Dual modulation of $mGlu_2$ and 5-HT_{2A} receptors as a novel approach to alleviate L-DOPA induced dyskinesia

Élodie Bourgeois-Cayer
260818167

Integrated program in neuroscience

McGill University, Montreal

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Abstract

L-3,4-dihydroxyphenylalanine (L-DOPA) remains the most effective treatment for Parkinson's disease (PD). However, chronic administration of L-DOPA leads to the emergence of motor complications such as dyskinesia in the vast majority of patients. Serotonin 2A (5-HT_{2A}) receptor blockade is a validated approach to alleviate dyskinesia, but its effectiveness appears to be of limited magnitude. Recently, we have demonstrated that activation of metabotropic glutamate 2 ($mGlu_2$) receptors reduce dyskinesia in the 6-hydroxydopamine (6-OHDA)-lesioned rat. Based on the fact that 5-HT_{2A} and $mGlu_2$ receptors form a functional hetero-complex, we hypothesised that combining EMD-281,014, the most selective 5-HT_{2A} antagonist commercially available with LY-354,740, a selective $mGlu_2$ orthosteric agonist (OA) and LY-487,379, the most selective $mGlu_2$ positive allosteric modulator (PAM) commercially available, would be more effective at alleviating dyskinesia than modulating 5-HT_{2A} and $mGlu_2$ receptors separately.

Rats were rendered hemi-parkinsonian by stereotaxic injection of 6-OHDA into the medial forebrain bundle. Following a recovery period, degree of parkinsonism was assessed using the cylinder test. Rats were then primed with daily administration of L-DOPA to induce stable axial, limb and oro-lingual (ALO) abnormal involuntary movements (AIMs). On experimental days, rats were administered L-DOPA in combination with previously determined effective doses of each of EMD-281,014 (vehicle, 0.03 and 0.1 mg/kg s.c), LY-354,740 (vehicle and 0.1 mg/kg s.c.), and LY-487,379 (vehicle and 0.1 mg/kg s.c.) after which ALO AIMs were assessed for 2 min, every 20 min, for 180 min. After a 3-day washout period, an acute low-dose of L-DOPA was administered with a combination of EMD-281,014/LY-354,740/LY-487,379, and the effect on L-DOPA anti-parkinsonian action was determined by the cylinder test.

Combining EMD-281,014 0.1 mg/kg and LY-487,379 0.1mg/kg to L-DOPA significantly reduced ALO AIMs duration, by 10% ($P < 0.05$), when compared to the vehicle. Other combinations, notably EMD-281,014 and LY-354,740, or EMD-281,014 with both LY-487,379 and LY-354,740, did not lead to an enhanced anti-dyskinetic effect. Combining EMD-281,014 with LY-354,740 and LY-487,379 did not have any effect on L-DOPA anti-parkinsonian action.

Our results suggest that an additional anti-dyskinetic benefit was achieved when combining EMD-281,014 and LY-487,379, but not when combining EMD-281,014 with an $mGlu_2$ OA or with an OA and a PAM, in the 6-OHDA-lesioned rat model of PD.

Keywords: Parkinson's disease, dyskinesia, $mGlu_2$, 5-HT_{2A}, L-DOPA, 6-OHDA-lesioned rat

Résumé

L-3,4-dihydroxyphenylalanine (L-DOPA) demeure la manière la plus efficace de traiter la maladie de Parkinson. Cependant, son administration chronique mène à l'émergence de complications motrices telles que les dyskinésies chez la majorité des patients. Le blocage des récepteurs sérotoninergiques 2A (5-HT_{2A}) est établi comme une méthode efficace pour soulager partiellement les dyskinésies, cependant, elle est limitée en ampleur. Récemment, nous avons démontré que l'activation des récepteurs métabotropiques du glutamate 2 ($mGlu_2$) réduit les dyskinésies chez le rat lésé à la 6-hydroxydopamine (6-OHDA). Se basant sur le fait que les récepteurs 5-HT_{2A} et $mGlu_2$ forment un hétéro-complexe fonctionnel, nous avons émis l'hypothèse qu'en combinant EMD-281,014, l'antagoniste de 5-HT_{2A} le plus sélectif disponible commercialement avec LY-354,740, un agoniste orthostérique sélectif et LY-487,379, un modulateur allostérique positif hautement sélectif, un soulagement plus efficace dyskinésies pourrait être obtenu.

Des rats ont été rendus hémi-parkinsoniens par injection stéréotaxique de 6-OHDA dans le faisceau longitudinal médian droit du télencéphale. Suite à une période de récupération, le degré de parkinsonisme a été évalué grâce au test du cylindre. Par la suite, les rats ont été sensibilisés avec une injection quotidienne de L-DOPA pour instaurer des mouvements involontaires anormaux (AIMs) stables et reproductibles. Lors des expériences, les rats ont reçu L-DOPA en combinaison avec EMD-281,014 (véhicule, 0.03 et 0.1 mg/kg s.c), LY-354,740 (véhicule et 0.1 mg/kg s.c.), et LY-487,379 (véhicule et 0.1 mg/kg s.c.) dont les doses efficaces avaient été prédéterminées. Suite à cela, les AIMs furent évalués pendant 2 min chaque 20 min pour 180 min. Après une période d'élimination de 3 jours, une basse dose de L-DOPA a été administrée en combinaison avec EMD-281,014/LY-354,740/LY-487,379 afin de déterminer, à l'aide du test du cylindre, leurs effets sur l'action antiparkinsonienne de L-DOPA

Combiner EMD-281,014 0.1 mg/kg et LY-487,379 (0.1mg/kg) à L-DOPA a significativement diminué la durée des AIMs de 10% ($P < 0.05$). Par contre, combiner EMD-281,014 avec LY-354,740 et LY-487,379 n'a pas eu d'effet sur l'action antiparkinsonienne de L-DOPA

Nos résultats suggèrent qu'un effet synergique maximal est atteint en combinant EMD-281,014 et LY-487,379, mais que l'ajout de LY-354,740 n'a pas d'effet supplémentaire.

Mots clés: Maladie de Parkinson, dyskinésie, $mGlu_2$, 5-HT_{2A}, L-DOPA, rat lésé à la 6-OHDA

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List of abbreviations

5-HT	serotonin
5-HT_1	5-hydroxytryptamine receptor 1
5-HT_{1A}	5-hydroxytryptamine receptor 1A
5-HT_{1B}	5-hydroxytryptamine receptor 1B
5-HT_2	5-hydroxytryptamine receptor 2
5-HT_{2A}	5-hydroxytryptamine receptor 2A
5-HT_{2C}	5-hydroxytryptamine receptor 2C
5-HT_3	5-hydroxytryptamine receptor 3
5-HT_4	5-hydroxytryptamine receptor 4
5-HT_5	5-hydroxytryptamine receptor 5
5-HT_6	5-hydroxytryptamine receptor 6
5-HT_7	5-hydroxytryptamine receptor 7
6-OHDA	6-hydroxydopamine
AADC	aromatic acid decarboxylase
AIMs	abnormal involuntary movements
AIMS	abnormal Involuntary Movement Scale
AL	axial limbs
ALO	axial limbs oro-lingual
COMT	catechol-O-methyltransferase
LID	L-DOPA induced dyskinesia
mGlu	metabotropic glutamate receptor
$mGlu_1$	metabotropic glutamate receptor 1
$mGlu_2$	metabotropic glutamate receptor 2
$mGlu_3$	metabotropic glutamate receptor 3
$mGlu_4$	metabotropic glutamate receptor 4
$mGlu_5$	metabotropic glutamate receptor 5
$mGlu_6$	metabotropic glutamate receptor 6

$mGlu_7$	metabotropic glutamate receptor 7
$mGlu_8$	metabotropic glutamate receptor 8
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NHP	non-human primate
OA	orthosteric agonist
PAM	positive allosteric modulator
PD	Parkinson’s disease

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1. Introduction

1.1 General introduction

Parkinson's disease (PD) is the second most widespread neurodegenerative disease, affecting approximately 0.3% of the general population, but around 1% of the population over the age of 60 (Tanner, 1992). It was first described as *the shaking palsy* by Dr James Parkinson in 1817 (Parkinson, 1817). Since then, dopaminergic neuron degeneration has been found to be one of the causes of PD motor symptoms, leading L-3,4-dihydroxyphenyalanine (L-DOPA) to be used as the most effective non-invasive treatment for the disease (P. Huot, Johnston, Koprich, Fox, & Brotchie, 2013).

Nevertheless, chronic intake of L-DOPA has been associated with a severe motor complication described as L-DOPA-induced dyskinesia (LID) which is defined as abnormal involuntary movements when on L-DOPA (Ahlskog & Muenter, 2001; Parkinson, 1817). The mean onset for LID is 4.2 years after the beginning of L-DOPA treatment and its manifestation is almost inevitable, with as many as 95% of patients harbouring LID after 15 years of treatment (Hely, Morris, Reid, & Trafficante, 2005). Despite LID high prevalence, there is currently no clinically available drug that can fully alleviate this motor complication, however, a few like amantadine and clozapine can help reduce it (Bennett, Landow, Dietrich, & Schuh, 1994; Paci, Thomas, & Onofrj, 2001).

1.2 Parkinson's disease

1.2.1 Brief history of Parkinson's disease

Old scripts have described symptoms similar to PD such as drooling, tremor and lack of movement meaning PD had been recognised long before Parkinson's initial description. An Egyptian papyrus from 1200 BC describes a king drooling with age. The Bible also makes a number of references to tremors (Lees, 2007). Sanskrit texts from 1500 BC also comprehensively

describe PD-like tremor and Ayurvedic texts describe Kampavata (their possible equivalent to PD) being treated with *Mucuna pruriens*, from which plant genus L-DOPA was originally isolated (Katzenschlager, Evans, et al., 2004). Other famous figures such as Leonardo da Vinci, Johannes Baptiste Sagar and Galen made reference to PD-like symptoms in patients (Lees, 2007).

However, the first detailed description of PD was made by the English physician James Parkinson 1817 in an essay on what he called the *shaking palsy* based on clinical description of 6 patients (Parkinson, 1817). The disease was later renamed as PD by the French neurologist Jean Martin Charcot, who recognised Parkinson's essential contribution to the disease's characterisation (Lees, 2007). Charcot himself contributed greatly to the understanding of PD by making a distinction between rigidity, weakness and bradykinesia (Charcot, 1902). Several others also contributed to the understanding of PD, like Armand Trousseau, who first described rigidity and other aspects of PD (J. M. Pearce, 2002), as well as Samuel Alexander Kinnier Wilson, who studied movement disorders, including akinesia and bradykinesia in PD and their relation to basal ganglia [BG] (Broussolle, Trocello, Woimant, Lachaux, & Quinn, 2013).

In the early 20th century, Friedrich Heinrich Lewy first described inclusion bodies, later named after him by Konstantin Tretiakoff, seen in PD. Tretiakoff himself showed that the substantia nigra (SN) was likely to be affected in PD (Holdorff, 2002), which was only widely accepted once it was later confirmed with further studies by Rolf Hassler (Lees, 2007). In the 1960s, Oleh Hornykiewicz showed that striatal dopamine deficiency was correlated with motor symptoms of PD. A finding which was only made possible by the work Arvid Carlsson made on dopamine in the previous decade (Fahn, 2008). This observation led to the use of the dopamine precursor L-DOPA as a potential way to treat PD. George Cotzias developed the therapy, still in

use today to introduce L-DOPA to patients (Cotzias, Van Woert, & Schiffer, 1967) and found the L- enantiomer to be safer and more efficient (Cotzias, Papavasiliou, & Gellene, 1969).

Despite the fact that its use is associated with considerable adverse effects such as nausea, orthostatic hypotension, hallucinations and LID (Connolly & Lang, 2014), L-DOPA remains the best non-invasive treatment for PD (Nagatsu & Sawada, 2009).

1.2.2 Epidemiology of Parkinson's disease

It has been approximated that about 90% to 95% of cases of PD are sporadic (Tysnes & Storstein, 2017) and the exact mechanisms by which neurodegeneration occurs are still not fully understood (Lang & Espay, 2018). The prevalence of PD is approximated to 1% in the population over 60 years old (Tanner, 1992), however, only about 80% to 90% of diagnosed cases of PD are confirmed at autopsy (Litvan et al., 2003). Recently, the cardinal signs for clinical diagnosis of PD were revised, now bradykinesia must occur in combination with rest tremor, rigidity or both (Postuma et al., 2015). Bradykinesia can be described as the slowness of a performed movement and is often associated with two other terms akinesia and hypokinesia (Berardelli, Rothwell, Thompson, & Hallett, 2001). Akinesia is described as a poverty of spontaneous movements, while hypokinesia refers to movements being smaller in amplitude than desired (Berardelli et al., 2001). Postural changes are now left out of the new diagnostic criteria (Tysnes & Storstein, 2017). Supportive criteria might include motor and non-motor symptoms, such as olfactory loss and the presence of LID (Tysnes & Storstein, 2017).

Despite the exact mechanisms causing the disease not being fully known, some factors are known to increase the risk of the disease. First degree family members of a patient with PD have a 2- to 3-fold increase risk of developing the disease compared to the general population (Savica, Cannon-Albright, & Pulst, 2016; Sveinbjornsdottir et al., 2000). Environmental factors increasing

the risk of PD can be alcohol, vitamin D exposure and urate levels, mostly agents with a mutagen factor (Tysnes & Storstein, 2017). There is also a higher prevalence of the disease in men than women (de Lau & Breteler, 2006).

