2002) and associated morbidity and mortality (Golfinos et al., 1995, Carvalho et al., 2009). The Brainsight™ neuronavigation (Vet Robot) system uses imaging data of an individual animal for precise anatomical brain targeting. Briefly, a 3D reconstructed skull surface of the marmoset, derived from the MRI or CT, is used along with a

laser light source to identify homologous points on the actual skull of the animal in surgery within a common coordinate space (Orringer et al., 2012). By using 3D coordinates that are unique to each animal, precision in localising targets is enhanced, which is crucial in procedures that use marmosets, whereas conventional stereotaxic atlases fail to address inter-individual variation between animals (Tokuno et al., 2009, Paxinos et al., 2012).

In the present study, we sought to describe the co-registration of imaging modalities to precisely locate a target in the marmoset brain for injection of alpha-synuclein pre-formed fibrils. This is the first time that the methodology behind frameless stereotaxic neuronavigation is presented in non-human primates. Moreover, generation of the skull point cloud and subsequent registration to CT allowed 3D reconstruction of the skull surface, which facilitated surgical planning to determine the approach to entry point and to optimise accurate reaching of surgical coordinates. The alpha-synuclein pre-formed fibrils model is an emerging model of Parkinson's disease and intra-striatal injection of these fibrils in rodents and non-human primates has induced progressive degeneration of the nigro-striatal system (Luk et al., 2012, Shimozawa et al., 2017, Chu et al., 2019, Patterson et al., 2019). Four months following the surgery, the extent of nigro-striatal denervation was assessed to provide a proxy of accurate target localisation.

Chapter 7 Experimental Procedures

Animals

Male common marmosets (*Callithrix jacchus*, $N = 2$, identified individually as subjects A and B) weighing 376 g and 454 g, respectively, were housed in pairs under conditions of controlled

temperature (24 ± 1°C), humidity (50 ± 5%) and a 12 h light/dark cycle (07:15 a.m. lights on). Age during surgical procedure was 2 years and 11 months for subject A and 5 years and 5 months for subject B. Animals had unlimited access to water and were fed twice daily with food, including fresh fruits, nuts, boiled eggs, boiled pasta, etc. Their home cages were enriched with perches and primate toys. Animals were acclimatised to handling and transfer to observational cages prior to the start of studies. All procedures were approved by the McGill University and Montreal Neurological Institute-Hospital (The Neuro) Animal Care and Use Committees, in accordance with guidelines established by the Canadian Council on Animal Care.

Computed topography scan

Animals were sedated with ketamine [20 mg/kg, intra-muscular (i.m.)] and scanned in a supine position in the sagittal plane using a Vimago L computed tomography (CT) scanner (Epica Medical Innovations, USA) according to a standard protocol: helical scanning, reconstructed slice thickness of 0.99 mm, 80 kV, 60 mA and 7 ms exposure with a voxel size of 0.30 mm × 0.30 mm × 0.30 mm. 398 CT images were acquired over 3 min.

Magnetic resonance imaging

Acquisition. Marmosets were lightly sedated with ketamine (20 mg/kg, i.m.). High-resolution T1-weighted 3D images were obtained from a 3 Tesla Siemens PrismaFit Scanner (Siemens Medical Systems, Germany) using a Magnetisation Prepared Rapid Acquisition Gradient Echo (MPRAGE) sequence (Mugler III and Brookeman, 1990) with the following parameters: 0.36 mm isotropic resolution (interpolated to 0.18 mm isotropic), repetition time (TR) = 2.2 s, echo time (TE) = 2.58 ms, inversion time (TI) = 706 ms, flip angle = 8 degrees,

matrix size = 256 × 324 × 384, three averages, time ~ 18 min (Hayashi et al., 2021). Images were collected using a marmoset-specific 16-channel receive-only marmoset head coil (Takashima Seisakusho Corporation, Japan) (Hori et al., 2018) with the marmoset positioned in an MRI-compatible testing chair that was designed specifically for the above coil (Rogue Research Inc, Canada).

Fixation of marmoset for MRI scanning. As illustrated in Fig. 1A, a 16-channel receive coil (inner diameter 38 mm × 48 mm × 28 mm) was placed over the head of the animal while the body was supported in the MRI-compatible chair. The chair (Fig. 1B, 1C) consisted of a polycarbonate frame and a plastic cradle to hold the head in place. Additional elastic straps were used to hold the forehead, arms and legs in place within the chair. The chair was then positioned into an MRI-compatible sled that fit into the bore in front of the MRI bed. This system allowed the animal to be oriented 15º from supine while the head of the animal remained at isocentre.

Processing. CT and T1-weighted MR images were converted to MINC format and then, MRI and CT data were overlaid using MINC tools (V2; https://bic-mni.github.io).

Positron Emission Tomography

Radioligand synthesis. The radiotracer was synthesised according to standard methods with a radiochemical purity of 98.57 to 99.72%. [^{11}C]-labelled carbon dioxide ([^{11}C]CO_2) was produced via the $^{14}N(p, \alpha)^{11}C$ reaction by proton bombardment of a 150 mL nitrogen gas target (gas mixture is 0.5% oxygen in nitrogen, research grade 99.9999%). The target gas was released to a methyl iodide (MeI) synthesis module in a closed hotcell where it was captured on a column

of activated molecular sieves and transformed to MeI gas method. After delivery of the radioactivity in the synthesis box, the hydrogenation process was started using H_2 gas and Ni-catalyst at 425 °C. The produced methane was transferred to the iodination unit where iodination was accomplished in the presence of I_2 at 740 °C. The produced MeI was transferred to the reactor. The reactor contained 0.5 mg desmethyl dihydrotetrabenazine (DTBZ) and 5 μL 5N sodium hydroxide (NaOH) in 0.5 mL dimethyl sulfoxide (DMSO). The reaction vessel was kept at room temperature for 5 min to form [^{11}C]DTBZ. 1.5 mL of high-performance liquid chromatography (HPLC) solvent mixture (0.01 M NH_4COONa/AcCN 65/35) was then added to this vessel and the resulting mixture was transferred into the injector loop of the HPLC system and purified on a Luna 10 μm C-18 column (250 mm × 10 mm, Phenomenex Inc, USA) at a flow rate of 5 mL/min. The desired product eluted at a retention time of 12-14 min (gamma peak). Unreacted MeI and reaction by-products eluted at earlier retention times, and were transferred into the waste container. The product peak was collected into a vial containing 15 mL of water. The solution was passed through a C18 Sep-Pak cartridge (Waters Corp, Canada). The cartridge was washed with an additional 10 mL of water and the product was then eluted from the cartridge into a product vial with 0.5 mL of ethanol followed by 9.5 mL of sterile phosphate buffer.

Acquisition. Positron emission tomography (PET) scans were performed using a CTI Concord R4 micro-PET scanner (Siemens CTI, USA) with a spatial resolution at the centre of the field of view of 1.84 mm full width at half maximum (FWHM) in the axial direction. Under anaesthesia with 2% isoflurane inhalation at 0.5 L/min oxygen flow, animals were placed on the PET scanner bed in a supine position. The brain was positioned in the centre of the field of view.

Animal A was injected with 15.614 MBq of [^{11}C]DTBZ for the baseline scan with a molar activity of 27.983 GBq/μmol, while animal B was injected with 23.569 MBq for the baseline scan with a molar activity of 25.386 GBq/μmol and 16.391 MBq for the 4 months post-op scans with a molar activity of 62.112 GBq/μmol, respectively. 90-minute dynamic PET scans were acquired with a concomitant bolus injection of [^{11}C]DTBZ via the tail vein. Then, 10-min transmission scans with a ^{57}Co rotating point source were acquired. All PET data were reconstructed using a maximum *a posteriori* (MAP) algorithm into 8 × 30 s, 6 × 60 s, 5 × 2 min, and 14 × 5 min frames with voxel size equal to 0.6 mm × 0.6 mm × 1.2 mm and 128 × 128 × 63 X, Y, and Z dimension size. Dead time, decay, scatter, random, and attenuation corrections were applied.

Processing. To aid the registration between the individual MRI and online template (http://www.marmosetbrain.org/reference) (Majka et al., 2016), we first generated a group averaged MRI template (Nitzsche et al., 2015). This group averaged template was then registered and transformed to the online template via affine and nonlinear transformations based on Advanced Normalisation Tools (ANTs). Following MRI registration to the online template, the individual PET was registered to the individual MR image based on a rigid body transformation using ANTs and then normalised to the online template based on the aforementioned transformations. Regions of the online template atlas such as the caudate nucleus and putamen were subsequently transformed back into the native PET space. Using Logan reference region graphical analysis with the cerebellum as a reference region (Logan et al., 1990), the outcome measure, distribution volume ratio (DVR) was calculated in the target regions (Doudet et al., 2006). The final PET images were smoothed using 1.4 mm FWHM

gaussian kernels. T1-weighted MR images were processed using the ANTs pipeline and reconstructed PET images were processed using the MINC-toolkits and PMOD 3.9 software.

Surgical planning

To assist with surgical planning, targets and trajectories to reach within the putamen were identified and saved in the Brainsight™ Vet neuronavigation software prior to surgery. In Fig. 2A, C, the injection trajectory through the putamen in reference to the caudate nucleus of subjects A and B was reconstructed in 3D from MR images. As shown in Fig. 2B, D, T1-weighted coronal MR images of subjects A and B are presented with the injection trajectory through the putamen. All regions of interest, including the brain, skull, and striatum were reconstructed in 3D and visualised in the software. A 3D printed model of the skull of each animal was constructed to scale from CT scans prior to surgery to go through the surgical rehearsal of the planned trajectories to ensure compatibility of the stereotaxic frame with the robotic arm and the attachments to the robotic arm such as the injector system (Fig. 3A-C).

Synthesis of alpha-synuclein pre-formed fibrils

Recombinant human alpha-synuclein was expressed using the Clearcoli™ BL21 (DE3) strain to minimise the presence of endotoxins because marmosets are highly vulnerable to endotoxins (unpublished observations). The pre-formed fibrils were generated from the purified alpha-synuclein based on a protocol (Jin et al., 2021, Tavassoly et al., 2021). The morphology, size and uptake of the fibrils were validated by electron microscopy, dynamic light scattering and immunofluorescence.

Surgical procedure

On the day of surgery, brains of animals were displayed as a 3D reconstruction in all anatomical planes along with the original DICOM MRI data, while the target site and trajectory into the putamen were also visualised. The path to target was determined using the neuronavigation software as previously described and displayed on the computer screen in real-time. The setup of the frameless stereotaxic neuronavigation system on the day of surgery is displayed in Fig. 3A-C.

Prior to surgery, animals were injected with ketamine (20 mg/kg, i.m.), atropine (0.5 mg/kg, i.m.) and midazolam (0.5 mg/kg, i.m.). After intubation, anaesthesia was maintained with 2-3% isoflurane inhalation with 100% oxygen administered at a rate of 1 L/min and animals were positioned in a custom stereotaxic frame that accompanies the Brainsight™ Vet Robot system. The physiological state (oxygen saturation, respiration rate and volume, pulse rate, temperature and end-tidal carbon dioxide) of animals was assessed by a monitoring system (Lifewindow LW9xVet monitor, Digicare Biomedical Technology, USA). A midline scalp incision was made to expose the skull, and soft tissue and muscle were retracted from the skull surface. The skull was then scanned with a red laser light source of 635 nm that was attached to the end of a robotic arm. The laser points reflected off the skull formed a point cloud that was then co-registered to the 3D reconstruction of the skull from the CT, as shown in Fig. 4, based on techniques similar to profilometry, using a least squares optimisation algorithm (Levenberg, 1944, Marquardt, 1963). Two landmarks were identified on the 3D reconstructed skull in Brainsight™ and the same homologous points were later identified with the laser source during registration of the animal's skull in the operating theatre. These points were considered the initial guess for the start of the iterative process of finding the true fit between the two data sets

(3D reconstruction and point cloud) using the Levenberg-Marquardt algorithm (Levenberg, 1944, Marquardt, 1963).

Based on the alignment of the skull with the scan, the trajectory path to the injection sites was determined in the software. The tip of the syringe and shaft were calibrated in the software. A 1 mm hole was drilled through the skull surface with a power drill (Foredom, USA), operated by a foot pedal that attached to the end of a robotic arm. Subsequently, the attachment to the robotic arm was exchanged for an adaptor that held a 100 µL Hamilton Syringe connected to a Quintessential Stereotaxic Injector (Stoelting Co, USA). Animals received 3 injections of alpha-synuclein pre-formed fibrils (1 mg/mL) into the left putamen at three depth levels (15 µL, 20 µL, 15 µL) for 50 µg of total protein. The syringe was left in place for an additional 5 min between injections and prior to slow retraction of the needle to minimise reflux along the needle track. Throughout surgery, animals received cephazolin (22 mg/kg, intra-venously) as antibiotic treatment, as well as one injection the next day. At the end of surgery, the incision was closed with staples and sprayed with OpSite Spray and animals were administered dexamethasone (1 mg/kg, i.m.) to prevent brain swelling and buprenorphine (0.01 mg/kg, i.m.) to minimise post-surgical pain.

Chapter 7 Results

Differences in skull and brain size of marmosets

From CT images of the skull, subject A and B measured 34.77 mm and 23.59 mm in length from the rostral end of the frontal bone to the caudal end of the parietal bone; the width between

the zygomatic arches measured 24.99 mm and 22.23 mm, respectively. As shown in MR images of subject A in Fig. 5A and subject B in Fig. 5CB, the bilateral volume of the putamen measured 104.0 mm^3 and 97.2 mm^3, respectively, a 7% difference.

Overlay of CT and T1-weighted MR images of marmosets

In Fig. 6A, 6C, CT scans superimposed with the MRI scans of the marmoset skulls of subjects A and B are displayed. Co-registration was verified by investigators as the CT images matched well to MR images. In Fig. 6B, 6D, MR images of subjects A and B are shown.

Overlay of T1-weighted MRI and [^{11}C]DTBZ images of marmosets

In Fig. 7A, the averaged para-sagittal and coronal PET scans of marmosets A and B to vesicular monoamine transporter 2 ($VMAT_2$) using the radiotracer [^{11}C]DTBZ are presented. Following co-registration of MR images to PET images, the superimposed images are presented in Fig. 7B, which demonstrates high uptake of [^{11}C]DTBZ that is specific to the striatum.

PET assessment of nigro-striatal denervation

Four months following the surgery, a PET scan of subject B was performed to assess the extent of nigro-striatal denervation. In Table 1, changes in DVR of the ipsilateral putamen, ipsilateral caudate nucleus, midbrain, 4 months post-op compared to baseline are presented.

Chapter 7 Discussion

In the present study, we have demonstrated that frameless stereotaxic neuronavigation using Brainsight™ is a promising approach to localise and perform injections in the target structure, the putamen, of two different marmosets. In particular, this is the first time that 3D reconstruction of the skull based on registration of the 3D point cloud of the skull to corresponding CT images has been described in non-human primates. Based on the co-registration of CT and MR images, the path to target in the putamen was calculated specifically for each individual animal. The 6-axis Brainsight™ robotic arm also automated stereotaxic guidance to localise and inject alpha-synuclein pre-formed fibrils into the putamen with minimal human intervention. Moreover, by co-registering MRI and PET images, the degree of nigro-striatal denervation was assessed at 4-months post-op.

Renewed interest in marmosets for neuroscience research, including large-scale initiatives to map their brain (Okano et al., 2016, Liu et al., 2020), depends on accurate localisation of brain structures. While these endeavours have largely relied on 2D stereotaxic atlases of the marmoset brain (Stephan et al., 1980, Tokuno et al., 2009, Paxinos et al., 2012), they fail to account for the variability in brain and skull size amongst animals due to a limited sample size (François et al., 1996, Deogaonkar et al., 2005, Miocinovic et al., 2007). These issues have led to the advent of averaged templates of the marmoset brain (Liu et al., 2018, Risser et al., 2019, Majka et al., 2021), which incorporate individual variability and consequently, provide a more accurate localisation of brain structures. However, they are less informative about subcortical structures and as such, a deviation of a few mm can significantly alter accurate localisation of targets, especially for small and deep nuclei (Basso et al., 2021). While we found a 32% and 11% difference in skull length and width between the two marmosets

studied, these findings are at odds with an earlier study that reported a lack of significant difference in skull measurements between female and male marmosets (Casteleyn et al., 2012). On the other hand, we reported a 7% difference in putamen volume between the two animals, which was consistent with other groups that found variations in marmoset brain sizes (Liu et al., 2021), as well as significant discrepancies in individual compartment size from template brain (Lin et al., 2019). It is important to note that our results were obtained from a small number of animals (N = 2) and await validation with a larger sample size and by independent groups. However, the paucity of studies on the marmoset skull and brain renders it difficult to comment on the relevance of our results. Nonetheless, an approach that takes into consideration the unique characteristics of a marmoset's skull and brain, could greatly enhance the accuracy and success of stereotaxic procedures.

Accurate identification of target coordinates may be compromised by individual variability in stereotaxic coordinates of landmarks in the marmoset skull (Hikishima et al., 2011), as well as operator-induced errors (Henderson et al., 2014). Multi-modality medical image registration of subjects to MR images is an established method to localise surgical targets in rodents and humans (Vaquero et al., 2001, Liu et al., 2019b). Extending its use in non-human primates may correct for individual skull and brain variability and achieve a higher degree of accuracy and precision than traditional stereotaxic atlases. An imaging-guided stereotaxic system to localise anatomical brain structures based on MR images has been published in the macaque (Frey et al., 2004) and marmoset (Mundinano et al., 2016). However, there are notable methodological differences from these previous studies, which highlight the contribution of our findings. While these studies relied on implantation of fiducial markers on the skull and a cranial marker on the sagittal suture, our approach was less invasive by using skull landmarks.

Moreover, the marmoset study required animals to be placed in an MRI-compatible stereotaxic frame during scans and subsequent surgical procedure (Mundinano et al., 2016). In addition to a frame-based system, other differences include calculation of stereotaxic coordinates, which becomes more complicated for trajectories that are not perpendicular to the skull or imaging plane (Basso et al., 2021), manual alignment of MR images with the animal's position in the stereotaxic frame based on fiducial markers. Due to the considerable time investment and limited ease of use of frame-based imaging, this system may not be favourable for some stereotaxic procedures and study endpoints; these shortcomings are improved upon with a frameless stereotaxic system.

The approach of a frameless imaging-guided stereotaxic system avoids targeting inaccuracies and inconveniences of manual alignment used in frame-based approaches. Thus, by using a laser to scan the marmoset skull and then construct a 3D skull point cloud to co-register with CT images, we obtained high-resolution imaging to accurately localise brain structures for surgical targeting. Importantly, subject-specific registration of imaging data circumvented issues of individual variability in skull and brain size of the two animals. Moreover, integration of Brainsight^{TM} with imaging modalities permitted automatic stereotaxic guidance, as well as injections from virtually any angle with the 6-axis robot. Due to the availability of 3D reconstructed putamen and 3D scale model of the skull, we determined a lateral approach to entry point would be optimal because it maximised targeting of the putamen. In contrast, other motorized systems offer a more limited range of motion with only 3 axes of movement. These systems also face reliability concerns due to their reliance on external skull landmarks to co-register a stereotaxic atlas to imaging data of individual animals. On the other hand, our system allowed adequate surgical planning as the optimal trajectory was verified for

feasibility and compatibility between the different components (vet robot, injector system, syringe, target and trajectory) of the setup prior to surgery. Thus, frameless imaging-guided systems allow surgical planning to surgical targets and trajectories with greater ease (Basso et al., 2021) and are associated with improvements in outcomes in non-human primates (Dubowitz and Scadeng, 2011) and in clinical practice (Käppler et al., 2015, Galvez et al., 2018).