1.2.3 Treatment of Parkinson's disease

Various pharmacological therapies are used to treat PD at different stages of the disease, however, no disease-modifying therapies are currently available (Connolly & Lang, 2014). L-DODA remains the most effective drug to treat the entire spectrum of motor symptoms (J. J. Ferreira et al., 2013), nonetheless, other pharmacological interventions are often prescribed (Schapira & Warren Olanow, 2005).

MAO-B inhibitors, such as selegiline (deprenyl) and rasagiline, used to be the first prescribed drug for early PD with mild symptoms because of their assumed neuroprotective efficacy. However, since then, it has been proven otherwise (Susan H Fox et al., 2018). MAO-B inhibitors inhibit dopaminergic breakdown, thereby enhancing dopamine availability in the brain. MAO-B inhibition can delay the initial L-DOPA therapy by 9-12 months. It can either be used in monotherapy or combined with L-DOPA. Patients that keep using selegiline following initial L-DOPA therapy may require lower levels of L-DOPA to manage their symptoms. Despite their symptomatic benefit being significant, it is overall relatively modest and they have various adverse effects such as dizziness, headache, confusion, depression, etc. (Connolly & Lang, 2014).

Another category of drugs is dopamine agonists (DA), such as pramipexole and ropinirole. As suggested by their name, DA bind to and directly activate dopamine receptors. These DA can be used either as monotherapy in early PD (Group, 1997; Korczyn et al., 1999; Schrag, Keens, & Warner, 2002; Shannon, Bennett, & Friedman, 1997) or as an adjunct treatment in advance PD (Guttman, 1997; Lieberman et al., 1998; Lieberman, Ranhosky, & Korts, 1997). DA efficacy is,

however, lower than L-DOPA, and their use is associated with a handful of adverse effects the most severe being hallucinations, impulse-control disorders, somnolence, peripheral edema, valvular heart disease, fibrosis, and heart failure (Borovac, 2016; Kataoka, Sawa, Sugie, & Ueno, 2014; Kataoka & Ueno, 2014; Lockett, DeBacker, & Cauthon, 2015; Stowe et al., 2008; Wood, 2010).

L-DOPA was the first dopaminergic drug developed for the treatment of PD and it remains the most potent one, providing effective relief of bradykinesia, rigidity and improvement of tremor in the majority of patients (Schapira & Warren Olanow, 2005). However, various other molecules can be combined with L-DOPA to achieve better clinical results. First, L-DOPA is administered in combination with an aromatic L-amino acid decarboxylase (AADC) inhibitor, which prevents the conversion of L-DOPA to dopamine in the bloodstream and allows for lower doses of L-DOPA to be administered (Birkmayer, Knoll, Riederer, & Youdim, 1983). Indeed, unlike dopamine and AADC inhibitors, only L-DOPA can cross the blood-brain barrier. Benserazide and carbidopa are two examples of widely used AADC inhibitors (Connolly & Lang, 2014). Another type of molecule are catechol-o-methyltransferase (COMT) inhibitors. They block the action of COMT enzymes that are responsible for the methylation of catecholamines such as dopamine, noradrenaline, adrenaline and L-DOPA. COMT inhibitors prevent the methylation of L-DOPA to 3-O-methyldopa (3-OMD) which increases the bioavailability of L-DOPA. 3-OMD has no therapeutic potential (Zhu, 2002) and competes with L-DOPA for the blood-brain barrier transport system (Lee, Chen, King, & Charlton, 2008). Three COMT inhibitors are available, entacapone, tolcapone and, more recently, opicapone (Almeida et al., 2013; Joaquim J Ferreira et al., 2016; Joaquim J Ferreira et al., 2015). Tolcapone can be hepatotoxic, however, unlike entacapone, it can cross the blood-brain barrier and prevent the conversion of L-DOPA to 3-OMD in the brain as

well (Connolly & Lang, 2014). Nonetheless, no matter the adjunct therapy used, none of them can prevent the development of LID.

1.3 Basal Ganglia

Despite PD affecting the entire central nervous system it is commonly referred to as a disease of the BG, meaning the main circuitry affected by the disease is the BG loop (Fearnley & Lees, 1991). The BG are a collection of nuclei within the brain which affect different functions such as motor control, eye movements, mood, reward, and executive functions (Kandel, Schwartz, Jessel, Siegelbaum, & Hudspeth, 2013).

1.3.1 Anatomy of the basal ganglia

As seen in Figure 1, the BG regroup four main structures; the striatum, globus pallidus (GP), SN and subthalamic nucleus [STN] (Alexander & Crutcher, 1990).

1.3.1.1 The striatum

The striatum is the main point of entry of cortical information to the BG (Bolam, Hanley, Booth, & Bevan, 2000). It receives prominent projections from the cerebral cortex, brainstem and thalamus (Kandel et al., 2013). It is composed of both projection neurons and populations of interneurons. 90-95% of the total population of striatal neurons are medium spiny neurons which utilise gamma-aminobutyric acid (GABA) as their major neurotransmitter (Dube, Smith, & Bolam, 1988). In most mammals, the striatum is divided into two parts, the caudate nucleus and the putamen, by the internal capsule (Albin, Young, & Penney, 1989). In others, like the rat, the two structures are fused (Hardman et al., 2002). The striatum also encompasses the nucleus accumbens. The putamen is the part of the basal ganglia that receives motor input. The caudate nucleus receives oculomotor input and executive/associative information, while the nucleus accumbens is linked to emotions and motivation (Kandel et al., 2013).

1.3.1.2 The globus pallidus

The GP is separated into two distinct nuclei, the internal (GPi) and external (GPe) segments (Kandel et al., 2013). The GPi is one of the major output structures of the basal ganglia, while the GPe is part of the circuit of the indirect pathway (Bolam et al., 2000). The GPe receives inhibitory input from the striatum and excitatory signals from the STN (Obeso et al., 2000). It sends GABAergic input to the STN, striatum, GPi and SN pars reticulata (SNr). As for the GPi, it receives GABAergic input from the striatum and GPe and glutamatergic input from the STN (Edwards, Stamelou, Quinn, & Bhatia, 2016). The GPi sends GABAergic projections to the thalamus (Parent & Hazrati, 1995).

1.3.1.3 The substantia nigra

The SN is subdivided into two distinct parts, the SN pars compacta (SNc) and the SNr (Kandel et al., 2013). The SN gets its name from the high concentration of neuromelanin within the SNc, a dark pigment present in dopaminergic neurons (Zucca et al., 2014). The SNc sends dopaminergic input to the striatum, modulating the circuitry of the direct and indirect pathways (Obeso et al., 2000). The SNr is one of the major output structures of the BG sending inhibitory projections to the thalamus (Edwards et al., 2016).

1.3.1.4 The subthalamic nucleus

The STN is a nucleus located ventrally to the thalamus, as its name suggests (Bolam et al., 2000). It is part of the indirect circuit of the BG (Kandel et al., 2013). It receives excitatory input from the frontal lobe, and inhibitory input from the GPe (Obeso et al., 2000). It sends glutamatergic projections to the GPi and SNr (Edwards et al., 2016).

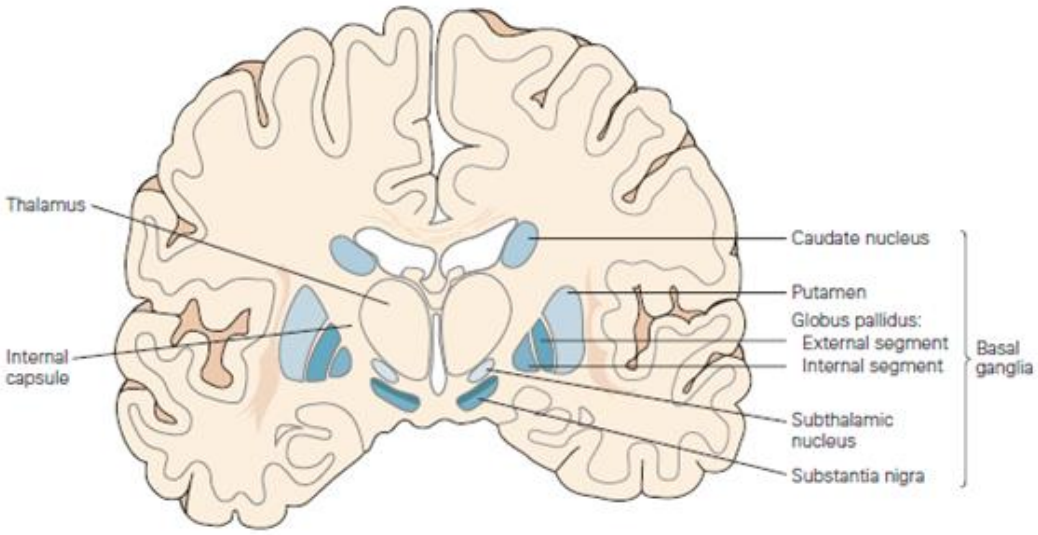

Figure 1: The human basal ganglia (Kandel ER, 2013)

1.3.2 Circuitry of the basal ganglia in physiological conditions in PD and in LID

1.3.2.1 Physiological conditions

The BG are associated with a large variety of functions such as motor, oculomotor, executive/associative and emotion/motivation (Kandel et al., 2013). Nevertheless, they are mostly known for their motor implications since various motor diseases, like PD and Huntington disease, are associated with malfunctioning of the BG circuitry (Albin et al., 1989). As seen in Figure 2, the motor circuit of the BG is divided into two main streams, the direct and indirect pathways (Purves, 2012). The direct pathway is responsible for allowing wanted movements to occur. It starts with the integration of motor information coming from various parts of the cerebral cortex to the striatum (Bolam et al., 2000). This entry of information is in the form of excitatory glutamatergic information (Alexander & Crutcher, 1990). The striatum conveys the information in the form of inhibitory GABAergic projections to the main output structures of the BG, the GPi and SNr (Calabresi, Picconi, Tozzi, Ghiglieri, & Di Filippo, 2014). Inhibition of the output structures leads to the disinhibition of the thalamus thus facilitating movement. The SNc modulates

the signal of the striatum by sending dopaminergic input that activates dopamine D_1 receptors, which further activates the GABAergic projection neurons of the striatum (Lanciego, Luquin, & Obeso, 2012).

The indirect pathway role is to suppress unwanted movements (Kandel et al., 2013). Like its direct counterpart, it starts with the integration of excitatory glutamatergic motor information from the cortex into the striatum (Alexander & Crutcher, 1990). The striatum then sends GABAergic projections to the GPe which in turn sends inhibitory information to both the STN and the output structures (Wei, Rubin, & Wang, 2015). The STN also receives excitatory projections directly from the cerebral cortex and sends excitatory projections to the output structures (Calabresi et al., 2014). All of this results in greater activation of the output structures, which translates into inhibition of the thalamus, less cortical activation and suppression of unwanted movements (DeLong & Wichmann, 2007). The SNc also modulates the indirect pathway by sending dopaminergic input that activates dopamine D_2 receptors, leading to more inhibition of the striatum (Lanciego et al., 2012).

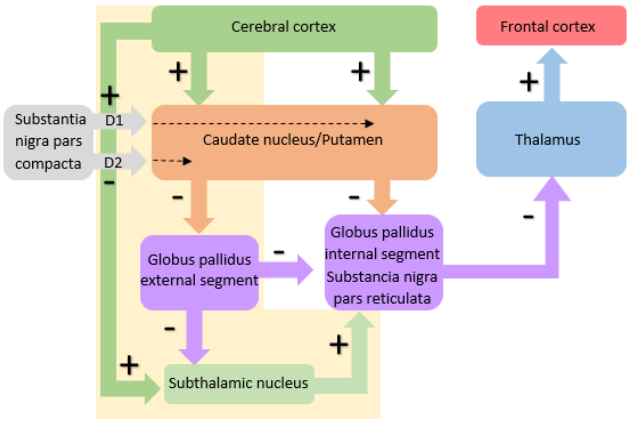

Figure 2: Motor circuit of basal ganglia

1.3.2.2 In Parkinson's disease state

In PD, the SNc dopaminergic neurons degenerate, which leads to a deficit of dopamine in the striatum. As mentioned above, in the direct pathway, dopamine binds to D_1 receptors, which are mainly excitatory, whereas in the indirect pathway, dopamine binds to D_2 receptors which are mainly inhibitory. This ultimately results in disinhibition of the output structures of the BG in both the direct and indirect pathways which translates into greater inhibition of the thalamus and fewer movements (Figure 3).

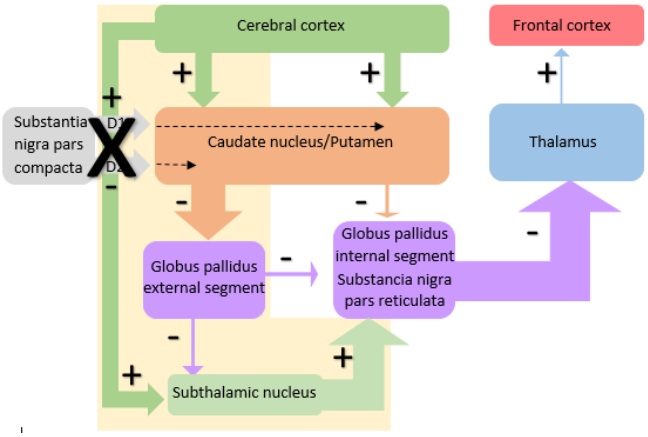

Figure 3: Motor circuit of basal ganglia in Parkinson's disease

1.3.2.3 In dyskinetic state

As illustrated in Figure 4, in the dyskinetic state, dopamine derived from L-DOPA induces over-activation of D_1 and D_2 receptors in striatum medium spiny neurons (Rodriguez-Oroz et al., 2009). This leads to inhibition of the output structures of the BG and translates into the disinhibition of the thalamus and enhances glutamatergic output to the cortex, resulting in abnormal involuntary movements (Purves, 2012).

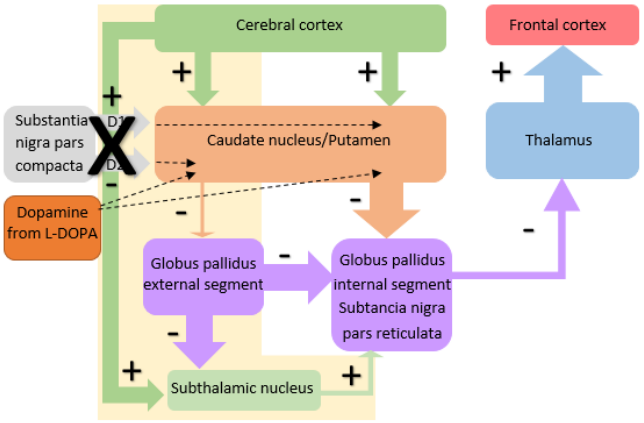

Figure 4: Motor circuit of the basal ganglia in L-DOPA induced dyskinesia

1.4 L-DOPA induced dyskinesia

LID is an important complication of chronic L-DOPA treatment and its occurrence is almost inevitable, as 95% of patients will harbour LID 15 years after the beginning of L-DOPA therapy (Hely et al., 2005). The apparition of LID is not constant and will vary from one patient to another. The mean onset is 4.2 years after the initial L-DOPA therapy (Hely et al., 2005), however, after 10 years, only 43% of patients will need medication adjustments to reduce the severity of LID (Van Gerpen, Kumar, Bower, Weigand, & Ahlskog, 2006). From a pharmacokinetic perspective, LIDs are classified into 3 different types: peak dose, off period and diphasic.

Peak dose LID mostly occurs when plasma levels of L-DOPA are the highest. They are usually characterised by choreic or ballistic movements of limbs and trunk and affect first the side most afflicted by PD (Hametner, Seppi, & Poewe, 2010), while neck and face movement are more of dystonic nature (Luquin, Scipioni, Vaamonde, Gershanik, & Obeso, 1992). They are most of the time less bothersome than other types of LID, however, certain drug adjustment can be done to manage peak dose LID.

Off period LID occurs when dopamine levels are low or decreasing (Bravi, Mouradian, Roberts, Davis, & Chase, 1993). It is present in about 20-30% of patients and is mostly associated with pain (Marconi et al., 1994). This type of LID occurs early morning before the first dose of L-DOPA is taken (Melamed, 1979). It mainly consists of dystonic postures affecting the lower limbs (Poewe, Lees, & Stern, 1988). It is usually managed by taking L-DOPA immediately after waking up or by taking fast action L-DOPA (Hametner et al., 2010).

Diphasic LID occurs when L-DOPA levels are either falling or rising. It is the least frequent type of LID (Luquin, Scipioni, et al., 1992; Marconi et al., 1994). It mostly affects the legs in alternating movements (Ruzicka, Zarubova, Nutt, & Bloem, 2011). Treatment for this type of dyskinesia is to increase the levels of L-DOPA. Diphasic LID mostly affects male patients with early onset PD (Chang et al., 2012).

1.4.1 Neurotransmission systems in LID

The multiple LID subtypes indicate that pathophysiology, pharmacokinetic and clinical presentation of LID is complex and might implicate various neurotransmission systems. This might also explain why targeting one type of receptor cannot fully alleviate LID and suggests that a combined drug approach might represent an interesting avenue.

1.4.1.1 Dopaminergic system

Dopaminergic depletion seems to facilitate the development of LID. Consequently, LID is more frequent in advanced PD, in which dopaminergic denervation is more severe (Hauser, McDermott, & Messing, 2006). In animal models, dopaminergic depletion of 88% in the rat (Schallert, Fleming, Leasure, Tillerson, & Bland, 2000) and 50% in the non-human primate (NHP) (Di Monte et al., 2000) was sufficient for the development of stable LIDs. However, despite consistent dopaminergic loss the development of LID is not identical across individuals,

suggesting there may be other factors to take into account (P. Huot et al., 2013). One of these factors may be L-DOPA administration. Indeed, normal NHPs can develop LID following consumption of high doses of L-DOPA (R. K. Pearce, Heikkila, Linden, & Jenner, 2001). This indicates that L-DOPA may be sufficient to induce LID but dopaminergic denervation may act as a facilitator to its development and may lead to more severe LID (P. Huot et al., 2013).

Other factors that contribute to the induction of LID might pertain to the pulsatile release of dopamine following L-DOPA intake. L-DOPA needs to be taken regularly because of its short half-life (Deleu, Northway, & Hanssens, 2002), leading to variations in L-DOPA plasma levels (Cedarbaum, 1987). In the healthy brain, dopamine release is tonic and never falls below a certain threshold (Grace, 1995), while in advanced PD L-DOPA ingestion leads to pulsatile, phasic release of dopamine (Nyholm, 2007). Pulsatile stimulation of dopaminergic receptors is thought to play an important role in the development of LID (Nutt & Holford, 1996). Indeed, DAs with shorter half-life apomorphine induced more abnormal involuntary movements (AIMs) in the 6-hydroxydopamine (6-OHDA) lesioned rat than DAs with a longer half-life pramipexole and pergolide (Papathanou, Rose, McCreary, & Jenner, 2011). However, we must take note that apomorphine binds to both D1 and D2 receptors while pramipexole and pergolide mostly bind to D2 receptors.

Dopaminergic receptors are divided into two groups. D_1-like receptors activate adenylyl cyclase activity and include D_1 and D_5 receptors, while D_2-like receptors reduce adenylyl cyclase activity and include D_2, D_3 and D_4 receptors (Lachowicz & Sibley, 1997). D_1 and D_2 receptors are the most abundant dopaminergic receptors in the striatum (Sokoloff & Schwartz, 1995) thus the most studied in PD and LID (P. Huot et al., 2013). It was demonstrated in a positron emission tomography study that levels of D_1 receptors were unchanged in PD patients with and without

LID, compared to healthy subjects, while D_2 levels were reduced in PD patients with and without LID (Turjanski, Lees, & Brooks, 1997). It was observed that there is an abnormal striatal distribution of D_1 receptors in dyskinetic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) NHPs, which might be attributable to hypoactivity of the 20s proteasomal subunit of striatal medium spiny neurons (Berthet et al., 2012).

D_1 receptors are also thought to be important for the priming process leading to LID development. Indeed, treatment with selective D_1 agonists led to the development of AIMs in 6-OHDA-lesioned rat (Dupre, Eskow, Negron, & Bishop, 2007; Jaunarajs et al., 2009) and dyskinesia in MPTP-lesioned primates (Blanchet, Grondin, & Bedard, 1996; Goulet, Grondin, Blanchet, Bedard, & Di Paolo, 1996). However, once the priming period is completed D_1 stimulation might become beneficial on LID, while D_2 activation may elicit more severe LID (Blanchet, Gomez-Mancilla, & Bedard, 1995). It was also demonstrated that D_2 stimulation with (+)-PHNO led to the development of LID in MPTP-lesioned NHPs (Luquin, Laguna, & Obeso, 1992). D_3 receptors are also thought to be implicated in LID development and expression. Indeed, it was shown that the D_3 agonist PD-128,907 elicits LIDs in MPTP-lesioned macaques (Blanchet, Konitsiotis, & Chase, 1997) and that the D_3 antagonist S-33,084 alleviated LID (Visanji et al., 2009). A possible cross-talk between D_1 and D_3 receptors is suspected, since stimulation of D_1 receptors led to increase striatal D_3 mRNA and binding levels (Bordet et al., 1997; Bordet, Ridray, Schwartz, & Sokoloff, 2000). Regarding D_4 receptors, a selective D_4 antagonist significantly reduced dyskinesia in MPTP-lesioned macaques (P. Huot et al., 2012) indicating a possible involvement of the receptors in the pharmacology of LID. Finally, the involvement of D_5 receptors in LID remain to be demonstrated.

1.4.1.2 Serotonergic system

The serotonergic system is believed to play a key role in LID, perhaps not directly by the modulation of serotonin (5-HT), but rather through the modulation of two other neurotransmitters, dopamine and glutamate. Indeed, the serotonergic system is thought to mediate abnormal glutamatergic and dopaminergic transmissions in the striatum (P. Huot et al., 2013).

There are 14 different 5-HT receptors that are divided into 7 subtypes, 5-HT_1 to 5-HT_7. All except the 5-HT_3 receptor are G-protein coupled receptors (Kandel et al., 2013). The most studied ones in the context of LID are 5-$HT_{1A/B}$ and 5-HT_{2A} receptors. 5-HT_{1A} and 5-HT_{1B} receptors are both coupled to a $G_{i/o}$ protein that inhibits adenylyl cyclase (Giulietti et al., 2014; Nichols & Nichols, 2008). 5-HT_{1A} activation leads to a reduction of corticostriatal glutamate release, as well as a reduction of dopamine release by serotonergic neurons in the striatum (P. Huot, Fox, & Brotchie, 2011). 5-HT_{1A} agonists like tandospirone, buspirone and eltoprazine showed anti-dyskinetic potentials, however, they interfered with L-DOPA antiparkinsonian effect (P. Huot, 2018). Eltoprazine activates 5-HT_{1B} and inhibits 5-HT_{2C} and provided some anti-dyskinetic benefit in a clinical trial (Svenningsson et al., 2015).

In advanced stages of PD, as dopaminergic loss increases, L-DOPA gets converted into dopamine by other AADC-containing cells. These cells might include dopaminergic neurons intrinsic to the striatum (P. Huot & Parent, 2007), noradrenergic neurons, serotonergic neurons, AADC-containing neurons, pericytes or glial cells (Nagatsu & Sawada, 2009). Although much remains unknown about the pathophysiology of LID, it has been demonstrated, in the 6-OHDA-lesioned rat, that the development of LID is dependent on the integrity and function of the serotonergic system (Carta, Carlsson, Kirik, & Bjorklund, 2007) because, if serotonergic neurons

have the necessary enzymes to convert L-DOPA into dopamine, they do not possess retroactive control, leading to unregulated dopamine release (Navailles, Bioulac, Gross, & De Deurwaerdere, 2010). In the context of dopamine denervation in PD, serotonergic neurons contribute increasingly to the conversion of L-DOPA to dopamine, which may partly explain the development of LID. Indeed, it has been demonstrated in the macaques and in rats that the integrity and function of the serotonergic system is necessary for the development of LID (Carta et al., 2007; Sgambato-Faure & Tremblay, 2018).

1.4.1.3 Glutamatergic system

Glutamate is the major excitatory neurotransmitter of the brain (Pin & Duvoisin, 1995) and a key component of the BG motor circuit (Alexander & Crutcher, 1990). Overactive glutamatergic activity is believed to be important in both LID development and expression (P. Huot et al., 2013). Thus, decreasing the levels of glutamate is thought to be a possible avenue for the treatment of LID. Glutamate receptors are divided into two main categories, ionotropic and metabotropic. Ionotropic receptors are ligand-gated channels on which glutamate binding directly opens the channel, while metabotropic receptors are G protein-coupled receptors that act through the production of second messengers (Kandel et al., 2013). Metabotropic glutamate receptors (mGlu) can be divided into three groups, sharing eight subtypes of receptors ($mGlu_{1-8}$). Group I receptors contain $mGlu_1$ and $mGlu_5$ and are coupled to activation of phospholipase C; group II comprises $mGlu_2$ and $mGlu_3$, whilst group III contains $mGlu_{4,6-8}$ (Conn & Pin, 1997). *N*-methyl-*D*-aspartate (NMDA) and $mGlu_5$ receptors have been the most studied in LID. The $mGlu_5$ antagonist MTEP reduced AIMs severity in 6-OHDA-lesioned rat without hindering L-DOPA anti-parkinsonian effect (Dekundy, Pietraszek, Schaefer, Cenci, & Danysz, 2006; Mela et al., 2007; Rylander et al., 2009). In the MPTP-lesioned macaque, MTEP also led to a reduction of LID, but also altered L-

DOPA anti-parkinsonian action (Johnston, Fox, McIldowie, Piggott, & Brotchie, 2010) indicating a small therapeutic window, which renders this target less attractive. NMDA receptor blockade is the purported mechanism of action of the only FDA-approved drug for LID, amantadine (Brigham et al., 2018). However, amantadine may elicit side effects like hallucinations and psychosis (Snow, Macdonald, McAuley, & Wallis, 2000) and, while it reduces LID, it does not abolish it (Perez-Lloret & Rascol, 2018), and LID still remains an unmet need.