Here, we have used subject-specific registration of CT, MRI and PET data to reliably target the putamen, as well as to determine the degree of nigro-striatal denervation. PET scans obtained four months post-op provide evidence of nigro-striatal denervation that reaches a ≈ 11% reduction in [^{11}C]DTBZ binding in the marmoset putamen. This is consistent with results obtained in the mouse, rat and non-human primate (Luk et al., 2012, Shimozawa et al., 2017, Chu et al., 2019, Patterson et al., 2019), and the extent of dopaminergic denervation specific to the putamen is likely induced by the injection of alpha-synuclein pre-formed fibrils in the putamen, which suggests that the marmoset putamen was accurately targeted during surgery. However, PET data only provides suggestive evidence of accurate surgical targeting and validation of the approach is still required, whether by means of using histology or CT imaging to visualise the needle track. On the other hand, co-registration of CT, MRI and PET images provides essential information for longitudinal studies that require precise localisation of targets at distant time points from initial imaging acquisition without the need for markers. Thus, by monitoring the progression of changes in brain innervation, this technique can be informative about selection of endpoints for terminal studies or for determining a timeline for pilot studies, and subsequently, the frequency of imaging acquisition could be modified. Moreover, procedures such as cranial implantation of electrodes or deep brain stimulation in marmosets, where targets are not reliably identified by stereotaxic atlases, might benefit from the enhanced

accuracy of this approach. Furthermore, once an experimental paradigm has been established, imaging may be used as a biomarker to assess the efficacy of therapies (Okano et al., 2016, Liu et al., 2020). In addition, procedures that target subcortical structures or targets that are smaller in volume can be more reliably targeted than when relying upon 2D stereotaxic atlases, as the variation in skull and brain size may lead to misidentification of target and compromise the success of procedures.

In summary, we have used an approach that integrates multi-modality medical imaging registration using CT, MRI and PET images with frameless stereotaxic neuronavigation to target the marmoset putamen. This approach may be beneficial for longitudinal studies to monitor endpoints at distal time points without the need of markers. Whereas 2D stereotaxic atlases and frame-based stereotaxic methods are limited in accuracy for procedures in non-human primates, the improved accuracy achieved by subject-specific registration and frameless stereotaxic neuronavigation may present a more reliable approach to procedures in non-human primates.

CONFLICT OF INTEREST STATEMENT

CK, MSK, SGN, JCG, DB, CLT, RH, KR, HB, AH, GM, JPS, WL, ECP, IS, TMD, EAF, PRN, SF and PH declare that they have no competing interests. SF is a fulltime-employee and partner in Rogue Research Inc, the manufacturer of the Brainsight^{TM} navigation system that was used for surgical procedures.

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AUTHORS' CONTRIBUTIONS

JCG, EAF and PH conceived the project. CK, SGN, JCG, AH, GM, JPS, TMD, EAF, SF and PH designed the project. SGN, DB, CLT, RH, KR, HB, WL, ECP and IS performed and collected data. CK, MSK, CLT, PRN and SF contributed to data analysis. CK wrote the first

draft of the manuscript. All the authors contributed to the final manuscript. All the authors read and approved the final manuscript.

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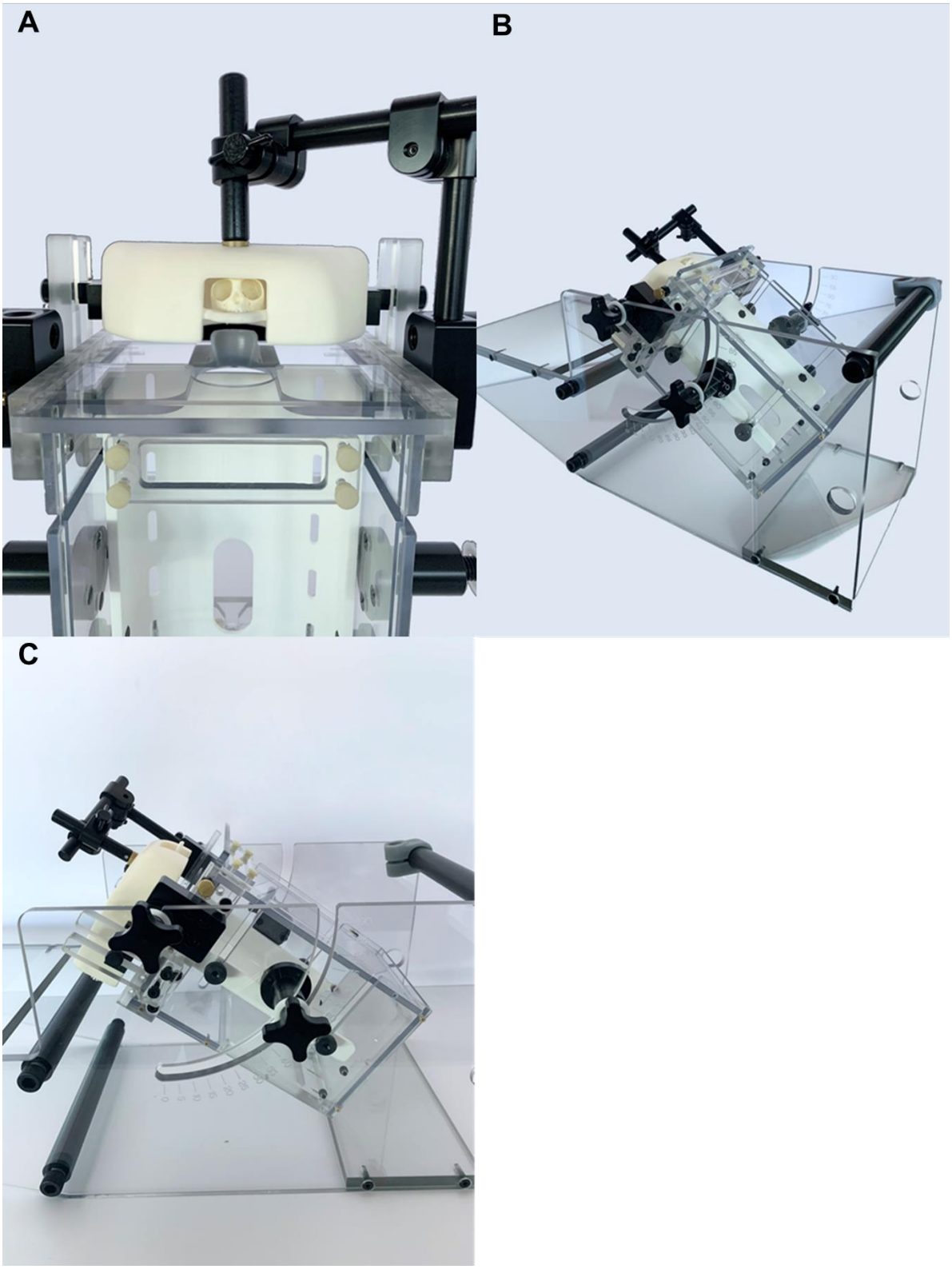
Chapter 7 Tables and Figures

Table 1. Regional changes in VMAT2 PET scan 4 months postsurgery. The VMAT2 PET scan of Subject B performed 4 months postop revealed declines in DVR in the ipsilateral putamen, caudate nucleus, and midbrain.

region	% change from DVR of baseline
ipsilateral putamen	-11%
ipsilateral caudate nucleus	-20%
midbrain	-6%
cerebellum (reference region)	-


A
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C

Fig. 1. Marmoset coil and MRI-compatible chair and sled. The 16-channel receive coil was placed over a head and body cradle that attached to the MRI-compatible chair (**A**). The MRI-compatible chair was positioned into the MRI-compatible sled that fit into the bore in front of the MRI bed (**B**). The chair consisted of a polycarbonate frame and a plastic cradle to hold the head of the animal in place. Elastic straps were used to hold the forehead, arms, and legs in place within the chair. A side view displaying how the MRI-compatible chair was fitted into the MRI-compatible sled (**C**).

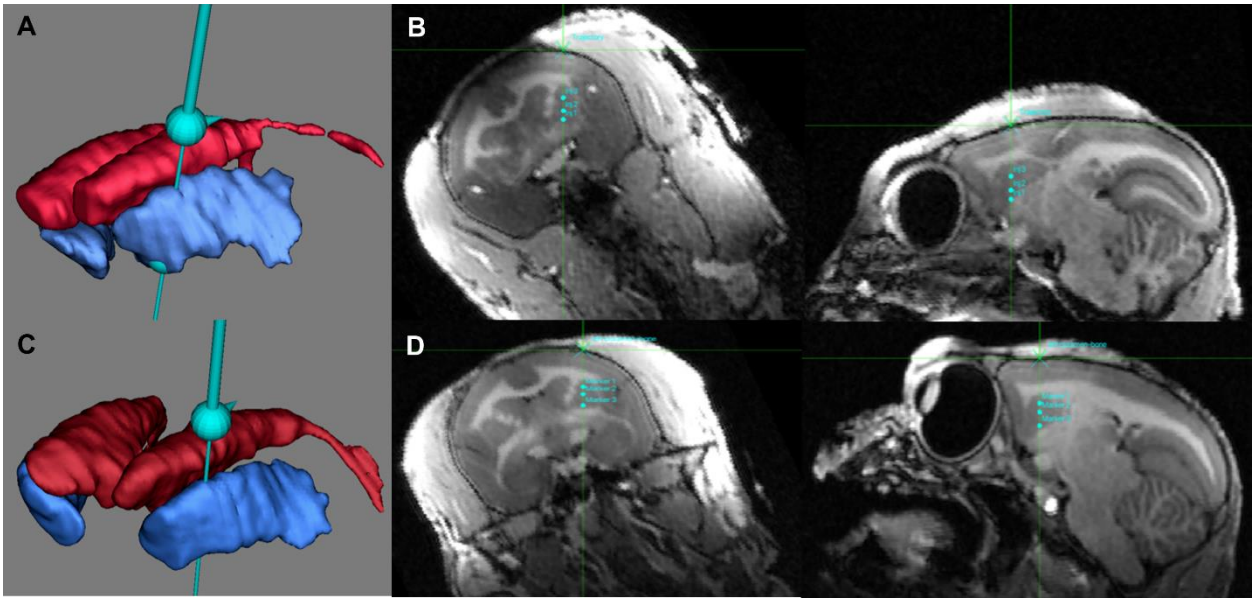

Fig. 2. Surgical planning. 3D reconstruction of the surgical target, putamen (blue), in reference to the caudate nucleus (red) in subject A from MR images (**A**). T1-weighted MR images of subject A with planned trajectory into the putamen in the coronal (left) and para-sagittal (right) planes (**B**). 3D reconstruction of the surgical target, putamen (blue), in reference to the caudate nucleus (red) in subject B from MR images (**C**). T1-weighted MR images of subject B with planned trajectory into the putamen in the coronal (left) and para-sagittal (right) planes (**D**). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

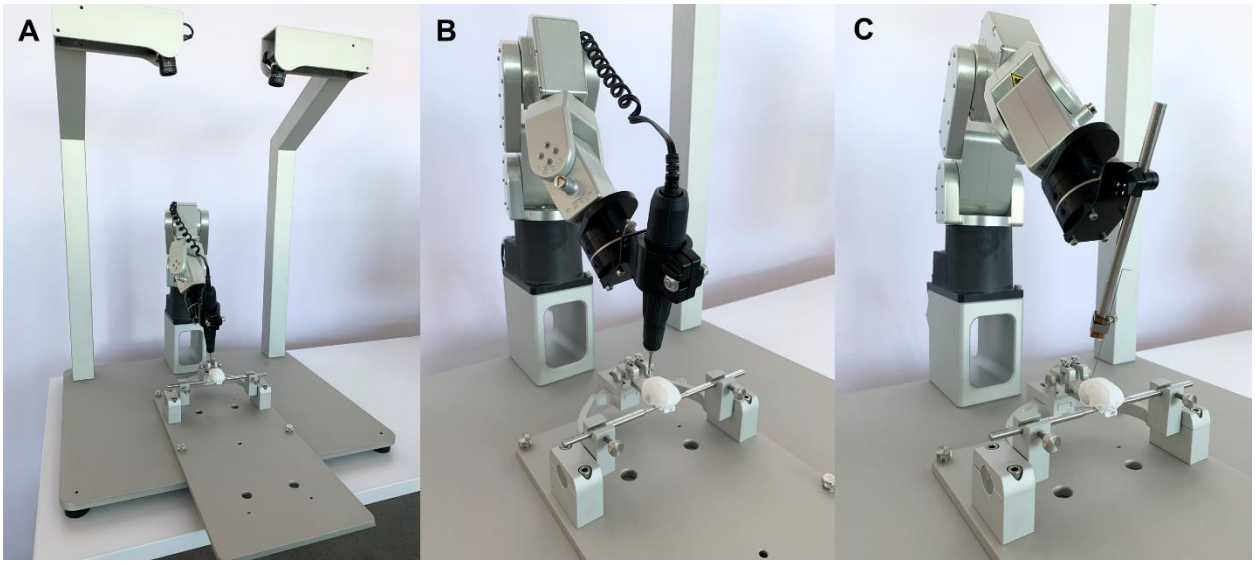

Fig. 3. Frameless stereotaxic neuronavigation surgical setup. Setup of the frameless stereotaxic neuronavigation system on the day of surgery. Stereo-cameras were positioned above the operative field on each side of the animal to obtain a view of the marmoset skull used for co-registration of the animal, as well as tool and robot calibrations (**A**). The Brainsight™ vet robot is versatile and is compatible with multiple attachments such as the handpiece of the drill (**B**) and syringe of the injector system (**C**).

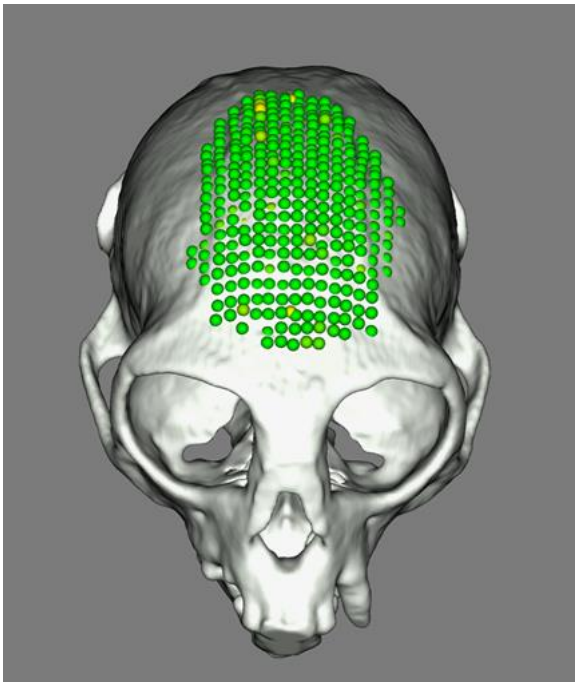

Fig. 4. Laser scanning of the skull surface. On the day of surgery, the animal was placed in the stereotaxic frame. After a midline incision was made, the bone was scraped and cleaned, and a red laser light source attached to the end of the robotic arm scanned the skull, generating a point cloud data set. The data from the laser were then superimposed onto the 3D reconstruction of the skull from MR/CT images, and homologous points were matched leading to the coregistration of the animal to the MR/CT volume.

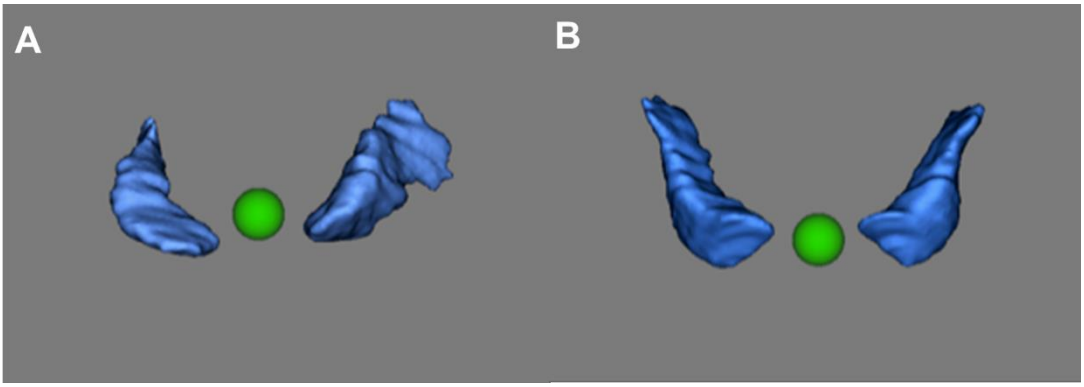

Fig. 5. Differences in putamen volume between marmosets. 3D reconstruction of the bilateral putamen of marmosets A (**A**) and B (**B**).

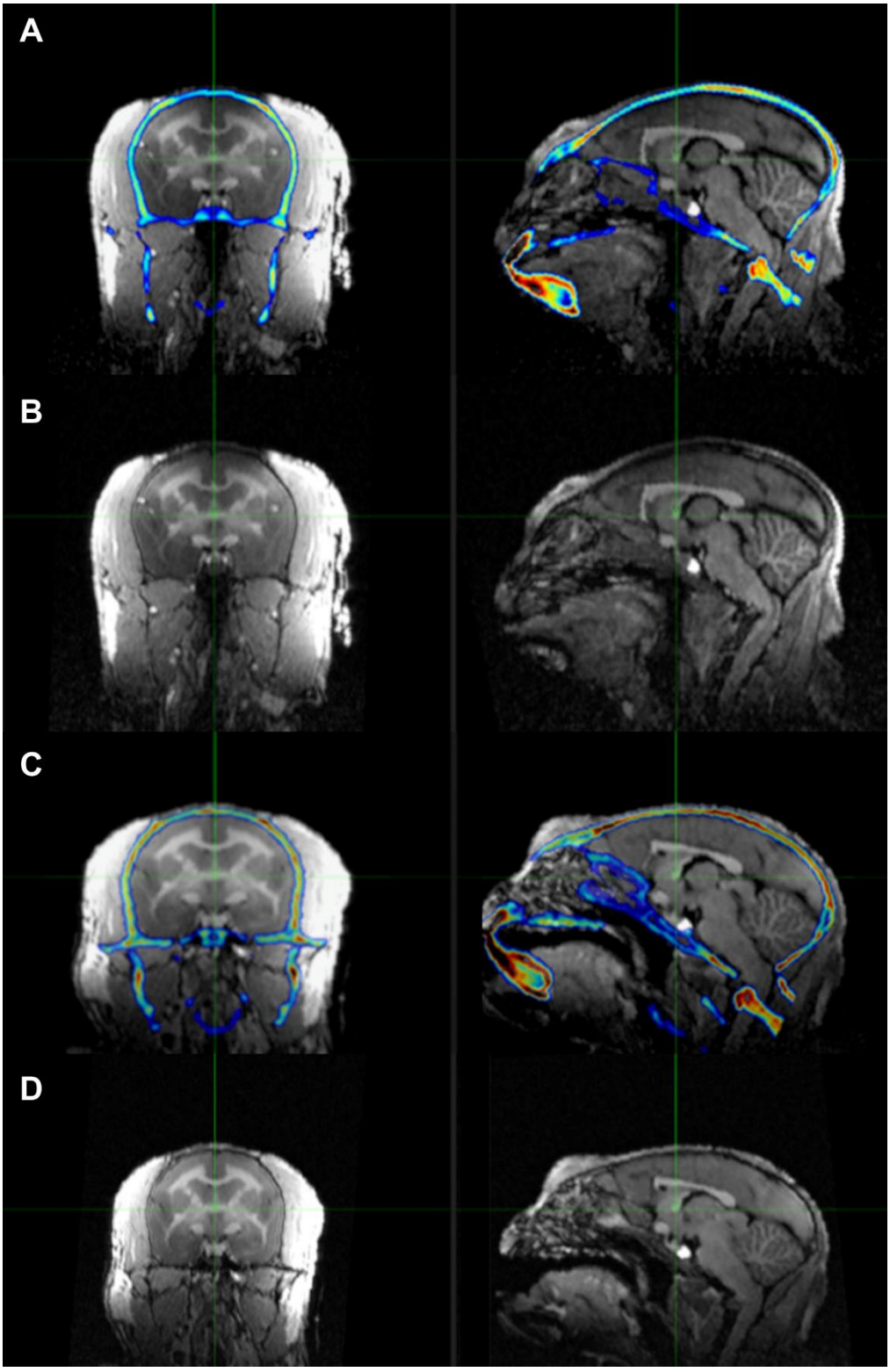
A
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Fig. 6. Overlay of CT and T1-weighted MR images of marmosets. CT scan superimposed with the MR image of marmoset A with coronal (left) and para-sagittal (right) views (**A**). T1-weighted MR images of marmoset A with coronal (left) and para-sagittal (right) views (**B**). CT scan superimposed with the MR image of marmoset B with coronal (left) and para-sagittal (right) views (**C**). T1-weighted MR images of marmoset B with coronal (left) and para-sagittal (right) view (**D**). The intersection of the green lines illustrates the anterior commissure. The jet colourmap was selected as the colour scheme to illustrate the skull.