1.4.1.4 5-HT_{2A} and $mGlu_2$ receptors

1.4.1.4.1 5-HT_{2A} receptors

5-HT_{2A} receptors are coupled to a $G_{\alpha q}$ protein which leads to excitatory transmission upon activation (Giulietti et al., 2014). 5-HT_{2A} receptors modulate the release of glutamate in the cortex and the striatum and dopamine in the striatum and SN (Aghajanian & Marek, 1999; Ansah, Ferguson, & Nayyar, 2011; Lucas & Spampinato, 2000). A variety of 5-HT_{2A} antagonists have elicited anti-dyskinetic benefits. For instance, clozapine reduced dyskinesia in MPTP-lesioned NHPs (Fox et al., 2010; Visanji et al., 2006) and 6-OHDA-lesioned rats (Lundblad et al., 2002), as well as in clinical settings (Bennett et al., 1994; Durif et al., 2004; Pierelli et al., 1998). However, clozapine also has high affinity for D_2 receptors as well as for alpha adrenoceptors 1 and 2 and H_1 histamine receptors, so the effects may not be mediated only through the 5-HT_{2A} receptor. It is also associated with a serious side-effect known as agranulocytosis (Alvir, Lieberman, Safferman, Schwimmer, & Schaaf, 1993).

Quetiapine alleviated LID in MPTP-lesioned NHPs (Oh, Bibbiani, & Chase, 2002; Visanji et al., 2006) and 6-OHDA rats (Oh et al., 2002), but had inconsistent effects on LID in clinical trials, as two studies reported improvement (Baron & Dalton, 2003; Gimenez-Roldan, Navarro, &

Mateo, 2003) and another showed no effect (Katzenschlager, Manson, Evans, Watt, & Lees, 2004). As for clozapine, quetiapine lacks selectivity and targets other serotonergic, dopaminergic and histaminergic receptors, as well as adrenoceptors.

Pimavanserin, which has a 40× selectivity for 5-HT_{2A} receptors over its next target 5-HT_{2C} receptors, reduced the severity of LID in MPTP-lesioned NHPs (Vanover et al., 2008) and in a Phase II clinical trial in patients with PD (Roberts, 2006a). However, volinanserin, a selective 5-HT_{2A} antagonist, had no effect on the severity of AIMs in the 6-OHDA-lesioned rat (Taylor, Bishop, Ullrich, Rice, & Walker, 2006). The reason(s) for these results are unclear as to whether it has to do with the high doses used which can lead to inhibition of 5-HT_{2C} as well or to the differences in the anatomy of the BG and/or localisation of 5-HT_{2A} receptors in the rat brain. These results are, however, consistent with those obtained with the highly-selective 5-HT_{2A} antagonist EMD-281,014, which led to a reduction of LID in the MPTP-lesioned marmoset (Hamadjida et al., 2018) but to no effect on AIMs severity in the 6-OHDA-lesioned rat (Frouni et al., 2018). In this study, EMD-281,014 was selected as the 5-HT_{2A} antagonist because of its selectivity and its great translational potential. Indeed, EMD-281,014, was clinically tested in Phase II for insomnia (Vanover & Davis, 2010).

1.4.1.4.2 Heterodimer between 5-HT_{2A} and $mGlu_2$

The $mGlu_2$ receptors are known to assemble into dimers (Maurel et al., 2008). It has been demonstrated that $mGlu_2$ and 5-HT_{2A} receptors interact closely through such a heterodimeric complex (Fribourg et al., 2011; Gonzalez-Maeso et al., 2008). The existence of this hetero-dimer was demonstrated by several methods, such as co-immunoprecipitation of human brain cortex neurons, cell lines and bioluminescence resonance energy transfer and fluorescence resonance energy transfer assays (Gonzalez-Maeso et al., 2008). It is believed that the anti-psychotic potential

of 5-HT_{2A} antagonists may be due to their capacity to modulate this very complex (Fribourg et al., 2011). It has also been demonstrated that stimulation of cells expressing the complex with an $mGlu_2$ agonist leads to structural rearrangements of 5-HT_{2A} receptors (Moreno et al., 2016). Positive or negative stimulation of either side of the 5-HT_{2A}/$mGlu_2$ complex modulates the Gi/Gq protein signalisation (Yin et al., 2014). As shown in Figure 5, the activation of $mGlu_2$ and inhibition of 5-HT_{2A} could both result in greater Gi signal and vice-versa for Gq signalling (Fribourg et al., 2011). Whether dual modulation of both halves of the 5-HT_{2A}/$mGlu_2$ hetero-complex would lead to greater anti-dyskinetic benefit has not been assessed.

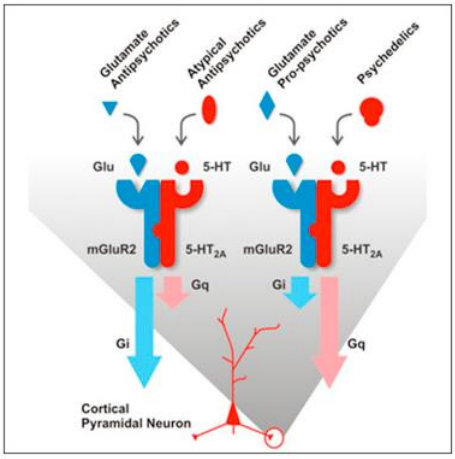

Figure 5: Model of action of the 5-HT_{2A}/$mGlu_2$ heterodimer (Fribourg et al., 2011)

1.4.1.4.3 Pharmacological modulation of $mGlu_2$ receptors

In this study, we chose to stimulate the $mGlu_2$ receptor using both a positive allosteric modulator (PAM) and an orthosteric agonist (OA). A PAM is a compound that does not directly activate a receptor, but rather enhances the effect of the endogenous ligand, by binding to a site other than the active site (Niswender & Conn, 2010) whereas an OA is an agonist that binds to the active site and activates a receptor directly (Niswender & Conn, 2010). A PAM and an OA can bind to $mGlu_2$ simultaneously (May, Leach, Sexton, & Christopoulos, 2007). The PAM used in

this study is LY-487,379, which is the most selective $mGlu_2$ PAM commercially available with no other known target (Poisik et al., 2005; Schaffhauser et al., 2003). It has been tested pre-clinically as an anti-psychotic and anxiolytic drug (Galici, Echemendia, Rodriguez, & Conn, 2005; Hermes & Renaud, 2011; Johnson et al., 2005; Wieronska, Stachowicz, Branski, Palucha-Poniewiera, & Pilc, 2012) and is known to reduce LID in both the 6-OHDA-lesioned rat (Kwan et al., 2017) and the MPTP-lesioned marmoset (Sid-Otmane et al., 2017). Its pharmacokinetic properties are known for both species (Gaudette et al., 2018). The OA selected for this study is LY-354,740. Also known as eglumegad, LY-354,740 is the most selective $mGlu_2$ OA commercially-available with approximately 6-fold selectivity over its closest target, $mGlu_3$ receptors (Schoepp et al., 1997) and entered clinical trials with anxiety-related endpoints (Bergink & Westenberg, 2005; Grillon, Cordova, Levine, & Morgan, 2003; Schoepp, Wright, Levine, Gaydos, & Potter, 2003) which gives it great translational potential. Its anti-dyskinetic potential has been demonstrated in both the 6-OHDA-lesioned rat (Hamadjida et al., 2017) and MPTP-lesioned marmoset (P. Huot, Nuara, Gourdon, & Hamadjida, 2017) and its pharmacokinetic profile is known in both species (Frouni et al., 2019; Gaudette et al., 2017).

1.4.1.4.4 $mGlu_2$ receptors

We believe that there might be two ways by which $mGlu_2$ receptors are implicated in LID. The first one is through regulation of glutamate release and re-uptake, the second one is through modulation of the $5\text{-}HT_{2A}$-$mGlu_2$ heterodimer that was discussed above.

The localisation of group II mGlu receptors is mainly pre-synaptic, where they modulate the glutamatergic input from the cortex to the striatum as well as the output from the STN to the GPi/SNr (Ohishi, Shigemoto, Nakanishi, & Mizuno, 1993; Yao et al., 2005). Activation of these receptors inhibits adenylyl cyclase (Conn & Pin, 1997), resulting in feedback inhibition of

glutamate release through inhibition of voltage-gated calcium channels (Byrnes, Loane, & Faden, 2009). In astrocytes, group II mGlu receptors increase glutamate uptake (Yao et al., 2005) which has been hypothesised to prevent glutamatergic-induced SNc neuron degeneration (Rouse et al., 2000). In the BG, the principal role of $mGlu_2$ receptor is to regulate glutamatergic transmission (Murray et al., 2002). As such, because glutamatergic transmission is overactive in LID (Calon, Rajput, Hornykiewicz, Bedard, & Di Paolo, 2003; Verhagen Metman, Del Dotto, Blanchet, van den Munckhof, & Chase, 1998), $mGlu_2$ activation, by reducing surrounding glutamate levels, could possibly reduce LID.

1.4.1.4.5 Possible synergy between 5-HT_{2A} blockade and $mGlu_2$ activation

This study is based on previous observation that a possible synergistic effect can be observed by combining 5-HT_{2A} blockade with $mGlu_2$ activation. Indeed, preliminary data from the lab show a greater reduction of LID in the MPTP-lesioned marmoset by combining either LY-354,740 or LY-487,379 with EMD-281,014. However, whether a combination of the three molecules would further reduce LID in the MPTP-lesioned marmoset has not been assessed yet. Whether a synergistic anti-dyskinetic effect would occur in the 6-OHDA lesioned rat is also unknown.

1.5 Animal models of Parkinson's disease and L-DOPA induced dyskinesia

Animal models are used in research to grasp a better understanding and/or to reproduce fully or partially a disease in vivo. For the study of LID, two models are typically used the 6-OHDA-lesion rat and the MPTP-lesion NHP.

1.5.1 MPTP-lesioned animal models

NHP or mice are the typical animals used in the MPTP-lesioned model. In the NHP, MPTP induces dopaminergic loss in the nigrostriatal pathway (Bezard et al., 2001; Burns et al., 1983; Guigoni et al., 2005; Jan et al., 2003; Jenner et al., 1984) and LID develops after L-DOPA priming (Nadjar, Gerfen, & Bezard, 2009). There are four commonly used NHP species in the literature: the squirrel monkey, the marmoset, the cynomolgus macaque and the rhesus macaque (Iderberg, Francardo, & Pioli, 2012). The cynomoglus and rhesus macaques develop behaviour very similar to human PD (Bezard & Przedborski, 2011; Langston, Quik, Petzinger, Jakowec, & Di Monte, 2000), however, they demand more complex and costly infrastructure to work with. The squirrel monkey is rather small which makes housing more convenient. However, normal squirrel monkeys can develop LID, making the model unreliable (R. K. Pearce et al., 2001). The marmoset is also small, which makes it convenient and has more literature on the PD pathophysiology than squirrel monkeys (Iderberg et al., 2012). The MPTP-lesioned mouse model has progressive denervation which makes it interesting to study the development of the disease, however, in order to develop dyskinesia, mice need to be aged and require large doses of L-DOPA (Gross et al., 2003; Nicholas, 2007).

1.5.2 6-OHDA-lesioned animal models

The 6-OHDA model is interesting since it requires a single administration of toxin. However, the injection is intracranial and requires a certain expertise and the appropriate material. 6-OHDA is usually used in rats or mice. Unilateral injection of 6-OHDA in mice induces stable, reproducible damage to the nigrostriatal tract and a high predictability in the degree and the course of degeneration (Iderberg et al., 2012). By varying the concentration of the toxin and the site of

lesion, it is possible to reproduce different stages of the disease (Blandini, Armentero, & Martignoni, 2008). However, because of their size, mice can be hard to use when studying detailed motor behaviours. In rats, 6-OHDA can be injected almost anywhere along the nigrostriatal tract and will result in different representations of the disease (Cenci, Whishaw, & Schallert, 2002). 6-OHDA damages all the catecholaminergic neurons in the rat, so in order to limit the degeneration to dopaminergic neurons, desipramine, an inhibitor of the noradrenaline transporter, is injected prior to the lesion (Iderberg et al., 2012). The most complete degeneration can be achieved by injecting into the medial forebrain bundle, which can lead to complete loss of dopaminergic terminals in the striatum (Winkler, Kirik, Bjorklund, & Cenci, 2002). By injecting the toxin unilaterally, the un-lesioned side can be used as a control (Lundblad et al., 2002).

1.6 Hypothesis and objectives

Following the discovery of the anti-dyskinetic potential of clozapine, numerous studies have suggested that 5-HT_{2A} antagonism may be an effective strategy to treat LID (Bennett et al., 1994; Denoordhout & Delwaide, 1986; S. H. Fox et al., 2018; Meco et al., 1988; Roberts, 2006b; Vanover et al., 2008). Although compounds like clozapine, quetiapine, ritanserin, pimavanserin and EMD-281,014 have demonstrated an anti-dyskinetic effect, the reduction of the severity of LID is limited in magnitude (Bennett et al., 1994; Denoordhout & Delwaide, 1986; Durif et al., 2004; Frouni et al., 2018; Hamadjida et al., 2018; Kwan et al., 2018; Oh et al., 2002; Vanover et al., 2008). In order to enhance the reduction of LID conferred by 5-HT_{2A} blockade, we looked at the heterodimer formed by 5-HT_{2A} and $mGlu_2$ receptors as a possible new target to achieve a greater reduction of LID.

Based on the fact that 5-HT_{2A} receptors form a hetero-complex with $mGlu_2$ receptors, we hypothesised that combining a selective 5-HT_{2A} antagonist with a selective $mGlu_2$ OA and a

selective $mGlu_2$ PAM, would be more effective at alleviating LID than modulating 5-HT_{2A} and $mGlu_2$ receptors separately. Thus, the present study seeks to determine and validate the potential synergistic effect of combined receptor modulations as a possible new approach to achieve a greater or a full suppression of LID. More precisely, we are looking at dual activation of the $mGlu_2$ receptor, with both a PAM and an OA, combined with a 5-HT_{2A} receptor antagonist as a possible way to alleviate LID.

To validate this hypothesis, we specifically aim to:

1. Determine whether synergy results from concurrent 5-HT_{2A} blockade, $mGlu_2$ positive allosteric modulation and $mGlu_2$ orthosteric stimulation and leads to further LID reduction, in the 6-OHDA-lesioned rat;
2. Determine the effect of the combination of EMD-281,014 with LY-487,379 and LY-354,740 on L-DOPA anti-parkinsonian action, in the 6-OHDA-lesioned rat.

2. Materials and methods

2.1 Animals

Female Sprague-Dawley rats (N = 30) were used for this study. Female rats were chosen over male rats because their weight does not vary over a long period of time. At arrival from Charles River (Saint-Constant, Canada), they weighed between 250-275g and were placed 3 per cage. The housing room temperature varied between 19-21°C, humidity was set at 55% and the light/dark cycles were 12 hours with the light cycle starting at 7:00 am. The rats also had unlimited access to food and water and were left undisturbed for 72 hours after arrival for acclimatisation. 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.

2.2 Induction of parkinsonism

Rats were pretreated with pargyline (5 mg/kg) and desipramine (10 mg/kg). After 30 minutes, rats were anaesthetised using isoflurane (2-4%) in 100% oxygen (1-2L/min). Then, rats were placed in a stereotaxic frame and were injected with 2.5 µL of 6-OHDA into the right medial forebrain bundle at a rate of 0.5 µL/min at the following coordinates: anteroposterior -2.8 mm, lateromedial -2.0 mm and dorsoventral -9.0 mm from Bregma and skull surface (Frouni et al., 2018). Animals were then allowed a 3-week recovery period, during which parkinsonism developed, after which the cylinder test was performed, to assess the severity of parkinsonian disability.

2.3 Cylinder test

For the cylinder test, rats were placed in a transparent cylinder and recorded for 15 minutes. Afterwards, the videos were analysed to assess the number of times rats make wall contact with the right, left, or both paws at the same time. Since the lesion affects the left side,

only the rats which had an overall use of the un-lesioned (right) forepaw over 70% of the time were selected, as this score correlates with 88% loss of dopamine in the striatum (Schallert et al., 2000).

2.4 AIMs assessment

Rats (N = 24) with severe rearing asymmetry at the cylinder test were primed with subcutaneous (s.c.) injections of L-DOPA/benserazide (10/15 mg/kg) for 14 days to induce stable AIMs. Only rats exhibiting reproducible and stable AIMs were selected to undergo further testing. AIMs were assessed using the abnormal involuntary movement scale (AIMs) (Cenci & Lundblad, 2007), which is used by most laboratories conducting research on the 6-OHDA-lesioned rat (Carta et al., 2007; Cenci, Lee, & Bjorklund, 1998; Dupre et al., 2007; Lane, Cheetham, & Jenner, 2005; Lane, Winkler, Brundin, & Cenci, 2006; Lundblad et al., 2002). Axial, limb and oro-lingual (ALO) AIMs were each rated according to their "duration" and "amplitude" on a 0 to 4 scale. On experimental days, rats received their respective treatments and were then placed in glass cylinders. Following baseline assessment and treatment administration, ALO AIMs were rated for two min every 20 min for 180 min according to validated scales. For the duration scale, 0 = no dyskinesia; 1 = occasional signs of dyskinesia, less than half of the observation time; 2 = frequent signs of dyskinesia, more than half of the observation time; 3 = dyskinesia is present during the entire observation time, but it is suppressible by external stimuli (*e.g.*, sudden, loud opening of the lid of the cage); 4 = continuous dyskinesia that is not suppressible by external stimuli (Cenci & Lundblad, 2007). However, a second way to calculate the duration was explored: the duration of the maximal amplitude of the AIMs which was calculated the same way as the duration but only for the highest amplitude movements. The two durations were then summed for a final duration scale

from 0 to 8. For the axial amplitude scale, 1 = sustained deviation of the head and neck, at ~ 30◦ angle; 2 = sustained deviation of the head and neck, angle ≤ 60◦; 3 = sustained twisting of the head, neck, and upper trunk at an angle > 60◦ but ≤ 90◦; 4 = sustained twisting of the head, neck, and trunk at maximal amplitude (angle > 90◦), causing the rat to lose balance from a bipedal position. Limb AIMs amplitude was rated as follows, 1 = tiny movements of the paw around a fixed position, 2 = movements resulting in a visible displacement of the whole limb either sideways or up-and-down, 3 = large displacement of the whole limb with visible contraction of shoulder muscles, 4 = vigorous limb displacement of maximal possible amplitude, with conspicuous contraction of both shoulder muscle groups and extensor muscles. Oro-lingual AIMs amplitude was rated as follows, 1 = twitching of facial muscles accompanied by small masticatory movements without jaw opening, 2 = twitching of facial muscles, accompanied by noticeable masticatory movements, occasionally leading to jaw opening, 3 = movements with broad involvement of facial muscles and masticatory muscles, frequent jaw opening and occasional tongue protrusion, 4 = all the above muscle categories are involved to the maximal possible degree. Integrated AIMs are defined as the multiplication of amplitude AIMs and duration AIMs (Ohlin et al., 2011). Cumulative AIMs represents the sum of all measured AIMs during any observation session.

2.5 Drug administration

On experimental days, rats were administered L-DOPA/benserazide (6/15 mg/kg s.c.) in combination with previously determined doses of each of EMD-281,014 (vehicle, 0.03 and 0.1 mg/kg s.c), LY-354,740 (vehicle and 0.1 mg/kg s.c.), and LY-487,379 (vehicle and 0.1 mg/kg s.c.). Drug administration was randomised according to a Latin square design. After a 3-day washout period needed to ensure complete elimination of the drugs, a low-dose of L-DOPA (3

mg/kg s.c.) was administered with a combination of EMD-281,014/LY-354,740/LY-487,379, and the effect on L-DOPA anti-parkinsonian action was determined by the cylinder test. Indeed, it is not possible, using the 6-OHDA-lesioned rat model of PD, to determine whether an anti-dyskinetic effect was achieved at the expense of L-DOPA anti-parkinsonian action, which is why a second test, here the cylinder test, was performed. Any drug interfering with L-DOPA anti-parkinsonian action might not be well tolerated by patients in clinical settings, which is why we assessed this parameter.

2.6 High performance liquid chromatography

In order to confirm the lesion severity, after extraction of the brain, the two hemispheres are separated and the striatal tissues were collected. They were placed in labelled 1.5 mL Eppendorf tubes and kept at -80°C. The samples were then shipped to Dr Lekha Sleno, from Université du Québec à Montréal, with whom our lab has an on-going collaboration. Dr Sleno's lab will process samples for specific quantification of dopamine and its metabolites by conducting high-performance liquid chromatography coupled with electro-chemical detection. These analyses are currently being conducted. By comparing dopamine levels in each hemisphere, it is possible to quantitate lesion severity.

2.7 Statistical analysis

Rearing asymmetry data collected via the cylinder test are presented as mean ± standard error (SEM) and were analysed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. ALO AIMs scores are presented as the median with interquartile range and were analysed using Friedman followed by Dunn's *post hoc* test. Finally, rearing data from the L-DOPA challenge are presented as the mean ± SEM and were analysed using one-way ANOVA

followed by Tukey's *post hoc* test. Statistical significance was set to $P < 0.05$. Statistical analyses were computed using GraphPad Prism 7.0d (GraphPad Software Inc, USA).

3. Results

3.1 Dopaminergic neurons denervation assessment

In order to assess the severity of the 6-OHDA lesion, the cylinder test was used as an asymmetry test. A right forepaw use of 70% correlates with 88% loss of dopamine in the striatum which is sufficient to induce stable and reproducible AIMs (Frouni et al., 2018). The rats selected in this study used the right forepaw in 82.5% ± 7.7% of wall contacts, compared to 15.5% ± 6.9% use of both forepaws and 2.0% ± 2.0% of the rears made with the left forepaw, as shown in Figure 6.

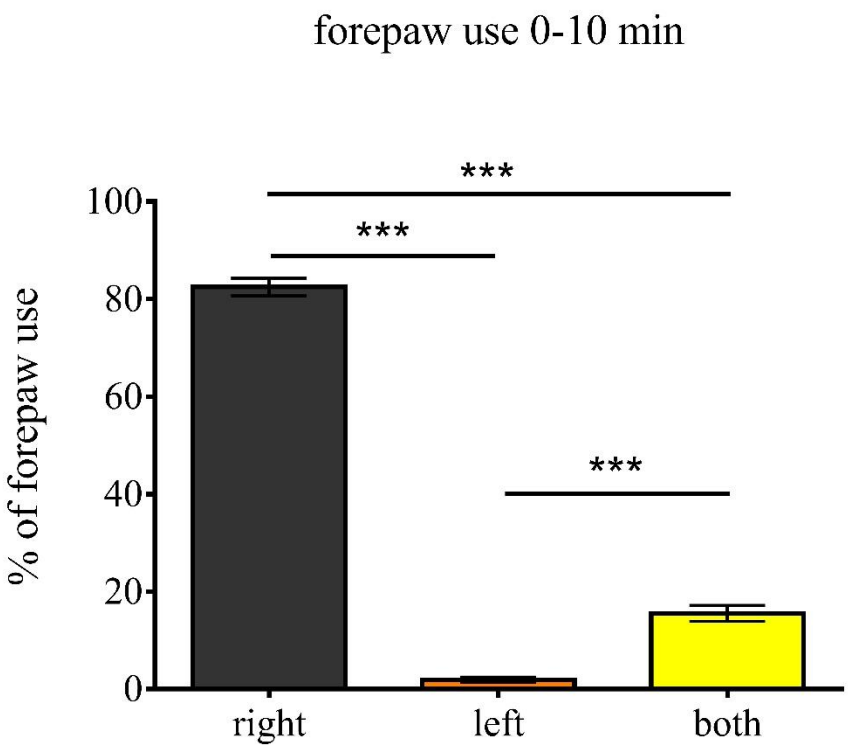

Figure 6: Representative graph of percentage of forepaws use over a 10-min time period by the rats selected to undergo the acute challenge experiments (N = 18).

Data are presented as mean ± SEM, ***: $P < 0.001$

3.2 Acute challenge study

During the course of the acute challenge study, rats were administered 6 mg/kg L-DOPA every day. There were always at least 48 hours between experimental days to ensure a complete washout of the drugs. Drugs were administered according to a Latin square within-subject design (see Appendix).

3.2.1 Cumulative axial AIMs

As illustrated in Figures 7 and 8, adding EMD-281,014 (0.03 and 0.1mg/kg) to L-DOPA, with either LY-354,740 or LY-487,379 or both, had no effect on axial AIMs throughout the behavioural sessions.

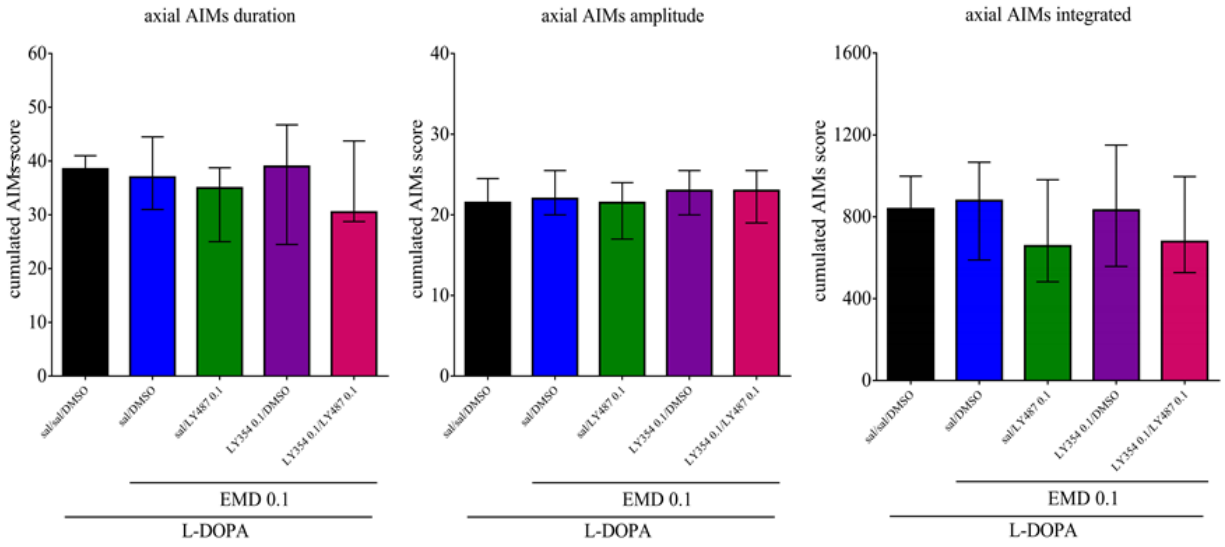

Figure 7: Representative graph of axial AIMs EMD-281,014 0.1 mg/kg
A. Representative graph of axial AIMs duration following injection of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle. **B.** Representative graph of axial AIMs amplitude after injection of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle **C.** Representative graph of integrated axial AIMs after administration of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle.