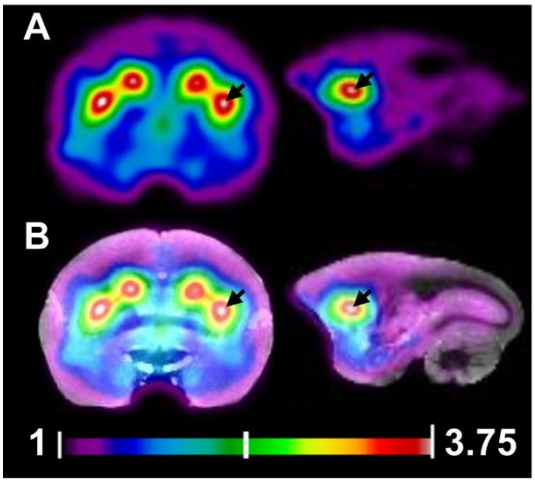

Fig. 7. Overlay of T1-weighted MR and [^{11}C]DTBZ images of marmosets. Averaged [^{11}C]DTBZ PET images of marmosets A and B with coronal (left) and para-sagittal (right) views (**A**). Averaged [^{11}C]DTBZ PET images were registered to averaged MR images of marmosets A and B and the superimposed coronal (left) and para-sagittal (right) images are presented (**B**). Areas of strong ligand uptake are indicated by yellow and red, whereas areas of lower ligand uptake are indicated by blue and green. There is high uptake of [^{11}C]DTBZ PET that is specific to the striatum. The arrowheads illustrate the surgical target.

Chapter 8 - Discussion

1. Recap of findings

In the 6-OHDA-lesioned rat, the 5-HT_3 antagonist granisetron alleviated the severity of dyskinesia without hindering L-DOPA anti-parkinsonian action. Taken together with favourable results obtained with the 5-HT_3 antagonist ondansetron, these results suggest that the family of 5-HT_3 antagonists represent an effective approach to alleviate L-DOPA-induced dyskinesia. Moreover, the anti-dyskinetic efficacy of ondansetron reported in the 6-OHDA-lesioned rat was confirmed in the MPTP-lesioned marmoset, although it enhanced L-DOPA anti-parkinsonian action in the latter model. Consistent with the antipsychotic efficacy of ondansetron reported in open-label clinical trials with PD subjects, it also suppressed psychosis-like behaviours in the MPTP-lesioned marmoset. Following these behavioural studies, an autoradiographic study determined that 5-HT_3 binding levels were significantly increased in the subthalamic nucleus, entopeduncular nucleus and thalamus of dyskinetic hemi-parkinsonian rats. These findings provide insight into the brain areas that may govern the anti-dyskinetic efficacy of 5-HT_3 antagonists, which may also be implicated in L-DOPA-induced dyskinesia. While these data support the translational potential of ondansetron and other 5-HT_3 antagonists in treatment-related complications in PD, they offer limited value as curative therapies in PD. Thus, preclinical models that recapitulate key features of the human condition, notably the propagation of abnormal alpha-synuclein, are crucial to the development and evaluation of disease-modifying therapies in PD. In order to establish non-human primate models based on intra-cerebral injection of alpha-synuclein PFFs, the methodology behind localisation and injection into a surgical target was developed in the marmoset. By relying on co-registration of imaging modalities, it was possible to determine stereotaxic coordinates unique to each animal, which circumvented issues of variability in skull and brain size between marmosets.

The results presented in the thesis were previously discussed in each individual chapter. This general discussion seeks to reconcile the results together and to offer a critical appraisal of their limitations and future directions.

2. Dose response curve of ondansetron

The U-shaped dose-response curve has often been ascribed to ondansetron (reviewed in Chapter 1) and some 5-HT_3 antagonists [1039, 1040] in preclinical paradigms of cognition, drug sensitisation, anxiety, and dyskinesia. In general, a higher magnitude of therapeutic benefit is achieved by relatively lower doses of antagonists in the microgram range [1041]. Contrary to these findings, we did not observe this shape of dose-response curve for the effects of ondansetron on dyskinesia or psychosis-like behaviours in the MPTP-lesioned marmoset. While a satisfactory explanation for the U-shaped dose-response curve of ondansetron is still lacking, speculative hypotheses put forth include mutual steric hindrance at 5-HT_3 receptors or varied distribution of 5-HT_3 receptors in the brain [1042]. Furthermore, dyskinesia and psychosis-like behaviours had not been previously studied, so it is difficult to comment on whether these mechanisms are also at play in these conditions. Nonetheless, we speculate that the discrepancy between the literature and our present findings may be attributed to the variety of indications, interspecies differences or the range of doses administered. Preclinical paradigms, such as cognition and dyskinesia, are mediated by different brain regions (e.g., frontal lobe vs. cortico-basal ganglia-thalamo-cortical loop) with varied expression of the 5-HT_3 receptor. Therefore, it is conceivable that regional differences in 5-HT_3 receptor binding may translate to varied dose-response curves depending on the endpoint studied. Indeed, whereas the pro-cognitive effect of ondansetron in the rat resembled a U-shaped dose-response curve [1043], its effect on anxiety-related behaviours was

dose-dependent in the mouse, rat, and marmoset [1044, 1045]. Of note, both the 6-OHDA-lesioned rat and MPTP-lesioned marmoset exhibit severe nigrostriatal denervation induced by neurotoxins [841], and these conditions may alter dose-response curves compared to others that employ healthy or drug naïve aged animals. Moreover, interspecies differences between the rat and marmoset, such as in the distribution of 5-HT_3 receptors and pharmacokinetics [1046-1048] may also contribute to altered dose-response curves of ondansetron between the two species. Last, the range of ondansetron doses examined in the MPTP-lesioned marmoset may have obscured capturing the full dose-response curve by presenting the upper limits of ondansetron doses. Although the effects of lower ondansetron doses on dyskinesia and psychosis-like behaviours in this model are unknown, dose selection was informed by pharmacokinetic analyses and translational potential.

The phenomenon of a U-shaped or inverse U-shaped dose-response curve is not unique to 5-HT_3 antagonists and has also been attributed to phytoestrogens [1049], antidepressants [1050], and antipsychotic drugs [1051, 1052]. The therapeutic implication of such a dose-response curve of ondansetron may be a narrow therapeutic window for PD that will be dependent on the endpoint. If treating dyskinesia is the primary concern, then lower doses of ondansetron should be administered, but if treating parkinsonian disability is the primary concern, then slightly higher doses of ondansetron may be more desirable. Furthermore, this type of dose-response curve may also have implications on the method of drug delivery of ondansetron wherein continuous infusion is preferred over bolus administration, in an attempt to ensure that the concentration of ondansetron is maintained within the therapeutic range [1053]. On the other hand, another 5-HT_3 antagonist, granisetron, did not present a U-shaped dose-response curve for its effect on dyskinesia in the hemi-parkinsonian rat. Although it remains unclear what features of the drug

contribute to this discrepancy with ondansetron, they may be related to its pharmacological differences, including its longer terminal half-life in humans (6.23 h for a 1 mg oral dose of granisetron vs 3.2-5.0 h for an 8 mg oral dose of ondansetron) [1054, 1055] and minimal binding to off-target receptors [1056], although both antagonists demonstrate high selectivity for the 5-HT_3 receptor considering the doses administered. Based on these considerations, granisetron may possess greater translational potential than ondansetron for PD and dyskinesia, as its more predictable dose-response curve would facilitate frequent adjustment of doses to determine the optimal dose for individual patients, a common practice in both conditions [399]. The mechanism underlying the dose-response curve of ondansetron and 5-HT_3 antagonists remains largely speculative due to the paucity of studies. Moreover, to our knowledge, they have not been studied in the context of PD or dyskinesia, and further developments in these research areas would inform dose selection of 5-HT_3 antagonists.

Pharmacokinetic studies found that ondansetron plasma levels associated with anti-dyskinetic efficacy doses in the rat and marmoset are below or within the same order of magnitude as plasma levels observed in the clinic [1055, 1057, 1058]. It remains difficult, however, to comment on whether plasma levels are a good indicator of ondansetron at its site of action, which is presumably in the central nervous system. Several brain regions have been implicated in dyskinesia, notably the striatum [578], globus pallidus [1059] and subthalamic nucleus [1060]; this involvement suggests that brain ondansetron levels may provide a more accurate measurement of its anti-dyskinetic efficacy instead of plasma levels. While we detected ondansetron in the rat striatum and primary motor cortex, they were only obtained in healthy animals at a single timepoint, i.e., peak L-DOPA action. Considering the time for drug distribution to tissues, as well as the time to cross the blood brain barrier [1061], it is conceivable that the time of maximal

ondansetron plasma levels may not fully correlate with maximal brain levels. By obtaining a more comprehensive brain-time concentration profile of ondansetron, it would be possible to determine whether brain levels correlate with its anti-dyskinetic activity and, as such, would serve as a better proxy for its anti-dyskinetic action than plasma levels. In terms of clinical relevance, measurements of ondansetron in vivo have been hampered by the lack of PET or SPECT radiotracer for the 5-HT_3 receptor [1062], as ligands had poor brain uptake and high nonspecific binding [1063-1065]. Therefore, clinical development of a radioligand could lead to the investigation of whether anti-dyskinetic or antipsychotic doses of ondansetron are associated with target engagement with the 5-HT_3 receptor. Based on the high selectivity of ondansetron (250-fold higher) for the 5-HT_3 receptor [1066] and the relatively low doses administered, it is unlikely that its therapeutic action might be attributed to binding to other receptors but further studies are required to clarify target engagement of ondansetron in vivo.

3. 5-HT_3 receptor blockade in treatment-related complications: a new therapeutic avenue

3.1. 5-HT_3 receptor blockade in dyskinesia

Blockade of the 5-HT_3 receptor represents a new therapeutic approach in L-DOPA-induced dyskinesia and, prior to the work presented in this thesis, only the 5-HT_3 antagonist ondansetron has been examined for its anti-dyskinetic potential in experimental parkinsonism. The current thesis has also demonstrated that another 5-HT_3 antagonist, granisetron, alleviated dyskinesia severity without worsening L-DOPA anti-parkinsonian action; these results provide support for a class effect of 5-HT_3 receptor blockade as both compounds are potent and structurally distinct 5-HT_3 antagonists. Intriguingly, the longer half-life of granisetron did not

translate to a longer duration of anti-dyskinetic benefit, which may suggest a limit to the benefit conferred by blockade of the 5-HT_3 receptor. Nonetheless, the magnitude of anti-dyskinetic efficacy of granisetron in the 6-OHDA-lesioned rat was ~ 25%-45%, comparable to that obtained with clinically-relevant doses of the anti-dyskinetic agent amantadine [1067], which may indicate a plateau to the therapeutic benefit that can be obtained in this model. For further demonstration of the selectivity of our therapeutic approach, it would be expected that 5-HT_3 receptor activation would likely worsen dyskinesia severity in these neurotoxin-based models of PD. However, due to the lack of selective and potent 5-HT_3 agonists [1068] and positive allosteric modulators [1069], as well as ethical concerns, these experiments have not been performed.

During the evaluation of potential anti-dyskinetic therapies in PD animal models, it is also necessary to determine whether their efficacy is attributed to interference with the action of L-DOPA [1070]. In the MPTP-lesioned marmoset, we found that ondansetron exerted anti-dyskinetic efficacy while enhancing L-DOPA anti-parkinsonian action at clinically relevant doses. These results are at odds with studies conducted in the 6-ODHA-lesioned rat, which found that ondansetron and granisetron had no significant effect on L-DOPA anti-parkinsonian action. Although it is unclear what is underlying the discrepancy between the two models, it may be related to differences in the characterisation of parkinsonism. Whereas forepaw use in the cylinder test is used as a measure of parkinsonism severity in the rat [868], in the marmoset, the rating scale for parkinsonism is more reminiscent of the scale used in PD patients [1071]. In fact, clinical characteristics of parkinsonism, such as bradykinesia, postural abnormalities, and range of movement, are incorporated into the behavioural repertoire of the MPTP-lesioned marmoset [623]. Moreover, pharmacological validation of the MPTP-lesioned non-human primate [513, 971] may afford it superior sensitivity to assess parkinsonism sensitivity [979]. In the clinic,

ondansetron did not alter the therapeutic benefit of L-DOPA, although the primary endpoint of these open-label trials was to assess its effect on PD psychosis and not dyskinesia [1016-1018]. Reconciling these results with those obtained in the parkinsonian marmoset, differences in the methodology (e.g., dosage, timing of administration, assessment of behaviours) may have contributed to the reported variability in the anti-parkinsonian action of ondansetron. Further research into the effects of ondansetron and other 5-HT_3 antagonists on the therapeutic efficacy of L-DOPA, particularly the mechanism governing these agents, will clarify the dearth of literature.

Based on the classic models of the cortico-basal ganglia-thalamo-cortical networks [323, 324], in dyskinesia, the subthalamic nucleus provides less glutamatergic innervation to the output nuclei, which disinhibits thalamo-cortical circuits [346]. Following administration of quipazine in rats, which exhibits affinity as a 5-HT_3 agonist [548], glucose use was significantly increased in the subthalamic nucleus and VL thalamus amongst several other brain areas [1072]. We speculate that in the subthalamic nucleus of dyskinetic animals, increased 5-HT_3 receptor densities due to enhanced inhibitory afferents [1073] may lead to further inhibition of the subthalamic nucleus and, in turn, disinhibit thalamo-cortical circuits, resulting in dyskinesia. These results, however, are at odds with those obtained with the 5-HT_3 antagonist ondansetron [1074], which failed to demonstrate a correlation between glucose metabolism and anatomical distribution of 5-HT_3 receptors. The discrepancy between the studies may be related to differences between activation and antagonism of 5-HT_3 receptors, or the relatively lower affinity of quipazine for 5-HT_2 receptors [1075] than for 5-HT_3 receptors [1076, 1077], although the functional effects of quipazine are not those expected of 5-HT_2 agonists [1072]. Nonetheless, further investigation will provide insight into the largely unknown role of the 5-HT_3 receptor in the subthalamic nucleus.

The paucity of literature on 5-HT_3 receptors in the thalamus renders it difficult to reconcile how an increase in 5-HT_3 receptor densities in the VA/VL thalamus may mediate dyskinesia. Surgical lesion of the motor thalamus has been successful in the treatment of dyskinesia in PD patients [1078], which provides support for the increased activity of the VA/VL thalamus in producing hyperkinetic movements [324, 346]. Studies found that a subpopulation of cortical 5-HT_3 receptor-expressing interneurons receive monosynaptic input from the thalamus that leads to strong depolarisation [1079-1081]. Based on the cortico-basal ganglia-thalamo-cortical circuitry [323, 324], it is possible that enhanced 5-HT_3 receptor-mediated GABAergic transmission may reduce the excitation of cortical interneurons. In turn, this may result in less inhibitory output to cortico-striatal neurons, and consequently, upregulated activity of output nuclei that contribute to the expression of dyskinesia. Therefore, administration of 5-HT_3 antagonists to dyskinetic animals may correct for the enhanced GABAergic activity in cortical neurons, culminating in an anti-dyskinetic benefit. As this theory remains speculative, further studies examining the functional significance of these 5-HT_3 receptor-expressing interneurons in relation to PD are warranted. Similarly, the anti-dyskinetic effect of pallidotomy and deep brain stimulation of the globus pallidus pars interna [1082, 1083] may be related to aberrations in its output signal that disrupt the activity of thalamo-cortical circuits [346, 1084]. To date, only two other studies have examined 5-HT_3 receptors in the globus pallidus. While one study failed to detect 5-HT_3 receptor mRNA [1085] in the mouse globus pallidus, an autoradiographic binding study reported moderate 5-HT_3 receptor densities in the rat entopeduncular nucleus and globus pallidus [1086]. Taken together, we speculate that the upregulation of 5-HT_3 receptors observed in the entopeduncular nucleus, the rodent homologue of the globus pallidus pars interna [1087], may contribute to alterations in its patterned activity, leading to enhanced thalamo-cortical output,

and the appearance of dyskinesia. As mentioned for the subthalamic nucleus and VA/VL thalamus above, the limited studies on the 5-HT_3 receptor in the entopeduncular nucleus, especially in the context of PD, hinder interpretation of our results and await further investigation.

The present thesis uncovered differences in 5-HT_3 receptor binding in the subthalamic nucleus, entopeduncular nucleus and thalamus that may underlie dyskinesia in the 6-OHDA-lesioned rat. As this was the first attempt to uncover the mechanism mediating the action of 5-HT_3 antagonists, the biological significance of these findings remains unclear as the role of the 5-HT_3 receptor has not been studied in these regions of interest. Nonetheless, using the same model, it would be intriguing to examine whether administration of 5-HT_3 antagonists to dyskinetic rats can prevent the upregulation of 5-HT_3 binding in the subthalamic nucleus, entopeduncular nucleus, or thalamus that is induced by L-DOPA treatment. Furthermore, brain ondansetron levels have not been assessed in the dyskinetic state but only in health animals, and studies undertaken to examine ondansetron levels in these brain regions would complement data obtained in the autoradiographic binding study.

An important consideration is that this is the sole study that examined the mechanism of action underlying 5-HT_3 antagonists in dyskinesia. While region-specific upregulation of 5-HT_3 binding has been linked to dyskinesia, it does not necessarily preclude the possibility of other processes that also mediate the anti-dyskinetic action of 5-HT_3 antagonists. For instance, the technique employed does not provide sufficient anatomical deta to be informative on the subcellular localisation of receptors [1088]. Under physiological conditions, 5-HT_3 receptors are largely present on the membrane surface [1089] but perhaps, in the dyskinetic state, 5-HT_3 receptor distribution is altered due to increased internalisation, and as a result, fewer receptors might be

present on the membrane to participate in downstream signalling. Indeed, administration of 5-HT_3 agonists significantly reduced membrane 5-HT_3 receptor expression, in part due to internalisation of receptors [1089] but this effect was blocked by the 5-HT_3 antagonist ondansetron [1090]. Therefore, beyond the regional changes in 5-HT_3 receptor binding observed in dyskinesia, alterations in the cellular localisation of the receptor may also contribute to the pathophysiology of the dyskinetic state. Furthermore, autoradiographic receptor labelling does not assess receptor function [1088] and consequently, cannot determine downstream processes that may be affected by changes in 5-HT_3 receptor signalling. Although there were no changes in the total number of 5-HT_3 receptors in the striatum of dyskinetic animals, there may have been changes in the regulation of receptor function, such as phosphorylation [1091], that may result in altered 5-HT_3 receptor activity. In turn, this may have shifted the balance between the direct and indirect pathways to favour disinhibition of the thalamus and cortex [316], leading to the development of dyskinesia [324, 1092]. Moreover, specific electrophysiological changes in the striatum have been reliably associated with dyskinesia, such as the inversion of firing rate changes in striatal medium spiny neurons [1093, 1094]. It remains to be ascertained whether 5-HT_3 receptor blockade in dyskinetic animals is associated with the stabilisation of dysregulated striatal activity [1095].

3.2. 5-HT_3 receptor blockade in PD psychosis

The behavioural repertoire of MPTP-lesioned marmosets chronically administered with L-DOPA also permits the evaluation of PD psychosis, another condition that affects a significant proportion of PD subjects [718, 753]. To address the lack of controls and concomitant medication in open-label studies conducted with ondansetron on PD psychosis, which may have confounded findings due to their role in psychosis [261, 1096, 1097], we assessed the effects of ondansetron on

psychosis-like behaviours using a randomised and blinded design in the MPTP-lesioned marmoset. Our results were in accordance with prior findings, having demonstrated a significant reduction in psychosis-like behaviours with ondansetron at doses associated with well tolerated plasma levels in the clinic. Furthermore, these results are timely as there is an ongoing double-blind, randomised, placebo-controlled Phase II trial assessing the efficacy of ondansetron treatment for visual hallucinations in PD (NCT04167813). While the primary outcome measure is visual hallucinations evaluated using the Scale for Assessment of Positive Symptoms, delusions will also be included as a secondary outcome measure using the same scale. The results of this trial will be enlightening as it will be the first large randomised placebo-controlled clinical trial to assess the antipsychotic benefit conferred by ondansetron in PD. Whereas positive findings from open-label trials were primarily conducted by the same group [1018] and replicated by another group [1098], one trial reported a lack of antipsychotic efficacy and tolerance to ondansetron in some subjects [1099]. Therefore, based on these promising data and the specificity of ondansetron for the 5-HT_3 receptor, clinical testing of ondansetron and other 5-HT_3 antagonists for the treatment of PD psychosis will be informative and either used to support the repurposing of clinically available 5-HT_3 antagonists or demonstrate the lack of translational potential for these agents.