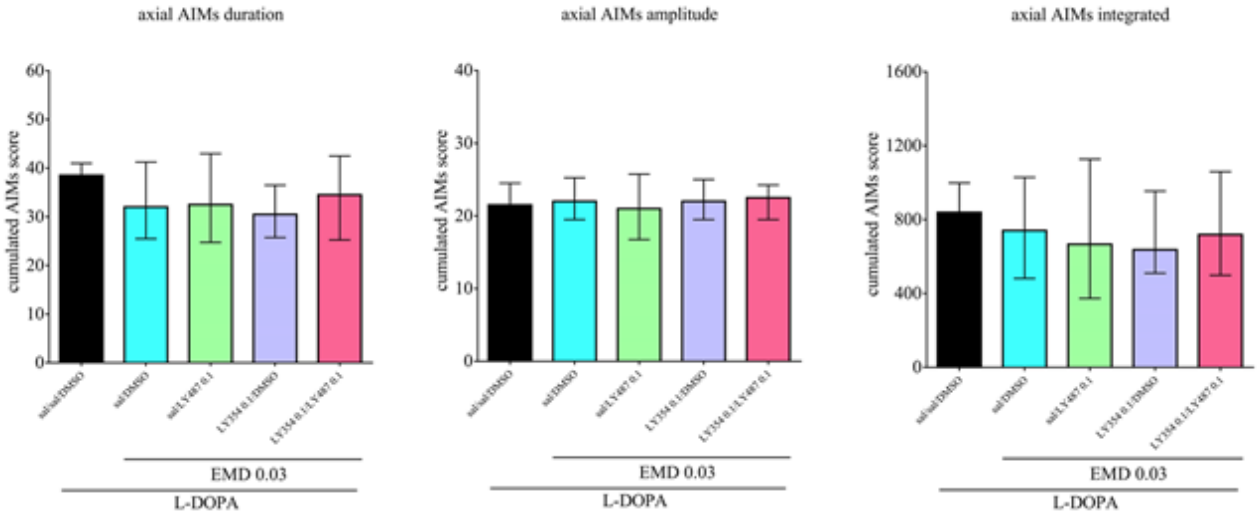

Figure 8: Representative graph of axial AIMs EMD-281,014 0.03 mg/kg
A. Representative graph of axial AIMs duration following administration of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle. **B.** Representative graph of axial AIMs amplitude after injection of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg8 s.c. with the respective vehicle **C.** Representative graph of integrated axial AIMs after administration of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle.

3.2.2 Cumulative limbs AIMs

As shown in Figure 9, combining EMD-281,014 (0.1mg/kg) and L-DOPA, with either LY-354,740 or with LY-354,740/LY-487,379, had no effect on limbs AIMs throughout the behavioural sessions. In contrast, administration of EMD-281,014 (0.1 mg/kg) in combination with LY-487,379 significantly decreased L-DOPA-induced limbs AIMs duration (by 22%, $P < 0.001$). However, as seen in Figure 10, combining EMD-281,014 0.03 mg/kg to L-DOPA, with either LY-354,740, LY-487,379 or with LY-354,740/LY-487,379, had no effect on limbs AIMs.

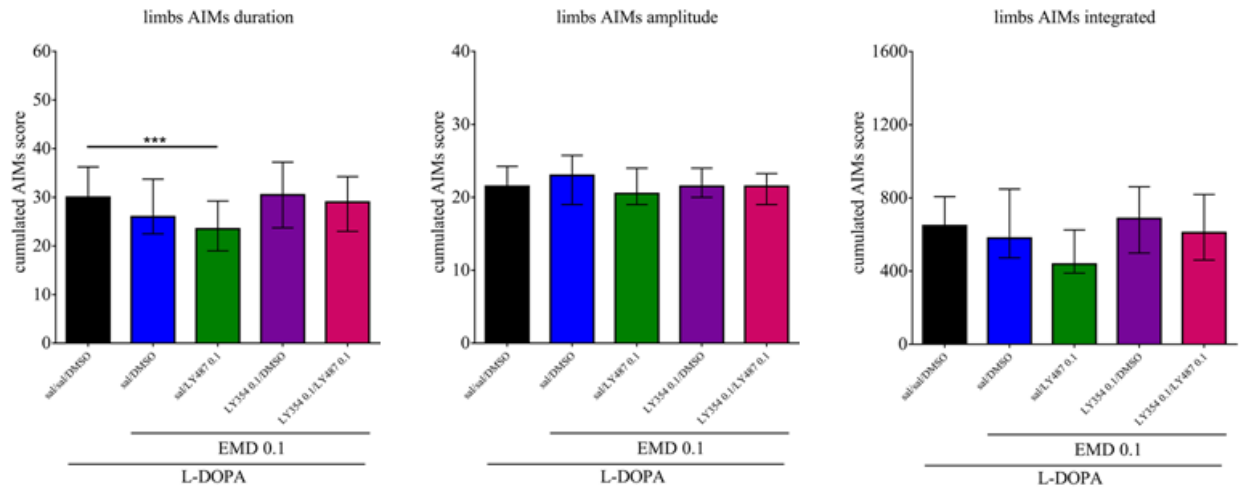

Figure 9: Representative graph of limbs AIMs EMD-281,014 0.1 mg/kg
A. Representative graph of limbs AIMs duration following injection of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle. **B.** Representative graph of limbs AIMs amplitude after injection of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle **C.** Representative graph of integrated limbs AIMs after administration of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle. ***: $P < 0.001$.

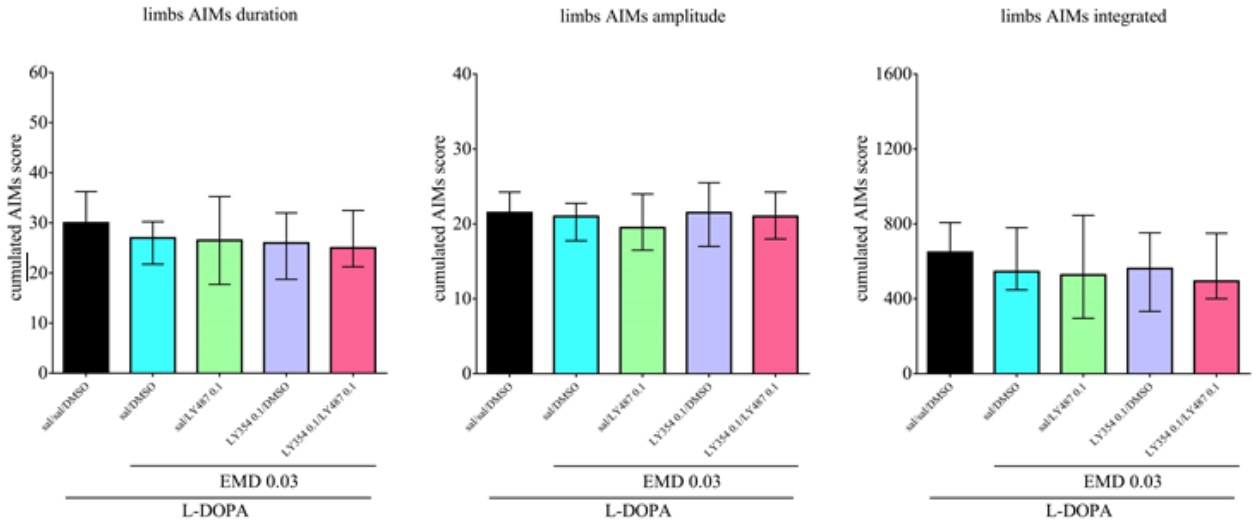

Figure 10: Representative graph of limbs AIMs EMD-281,014 0.03 mg/kg
A. Representative graph of limbs AIMs duration following administration of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle. **B.** Representative graph of limbs AIMs amplitude after injection of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle **C.** Representative graph of integrated limbs AIMs after administration of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle.

3.2.3 Cumulative oro-lingual AIMs

As illustrated in Figures 11 and 12, adding EMD-281,014 (0.03 and 0.1mg/kg) to L-DOPA, with either LY-354,740 or LY-487,379 or both, had no effect on oro-lingual AIMs throughout the behavioural sessions.

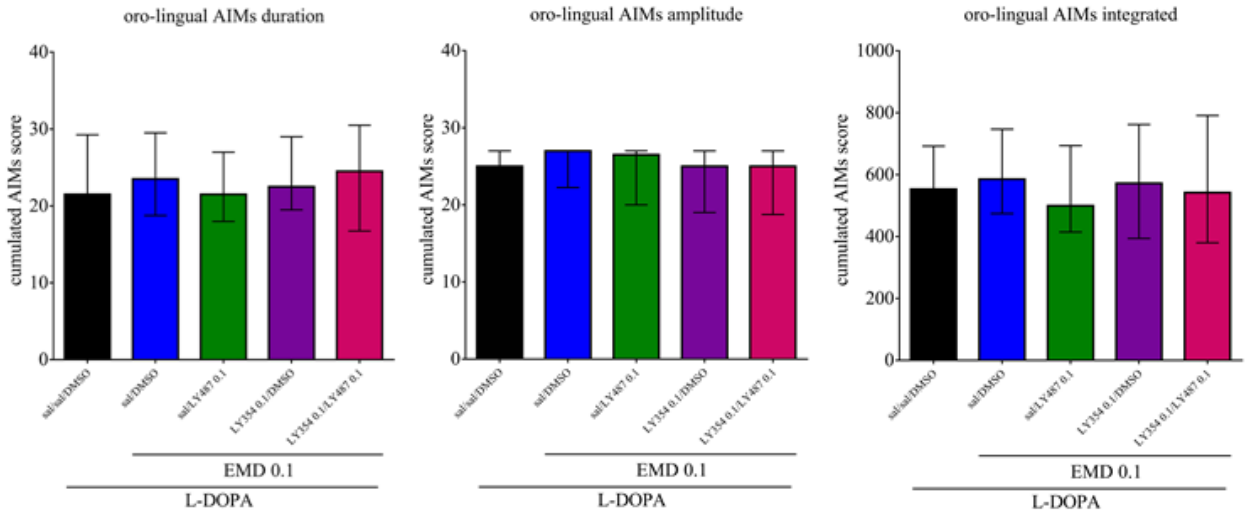

Figure 11: Representative graph of oro-lingual AIMs EMD-281,014 0.1 mg/kg
A. Representative graph of oro-lingual AIMs duration following injection of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle. **B.** Representative graph of oro-lingual AIMs amplitude after injection of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle **C.** Representative graph of integrated oro-lingual AIMs after administration of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle.

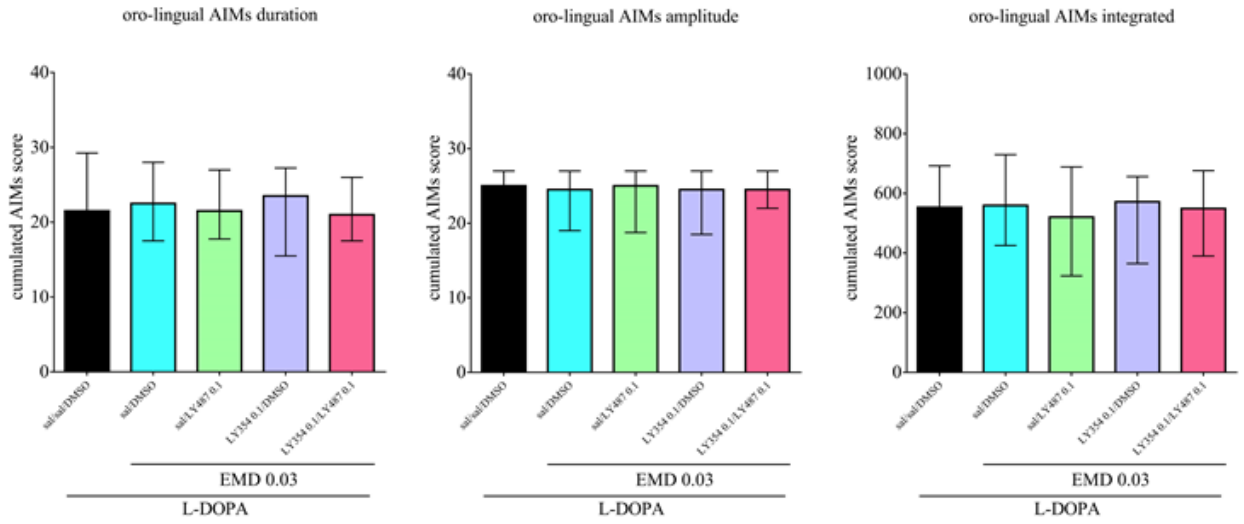

Figure 12: Representative graph of oro-lingual AIMs EMD-281,014 0.03 mg/kg

A. Representative graph of oro-lingual AIMs duration following administration of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle. **B.** Representative graph of oro-lingual AIMs amplitude after injection of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle **C.** Representative graph of integrated oro-lingual AIMs after administration of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle.

3.2.4 Cumulative AL AIMs

As seen in Figure 13, combining EMD-281,014 (0.1mg/kg) and L-DOPA, with either LY-354,740 or with LY-354,740/LY-487,379, had no effect on AL AIMs throughout the behavioural sessions. In contrast, administration of EMD-281,014 (0.1 mg/kg) in combination with LY-487,379 significantly decreased L-DOPA-induced AL AIMs duration (by 16%, $P < 0.001$). However, as illustrated in Figure 14, combining EMD-281,014 0.03 mg/kg to L-DOPA, with either LY-354,740, LY-487,379 or with LY-354,740/LY-487,379, had no effect on AL AIMs.

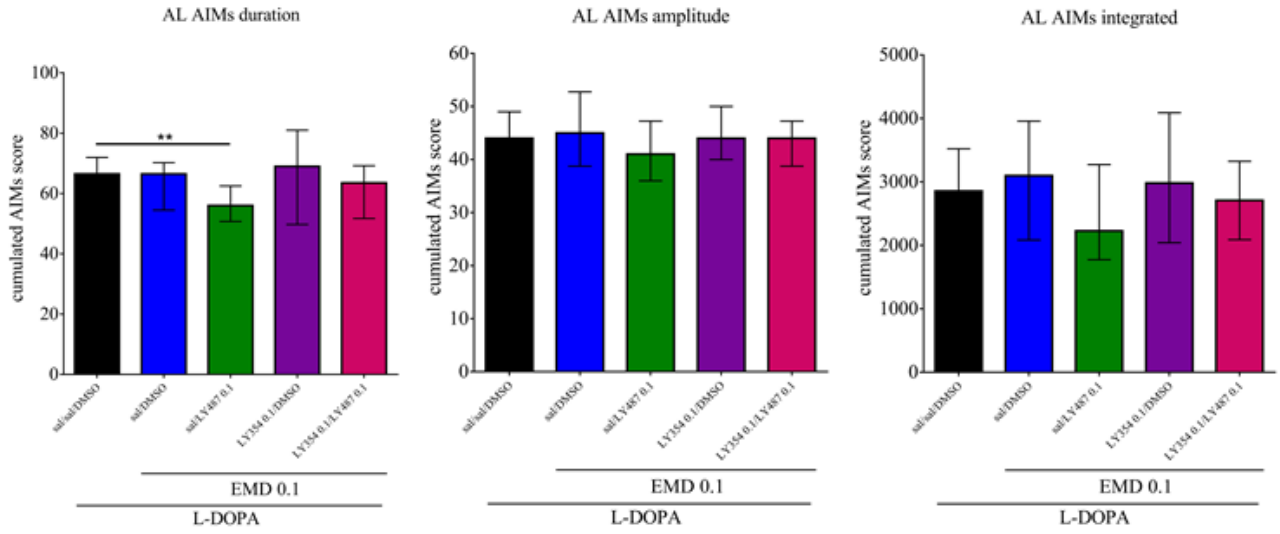

Figure 13: Representative graph of AL AIMs EMD-281,014 0.1 mg/kg
A. Representative graph of AL AIMs duration following injection of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle. **B.** Representative graph of AL AIMs amplitude after injection of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle **C.** Representative graph of integrated AL AIMs after administration of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle. **: $P < 0.01$.

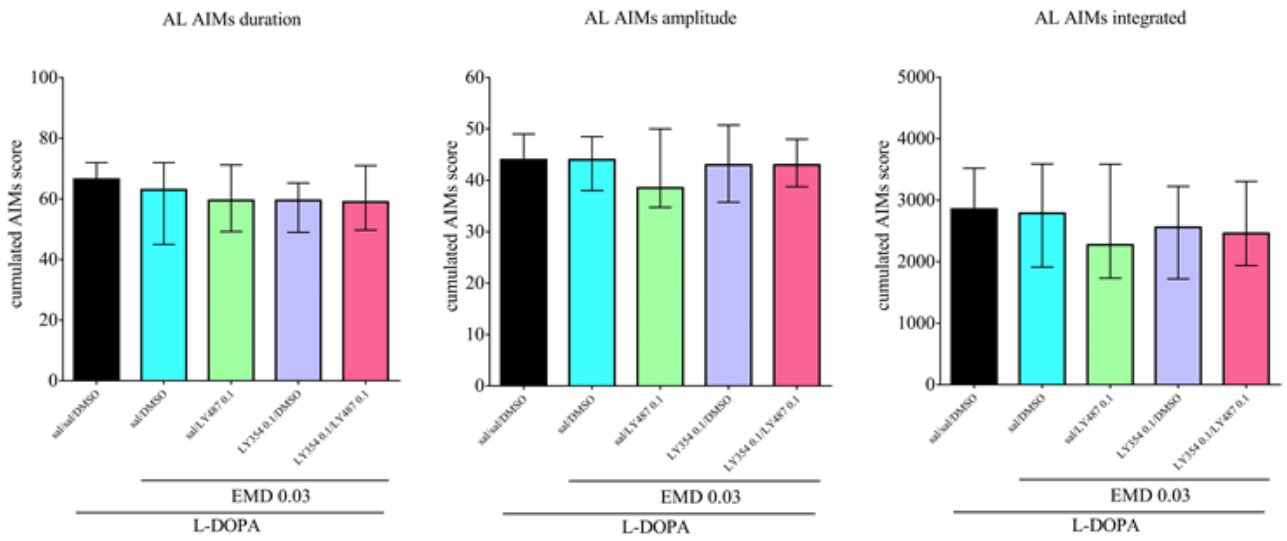

Figure 14: Representative graph of AL AIMs EMD-281,014 0.03 mg/kg
A. Representative graph of AL AIMs duration following administration of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle. **B.** Representative graph of AL AIMs amplitude after injection of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle **C.** Representative graph of integrated AL AIMs after administration of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle.

3.2.5 Cumulative ALO AIMs

As illustrated in Figures 15 and 16, when using 18 rats, adding EMD-281,014 (0.03 and 0.1mg/kg) to L-DOPA, with either LY-354,740, LY-487,379 or with LY-354,740/LY-487,379, had no effect on ALO AIMs throughout the behavioural sessions. In contrast, administration of EMD-281,014 (0.1 mg/kg) in combination with LY-487,379 significantly decreased L-DOPA-induced ALO AIMs duration (by 10%, $P < 0.05$).

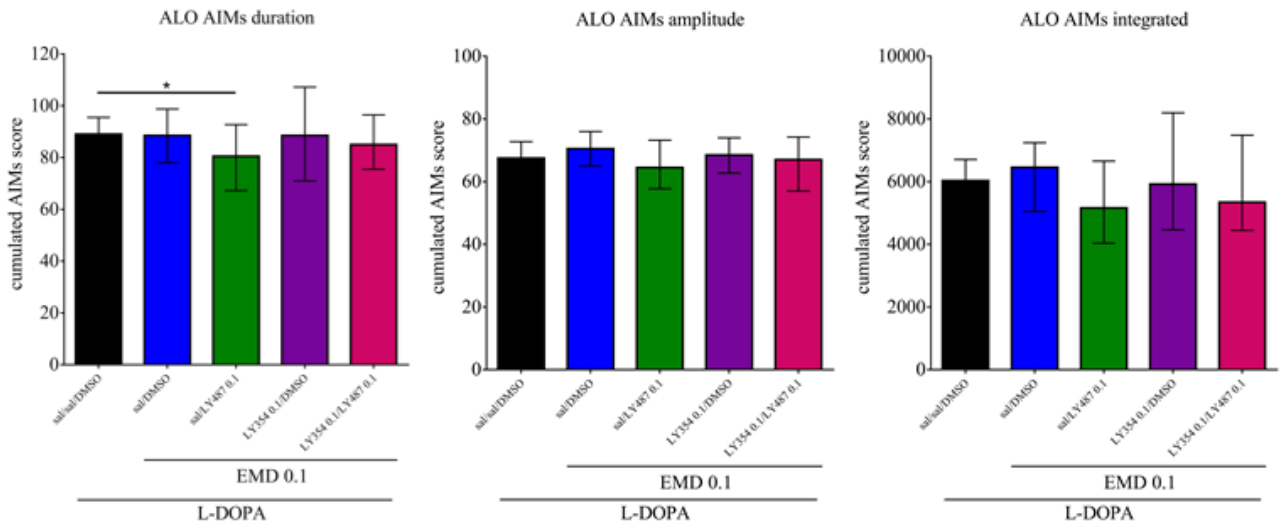

Figure 15: Representative graph of ALO AIMs EMD-281,014 0.1 mg/kg (N=18)
A. Representative graph of ALO AIMs duration following injection of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle. **B.** Representative graph of ALO AIMs amplitude after injection of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle **C.** Representative graph of integrated ALO AIMs after administration of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle. *: $P < 0.05$.

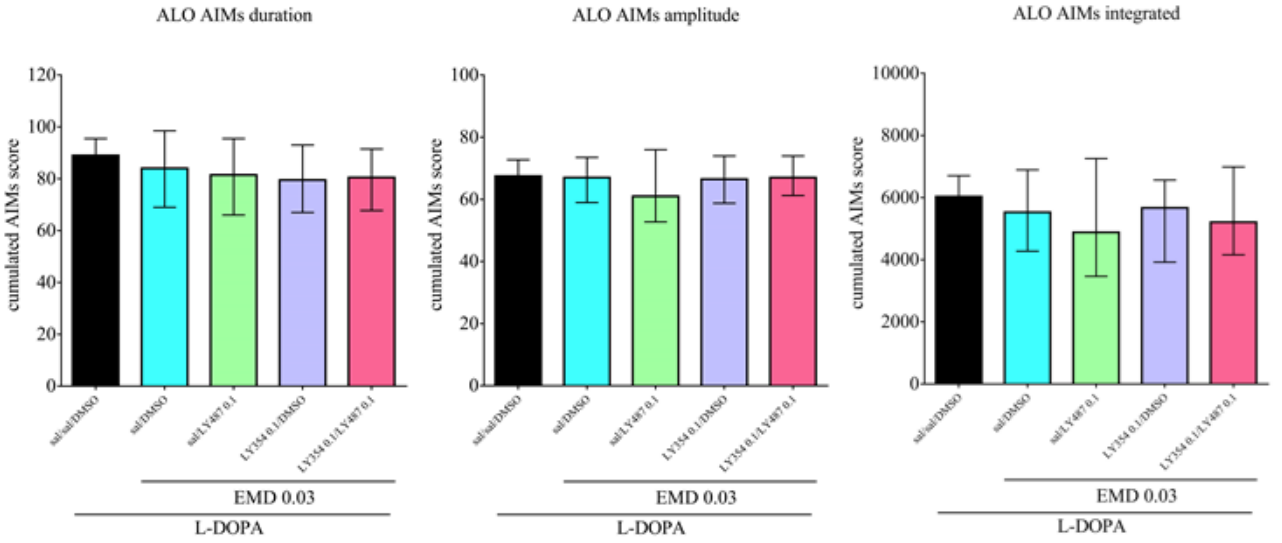

Figure 16: Representative graph of ALO AIMs EMD-281,014 0.03 mg/kg (N=18)
A. Representative graph of ALO AIMs duration following administration of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle. **B.** Representative graph of ALO AIMs amplitude after injection of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle **C.** Representative graph of integrated ALO AIMs after administration of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle.

3.2.6 Cumulative ALO AIMs

The same experiments were redone with 9 additional rats for a total of 27. As illustrated in Figures 17 and 18, adding EMD-281,014 (0.03 and 0.1mg/kg) to L-DOPA, with either LY-354,740, or LY-354,740/LY-487,379, had no effect on ALO AIMs throughout the behavioural sessions. In contrast, administration of EMD-281,014 (0.1, but not 0.03, mg/kg) in combination with LY-487,379 significantly decreased L-DOPA-induced ALO AIMs duration (by 7.5%, $P < 0.05$) and integrated ALO AIMs (by 21%, $P < 0.05$).

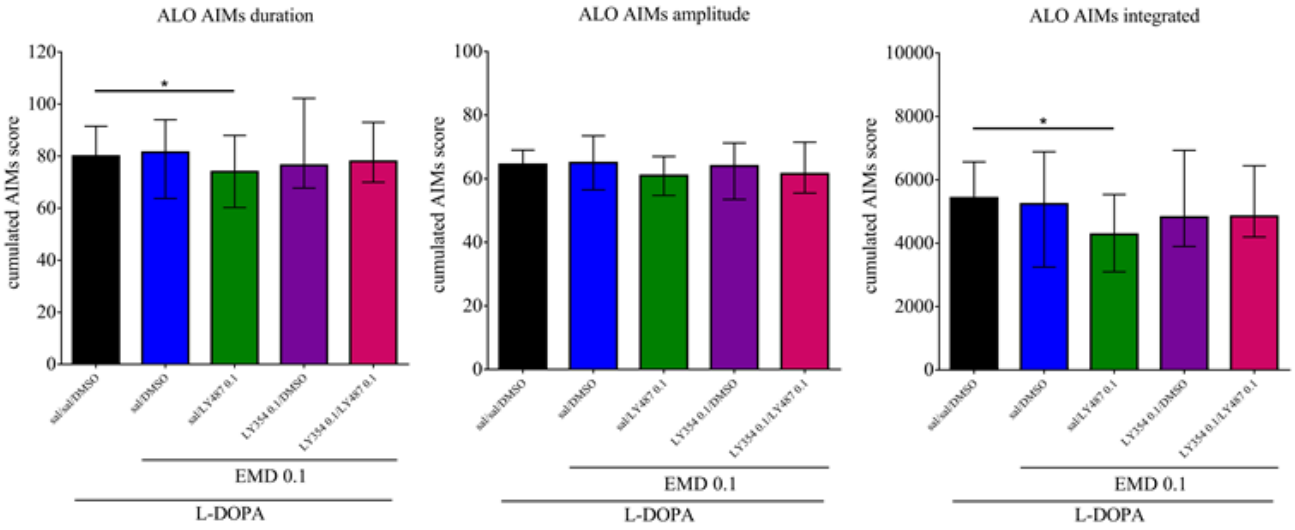

Figure 17: Representative graph of ALO AIMs EMD-281,014 0.1 mg/kg (N=27)
A. Representative graph of ALO AIMs duration following injection of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle. **B.** Representative graph of ALO AIMs amplitude after injection of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle **C.** Representative graph of integrated ALO AIMs after administration of EMD-281,014 0.1 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle. *: P < 0.05.