A large body of evidence has implicated a role for the 5-HT system in PD psychosis [1100] that may be related to an imbalance in dopaminergic and serotonergic transmission [830]. Despite the antipsychotic efficacy of ondansetron encountered in PD psychosis, the underlying anatomical substrate(s) remains to be elucidated as no study has sought to understand its mechanism of action. Based on the antagonistic role of atypical antipsychotics at 5-HT_3 receptors, we speculate that their benefit in PD psychosis may be mediated, at least in part, by

blockade at this site of action. The atypical antipsychotic clozapine has demonstrated antipsychotic efficacy in two randomised placebo-controlled trials [1101, 1102] and is considered "clinically efficacious" in the treatment of PD psychosis [812]. Amongst its actions at multiple receptors [809], therapeutic doses of clozapine have also displaced binding of the 5-HT_3 antagonist [^{3}H]GR65630 in human embryonic kidney 293 cells, as well as reduced inward current mediated by 5-HT_3 receptors [1103, 1104], suggesting it acts as a functional antagonist at 5-HT_3 receptors [1105]. Moreover, the atypical antipsychotic risperidone, which significantly improved psychotic symptoms in PD [1106], also demonstrated activity as a 5-HT_3 antagonist [1104]. Given the ability of 5-HT_3 receptors to modulate dopaminergic mesolimbic activity [1104, 1107], the inhibitory action of atypical antipsychotics at 5-HT_3 receptors may be responsible, at least in part, for their efficacy in the treatment of PD psychosis.

The dopamine hypothesis of PD psychosis posits that chronic stimulation of dopamine D_2 receptors by dopaminergic drugs causes a hypersensitisation of dopaminergic receptors, particularly in limbic structures, which may increase susceptibility to develop psychotic symptoms in PD [781, 801]. As iterated above, the mechanism underpinning the antipsychotic action of ondansetron has not been studied in the context of PD psychosis but there is evidence suggesting that it may be related to modulation of dopaminergic mesolimbic activity. Indeed, autoradiographic binding studies conducted in post mortem human tissue have detected moderate to high levels of 5-HT_3 binding in the ventral tegmental area and nucleus accumbens [1108-1111]. Furthermore, 5-HT_3 receptors in mesolimbic structures also receive input from dopamine neurons in the ventral tegmental area [1112]. In line with 5-HT_3 expression in limbic areas, stimulation of 5-HT_3 receptors in either the ventral tegmentum or nucleus accumbens enhanced dopaminergic output [1113, 1114], suggesting that the effect of 5-HT_3 receptors may be

related to modulation of mesolimbic dopamine release. For instance, ICS 205-93, a 5-HT_3 antagonist, suppressed morphine-induced dopamine release in the nucleus accumbens in awake freely-moving rats [1115]; similar results were obtained by other groups [1116, 1117]. Reduced mesolimbic dopamine transmission following blockade of 5-HT_3 receptors [1118] has also been reported following intraperitoneal injections of alcohol [1113, 1119] and cocaine [1120], and subcutaneous injection of morphine [1116, 1117]. At the cellular level, electrophysiological studies have found that chronic application of the 5-HT_3 antagonists LY 277359, MDL 75147EF, zatosetron and DAU 6215 decreased the number of spontaneously active nigrostriatal [1121, 1122] and ventral tegmental dopamine neurons [1123, 1124], which may be predictive of antipsychotic action [1125, 1126]. However, granisetron, another 5-HT_3 antagonist failed to alter the number of spontaneously active dopamine neurons in both regions [1127]. Although the reason for the discrepancy is unknown, it may be related to differences in doses of 5-HT_3 antagonists [1124], but potency differences are unlikely as the drugs studied exhibit similarly high affinity for the 5-HT_3 receptor [1056, 1121, 1123].

The functional role of 5-HT_3 receptors on dopaminergic mesolimbic activity is substantiated by evidence from behavioural studies. Amphetamine-induced locomotor hyperactivity is enhanced by application of the 5-HT_3 agonist 2-methyl-5-hydroxytryptamine in the nucleus accumbens [1128] but attenuated by ondansetron and other 5-HT_3 antagonists in rat and non-human primate models [1128-1130]. Moreover, injection of ondansetron into the nucleus accumbens also inhibited cocaine-induced stimulation of locomotion in rats [1131]. These results collectively suggest that 5-HT_3 receptor antagonists such as ondansetron may dampen the mesolimbic dopaminergic transmission, possibly by decreasing dopamine release, to alleviate psychosis symptoms. The localisation of 5-HT_3 receptors to the mesolimbic dopamine areas [1112]

and lack of antagonistic action at dopamine D_2 receptors [1066] may explain why the antipsychotic action of ondansetron in PD patients did not worsen motor symptoms [1017]. Alternatively, 5-HT_3 antagonists may exert antipsychotic efficacy by inhibition of dopamine binding at the 5-HT_3 receptor. In fact, dopamine displays affinity for the 5-HT_3 receptor in the micromolar range, acting as a partial agonist at the receptor [1132-1135]. For instance, in oocytes expressing human 5-HT_3 receptors, dopamine induced fast inward currents but this effect was blocked by the 5-HT_3 antagonist LY-278584 [1135]. Based on the action of dopamine as a partial agonist at 5-HT_3 receptors, we speculate that potent 5-HT_3 antagonists with nanomolar affinity [1136, 1137] outcompete and prevent dopamine binding in mesolimbic dopamine areas. In turn, this leads to attenuation of dopamine hyperactivity in these brain areas and improvement on the severity of psychosis symptoms. However, the potency and efficacy of dopamine effects are highly variable [1135], and further research is required to determine whether they mediate the antipsychotic efficacy of 5-HT_3 antagonists in neurotoxic models of PD.

The MPTP-lesioned non-human primate is a well-established model to understand the underpinnings of behavioural and symptomatic components of PD [1138], in part due to its neuroanatomical and behavioural similarities to the human condition [1139, 1140]. Moreover, key features of the disease are recapitulated, such as marked degeneration in the substantia nigra pars compacta [1141], oxidative stress, and mitochondrial dysfunction [99]. Amongst the frequently used different MPTP-lesioned non-human primate series, the marmoset has the highest predictive value for antipsychotic efficacy in PD [941]. Agents that demonstrated clinical effectiveness (clozapine, mianserin, mirtazapine, and quetiapine) [818, 819, 823, 826, 827, 1101, 1102, 1142] also showed antipsychotic efficacy in the MPTP-lesioned marmoset [513, 556, 1143], as well as findings on ondansetron presented in this thesis. Of note, psychosis-like behaviours were absent

in marmosets induced with a partial MPTP lesion [1138], suggesting that more extensive nigrostriatal denervation may be required for their appearance. This is line with our current understanding of the aetiology of PD psychosis [803], whereby the underlying disease and dopaminergic therapy act in concert [721, 752]. As the MPTP-lesioned marmoset demonstrates high construct, face, and predictive validity, it plays a crucial role as the translational bridge between early preclinical research and clinical testing [971]. In fact, we described novel stereotypical behaviours in this model that shared features reminiscent of punding in PD patients [1021]. Although punding may affect nearly 14% of subjects with PD [1021, 1144], this number is likely underreported due to poor characterisation and awareness [1145]. Further characterisation of these behaviours in the MPTP-lesioned marmoset, particularly in a more quantitative manner, may advance our understanding of the mechanisms underpinning punding, as well as testing the efficacy of pharmacological agents. Given the poor outcomes and quality of life associated with PD psychosis and neuropsychiatric symptoms in general [1145, 1146], there is a crucial need to address this knowledge gap, and the extensive behavioural repertoire of the MPTP-lesioned marmoset model provides the opportunity to do so.

4. Towards a new alpha-synuclein propagation-based animal model of Parkinson's disease

4.1. Limitations of neurotoxic models of PD

Neurotoxic models of PD, such as the 6-OHDA-lesioned rat and MPTP-lesioned non-human primate are well-established and pharmacologically validated models for the assessment of parkinsonism and dyskinesia [511, 938]. Furthermore, the MPTP-lesioned marmoset has the additional advantage of exhibiting a larger repertoire of behaviours, which enables the study of

psychosis-like behaviours [513]. Using these models, we have demonstrated the high translational potential of ondansetron and 5-HT_3 antagonists for treatment-related complications in PD. However, our results are limited to the context of advanced PD as we modelled severe nigrostriatal denervation and drug sensitisation [841, 844]. As iterated earlier in Section 4.2, these models have shortcomings in understanding the mechanistic processes underpinning PD as they fail to reproduce some key features of the human conditions, particularly the progressive nature of the disease and Lewy pathology. A major impediment in the clinical development of disease-modifying therapies in PD is that current animal models do not allow the simultaneous study of pathological mechanisms underlying the disease and assessment of symptomatic therapies. This may explain, in part, the failure of neuroprotective drug candidates in clinical trials despite their initial success in preclinical studies [1147]. Therefore, development of an animal model that recapitulates core pathogenic processes in the human condition [147, 985], and has closer genetic and anatomical proximity to humans, will be crucial in the quest to develop disease-modifying therapies for PD.

4.2. Alpha-synuclein propagation-based models of PD

In the brain of patients with advanced PD, there is widespread dissemination of inclusion bodies [67], and the main constituent of these inclusions is the protein alpha-synuclein [1027]. Although its role is not well understood, emerging evidence suggests that alpha-synuclein is crucial to the development of PD. From observations of post mortem brain tissue of PD subjects, Braak and colleagues formalised a hypothesis to describe the spreading of Lewy pathology based on the correlation between neuropathological findings with preclinical and clinical phases of the disease [67, 68]. In early stages, Lewy bodies are confined to the brain stem and olfactory

bulb, before spreading to the midbrain, and by later stages, throughout the lower forebrain and cortex. Further evidence in support of the Braak hypothesis came from clinical trials that grafted embryonic dopaminergic neurons into the brains of PD patients [70, 71]. Over 10 years after transplantation revealed the presence of pathological inclusions in the healthy donor neurons of these patients, which suggests that Lewy pathology can spread from host to donor and aggregated alpha-synuclein seeded the misfolding of endogenous alpha-synuclein [72].

Although the mechanisms underlying the initiation and spreading of pathological alpha-synuclein in PD are not well understood, converging evidence suggests that a prion-like propagation may explain this phenomenon [1148]. Studies in cultured neurons have demonstrated that alpha-synuclein can be secreted and taken up from the extracellular space [1149, 1150]. Once inside a neuron, alpha-synuclein can oligomerise with endogenous alpha-synuclein and seed the formation of aggregates [88, 1149]. Once healthy monomeric alpha-synuclein becomes misfolded, it acts as seeds to recruit endogenous synuclein, converting the latter into insoluble pathological polymers, and, ultimately, Lewy bodies [88]. This pathological alpha-synuclein then spreads throughout the brain in interconnected and neighbouring areas, eventually propagating to the entire brain [89]. Moreover, Lewy pathology requires the expression of endogenous alpha-synuclein as propagation does not occur in in vitro [986] and in vivo alpha-synuclein knockout cells [995], providing further support for a prion-like propagation.

Models that reproduce the abnormal propagation of pathological alpha-synuclein have been developed in rodents and non-human primates (reviewed in Section 4.3). We sought to address some limitations of these previous studies by developing a model in the marmoset based on injection of human alpha-synuclein PFFs, followed by characterisation of the model using in vivo and post mortem techniques. An important step in establishing this novel animal model

was to develop the methodology to accurately identify the surgical target for PFF injection in the marmoset brain. In Chapter 6, we described the co-registration of imaging modalities to reliably identify and inject PFFs into the putamen of two marmosets. This was the first time that the frameless stereotaxic neuronavigation procedure was described in non-human primates and it addressed the shortcomings of traditional approaches, such as limited sample size and restricted sex of animals [1033-1035] and limited regions of interests [1151-1154], that relied upon stereotaxic atlases. Whereas PET imaging revealed nigrostriatal denervation 4-months after PFF injection, these results only provide suggestive evidence that surgical targeting was accurate and await further confirmation by histology or CT imaging.

Once this marmoset model has been well-established, it may be used as a paradigm to evaluate the efficacy of disease-modifying therapies in PD, either by preventing or mitigating alpha-synuclein toxicity [1155], to address the translational gap of neurotoxic models. Indeed, current therapies in the PD pipeline targeting alpha-synuclein use a range of approaches including inhibition of alpha-synuclein misfolding and aggregation (NCT04685265), clearance of aggregates using antibodies [1156], and prevention of cell to cell transition within the brain (NCT03858270) [1155]. An additional advantage of using subject-specific registration data is that it permits image acquisition at distal time points. Therefore, in our model, we can monitor the progression of nigrostriatal denervation in vivo, which will allow us to determine endpoints for studies on disease-modifying therapies. Moreover, ongoing efforts in the lab are dedicated to characterisation of the behavioural deficits exhibited by these marmosets, particularly motor activity, providing a more detailed and clinically relevant analysis of the manifestation of parkinsonism in the model. Along with initiatives to develop biomarkers in PD, richer characterisation of the model will improve monitoring of disease progression, as well as the

evaluation of the efficacy of potential therapies [1157]. Last, our approach takes into consideration the unique features of a marmoset's skull and brain, and can thus be used to localise and inject into surgical targets in the brain besides the putamen, which may be especially beneficial for small and deep nuclei [1158]. Braak staging suggests that PD is unlikely to start within the striatum (our injection site) but more likely in the dorsal motor nucleus of the vagus nerve or the olfactory bulb [67]. Injection of alpha-synuclein PFFs into these brain regions and subsequent monitoring of the synucleinopathy will be informative on the mechanisms underpinning PD. The development of such a PFF-induced synucleinopathy model would enhance our understanding of critical pathogenic features of the disease and permit the assessment of novel disease-modifying therapies [988]. As patients with PD continue to live with the progressive nature of their disease, establishment of an excellent preclinical model, accompanied by efforts to fulfil knowledge gaps of the disease, represent important strides towards developing therapies with curative potential and improving their quality of life.

5. Impact and conclusion

The present thesis presented data that contributed to original knowledge in PD research and met the objectives iterated earlier. First, ondansetron plasma and brain levels were determined following subcutaneous administration in the rat for the first time. These data enhanced understanding of the central effects of ondansetron in the rat, including its anti-dyskinetic efficacy in the hemi-parkinsonian rat, as the literature did not examine the therapeutic relevance of relatively small doses of ondansetron. Second, in the 6-OHDA-lesioned rat, the 5-HT_3 antagonist granisetron significantly improved dyskinesia without worsening the therapeutic efficacy of L-DOPA. When taken together with results obtained with the 5-HT_3 antagonist

ondansetron, these data suggest that the anti-dyskinetic efficacy of 5-HT_3 antagonists is a class effect that is mediated by selective blockade of the 5-HT_3 receptor. Furthermore, the efficacy of this approach was confirmed in the MPTP-lesioned marmoset, wherein ondansetron alleviated dyskinesia severity while also enhancing L-DOPA anti-parkinsonian action. Considering the higher face and predictive value of this model, these favourable data support the translation of clinically available 5-HT_3 antagonists, namely ondansetron, to clinical testing for L-DOPA-induced dyskinesia in PD. In addition to parkinsonism and dyskinesia, the MPTP-lesioned marmoset model also permits the assessment of psychosis-like behaviours. Thus, ondansetron treatment led to the suppression of psychosis-like behaviours in this model, which aligns with findings on the antipsychotic efficacy of ondansetron in open-label trials with PD subjects. These results are timely as there is an ongoing Phase II trial examining the effect of ondansetron on visual hallucinations in PD (NCT04167813), indicating that ondansetron, and possibly other 5-HT_3 antagonists, may be repurposed for the treatment of PD psychosis. In addition, the existing behavioural repertoire of psychosis-like behaviours in MPTP-lesioned marmosets was expanded, suggesting that they are idiosyncratic and stereotyped, reminiscent of punding observed in some PD patients. Further characterisation of the MPTP-lesioned marmoset enhances the value of the model for the evaluation of symptomatic therapies in PD, which is especially crucial for PD psychosis as it is the sole validated model.

Whereas these behavioural studies have suggested evidence of anti-dyskinetic efficacy of 5-HT_3 antagonists, they failed to reveal the mechanisms underlying their effects. In an attempt to uncover possible mechanisms of actions, an autoradiographic study found a selective upregulation of [^{3}H]GR65630 binding in the subthalamic nucleus and ipsilateral entopeduncular nucleus and motor thalamus of dyskinetic 6-OHDA-lesioned rats but no significant alterations

in other brain regions examined. This was the first time that the role of the 5-HT_3 receptor was assessed in the context of L-DOPA-induced dyskinesia, and these results provide insight into the brain regions that mediate the efficacy of 5-HT_3 antagonists in dyskinesia that are worth further investigation. Current management of L-DOPA-induced dyskinesia remains inadequate and uncovering its molecular underpinnings as well as those of new therapies will facilitate the clinical development of these agents. The data presented in this thesis provide support for the potential of ondansetron and other 5-HT_3 antagonists in L-DOPA-related complications in PD, namely L-DOPA-induced dyskinesia and PD psychosis. Considering the substantial costs and barriers to drug discovery and development, the approval of several 5-HT_3 antagonists as anti-emetics may greatly accelerate their transition to clinical testing, leading to lower expenditures for repurposing in the treatment of PD.

On the other hand, the findings on 5-HT_3 antagonists and the role of the 5-HT_3 receptor in PD are limited to symptomatic therapies. As iterated previously, toxin-based models of PD fail to recapitulate key molecular processes, particularly Lewy pathology, and consequently, lack disease-modifying potential. Development of an animal model that reproduces critical features of the human condition, particularly the abnormal propagation of alpha-synuclein, is instrumental to the development of disease-modifying therapies in PD. A step in that direction is the establishment of an intra-cerebral injection of alpha-synuclein PFFs model in the marmoset to model the progression of PD. To address issues of inter-individual variability in the brain and skull size of marmosets, the methodology to localise and inject into a surgical brain target was described. This was the first time that such a frameless stereotaxic approach was described in non-human primates, which relied on co-registration of multiple imaging modalities to determine unique stereotaxic coordinates of individual marmosets. Publishing the

methodology of such an approach facilitates development of an animal model with greater translational potential as well as accurate targeting of brain structures for other procedures, such as deep brain stimulation or electrophysiology. It is the ultimate hope that data presented in this thesis will help fill knowledge gaps of PD, in the quest to develop curative therapies for patients to alleviate their suffering.

Chapter 9 - References

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Chapter 10 - Appendix

Table I: Duration rating scale of ALO AIMs in the 6-OHDA-lesioned rat

Parameter	Score
axial	0: no dyskinesia 1: occasional signs of dyskinesia, present < 50% of observation time 2: frequent signs of dyskinesia, present > 50% of the observation time 3: dyskinesia present during the entire observation period, but suppressible by external stimuli 4: continuous dyskinesia not suppressible by external stimuli
limbs	0: no dyskinesia 1: occasional signs of dyskinesia, present < 50% of observation time 2: frequent signs of dyskinesia, present > 50% of the observation time 3: dyskinesia present during the entire observation period, but suppressible by external stimuli 4: continuous dyskinesia not suppressible by external stimuli
orolingual	0: no dyskinesia 1: occasional signs of dyskinesia, present < 50% of observation time 2: frequent signs of dyskinesia, present > 50% of the observation time 3: dyskinesia present during the entire observation period, but suppressible by external stimuli 4: continuous dyskinesia not suppressible by external stimuli

Table adapted with permission from Cenci and Lundblad 2007 [905].

Table II: Amplitude rating scale of ALO AIMs in the 6-OHDA-lesioned rat

Parameter	Score
axial	0: no dyskinesia
	1: sustained deviation of the head and neck at about a 30º angle
	2: sustained deviation of the head and neck between an angle of 30º and 60º
	3: sustained twisting of the head, neck and upper trunk, at an angle between 60º and 90º
	4: sustained twisting of the head, neck and trunk at maximal amplitude, causing the rat to lose balance from a bipedal position
limbs	0: no dyskinesia
	1: tiny movements of the paw around a fixed position
	2: displacement of the whole limb (horizonal or up-and-down)
	3: large displacement of the limb with visible contraction of shoulder muscles
	4: vigorous limb displacement of maximal amplitude, with contraction of both shoulder groups and extensor muscles
orolingual	0: no dyskinesia
	1: twitching of facial muscles accompanied by small masticatory movements without jaw opening
	2: twitching of facial muscles accompanied by masticatory movements, occasional jaw opening
	3: movements involving facial muscles and masticatory muscles, frequent jaw opening and occasional tongue protrusion
	4: involvement of all of the above muscles to the maximal possible degree

Table adapted with permission from Cenci and Lundblad 2007 [905].