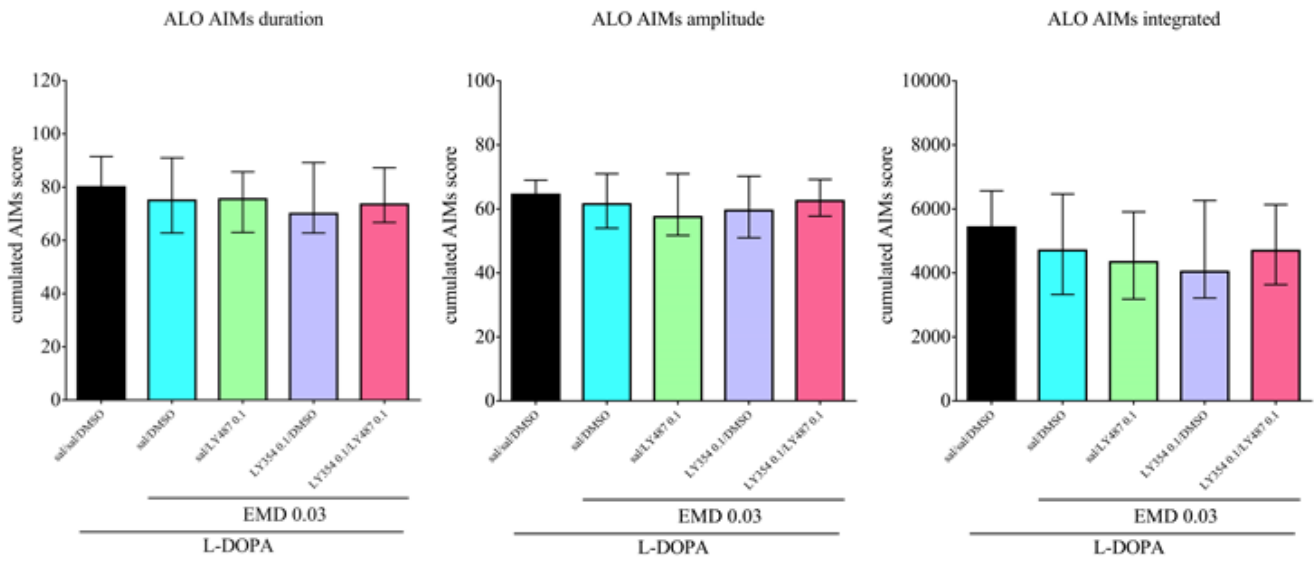

Figure 18: Representative graph of ALO AIMs EMD-281,014 0.03 mg/kg (N=27)
A. Representative graph of ALO AIMs duration following administration of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle. **B.** Representative graph of ALO AIMs amplitude after injection of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle **C.** Representative graph of integrated ALO AIMs after administration of EMD-281,014 0.03 mg/kg s.c. in combination with LY-354,740 0.1 mg/kg s.c. and LY-478,379 0.1 mg/kg s.c. with the respective vehicle.

3.3 L-DOPA challenge

6-OHDA-lesioned rats used mainly the right (un-lesioned) forepaw when rearing. Administration of L-DOPA resulted in a significant reduction of the use of the right forepaw, a correlate of anti-parkinsonian action ($F(3,327, 56,56) = 13,38$, $P < 0.001$; one-way RM ANOVA). Tukey's *post hoc* test revealed that, when compared to the lesion, there was a significant decrease in the right forepaw use when L-DOPA was combined with the vehicle (23%, $P < 0.05$), EMD-281,014 0.03 mg/kg alone (47%, $P < 0.01$) or with LY-354,740 (82%, $P < 0.001$), LY-487,379 (74%, $P < 0.001$) or both (60%, $P < 0.01$). Similar reductions of rearing with the right forepaw were observed when L-DOPA was administered with EMD-281,014 0.1 mg/kg alone (53%, $P < 0.001$) or with LY-354,740 (57%, $P < 0.001$), LY-487,379 (52%, $P < 0.01$) or both (61%, $P < 0.001$). There was also a significant decrease in the number of rears made with the right forepaw compared to L-DOPA/vehicle when L-DOPA was combined with EMD-281,014 0.03 mg/kg with

LY-354,740 (76%, $P < 0.001$) or LY-487,379 (66%, $P < 0.01$), as well as with EMD-281,014 0.1 mg/kg with LY-354,740 (44%, $P < 0.01$).

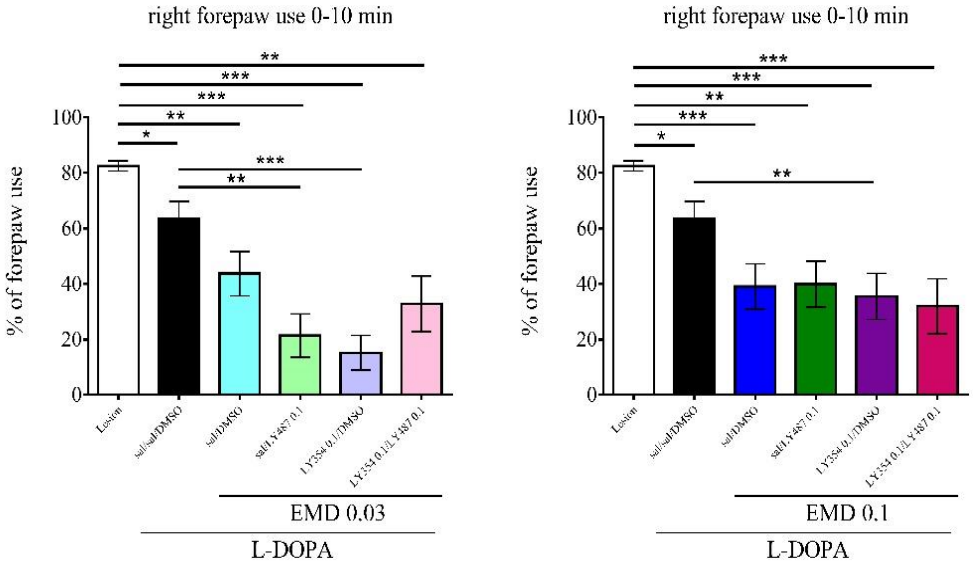

Figure 19: Representative graph of right forepaw use
A. Representative graph of right forepaw use over a 10 min period in the cylinder test after administration of different combinations of EMD-281,014 0.03 mg/kg, LY-354,740 and LY-487,379. **B.** Representative graph of right forepaw use over a 10 in period in the cylinder test after injection of different combinations of EMD-281,014 0.1 mg/kg, LY-354,740 and LY-487,379. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

4. Discussion

The results in this study indicate a small reduction of AIMs duration with the combination of an $mGlu_2$ PAM and 5-HT_{2A} antagonist. These results also show a possible anti-parkinsonian effect of the combination of EMD-281,014 with either LY-487,379 or LY-354,740. This indicates that dual modulation of the $mGlu_2$-5-HT_{2A} complex may enhance the effect of a low dose of L-DOPA. Interestingly, these results show that combined PAM and OA $mGlu_2$ activation with 5-HT_{2A} blockade does not significantly reduce L-DOPA induced AIMs in the 6-OHDA rat model nor significantly enhance L-DOPA anti-parkinsonian effect. The following discussion will describe the possible ways by which $mGlu_2$ PAM combined with 5-HT_{2A} is a possible avenue for reduction of LID and to enhance the anti-parkinsonian effects of L-DOPA and will also speculate on why such benefits were not achieved with 5-HT_{2A} blockade combined with $mGlu_2$ activation by simultaneous positive allosteric modulation and orthosteric stimulation.

4.1 Effect of drugs on L-DOPA induced dyskinesia

The results obtained here are in agreement with previous studies conducted in the 6-OHDA-lesioned rat that found no anti-dyskinetic benefit upon selective 5-HT_{2A} blockade (Frouni et al., 2018; Taylor et al., 2006). Thus, EMD-281,014, either 0.03 or 0.1 mg/kg did not reduce L-DOPA-induced AIMs when administered alone. In this study, the only treatment that led to a significant reduction of LID was the combination of the $mGlu_2$ PAM LY-487,379 with the 5-HT_{2A} antagonist EMD-281,014 0.1 mg/kg. The reasons why the other combinations did not result in a significant reduction of LID are unknown, however, the following paragraphs will attempt to provide explanations for these results.

A possible reason why the combination of an $mGlu_2$ PAM with a 5-HT_{2A} antagonist produced an anti-dyskinetic effect at a dose of 0.1 mg/kg for EMD-281,014 but not 0.03 mg/kg

could be explained by the maximal plasma concentration (C_{max}) and the percentage of receptor occupancy. The C_{max} achieved by injecting 0.03 mg/kg of EMD-281,014 to a rat is 5.70 ng/mL while it goes up to 29.67 ng/mL with a dose of 0.1 mg/kg (Frouni et al., 2018). There are no PET scan studies on rats for EMD-281,014 but if we were considering a similar receptor count 0.03 mg/kg would lead to a 89% receptor occupancy while 0.1 mg/kg leads to a 100% receptor occupancy (Mamo et al., 2004). This could explain why the 0.1 mg/kg dose, but not 0.03 mg/kg, was effective, when combined with an $mGlu_2$ PAM. It should be pointed out that the anti-dyskinetic benefit conferred by the combination 5-HT_{2A} antagonist / $mGlu_2$ PAM might be conferred by the $mGlu_2$ PAM and not result from an interaction between the 2 targets, as 5-HT_{2A} blockade alone did not lead to a LID reduction.

An intriguing result was that a reduction of LID was obtained upon combining a 5-HT_{2A} antagonist with an $mGlu_2$ PAM, but not an OA. As presented above, an OA and a PAM do not bind to the same site on a receptor. Possibly, when a 5-HT_{2A} antagonist binds to the 5-HT_{2A} protomer of the 5-HT_{2A}/$mGlu_2$ complex, it may induce conformational changes to the $mGlu_2$ active site, making it harder for the OA to bind to the complex. Accordingly, it was shown that LY-354,740 OA potential varied in function of which protomer it binds to (Moreno Delgado et al., 2017). This could reduce the availability of the orthosteric binding site on $mGlu_2$ protomers availability. Theoretically, this would result in more LY-354,740 remaining in the plasma, where it could interact with other targets.

We have recently shown that high doses of LY-354,740 do not reduce AIMs in the 6-OHDA-lesioned rat (Frouni et al., 2019). LY-354,740 is 6-fold less potent in activating the $mGlu_3$ receptor as it is for activating the $mGlu_2$ receptor in humans (Schoepp et al., 1997). We propose that, if LY-354,740 does not bind to $mGlu_2$ receptors, it may activate $mGlu_3$ receptors. $mGlu_3$

receptors were recently demonstrated to form a heterodimer with the $mGlu_5$ receptor (Di Menna et al., 2018) in which activation of $mGlu_3$ receptors leads to allosteric activation of $mGlu_5$ receptors. As discussed above, antagonising $mGlu_5$ results in a reduction of dyskinesia in both the rat and macaque (Dekundy et al., 2006; Johnston et al., 2010; Mela et al., 2007; Rylander et al., 2009). Here, the effect of LY-354,740 on $mGlu_3$ receptors might cross-activate mGlu5 receptors. Hypothetically, this $mGlu_5$ cross-activation might off-set the benefits conferred by $mGlu_2$ activationTo summarise, we propose that activation of the $mGlu_3/mGlu_5$ complex might explain why LY-354,740 combined with EMD-281,014 did not result in a reduction of LID.

We also found that the combination of EMD-281,014 and LY-487,379 alleviates LID, while EMD-281,014 plus LY-487,379 and LY-354,740 did not provide any benefit. A few hypotheses can be put forward to explain the lack of anti-dyskinetic effect of combined $5\text{-}HT_{2A}$ blockade and $mGlu_2$ positive allosteric modulation and orthosteric stimulation. The phenomenon of steric hindrance might play a role. Thus, PAMs are expected to enhance the effect of the endogenous ligand, however, LY-354,740 is not the endogenous ligand which in this case is glutamate. LY-354,740 is in fact quite bigger than glutamate with three extra atoms of carbon and hydrogen and it cannot be ruled out that its relatively big size compared to glutamate might prevent the binding of LY-487,379 to the orthosteric site. This could prevent the co-binding of LY-354,740 and LY-487,379 and lead to competitive binding of the two molecules (Schaffhauser et al., 2003), which would prevent LY-487,379 from eliciting its therapeutic effect. Another possibility might be that co-binding of a PAM and an OA can bias the signalling of a receptor (Ellaithy, Younkin, Gonzalez-Maeso, & Logothetis, 2015), with the resulting effect that a molecule that usually acts as a PAM can behave as a NAM if the conditions vary. In agreement with this possibility, a variety of asymmetrical rearrangement can occur in both halves of a dimer following the binding of a

molecule (Sleno & Hebert, 2019), implying that the activity of a molecule at a receptor may not be fixed but rather variable in function of the context. The lack of anti-dyskinetic reduction by the combination of the three drugs could also possibly be explained by the Gi/Gq ratio and the concept of balance index (BI), which was recently introduced to describe this ratio and predict the anti-psychotic properties of a drug (Fribourg et al., 2011; Kondo & Sawa, 2011). Fribourg used the BI in the case of the $mGlu_2$/5-HT_{2A} complex and demonstrated in *Xenopus* oocytes that the assembly of the complex enhances Gi signalling and reduces Gq signalling, and that binding of a molecule to either side of the complex leads to a change in the BI (Fribourg et al., 2011). The importance of this ratio on psychosis could potentially be transferred to LID. Indeed, the manifestation of LID could occur when the ratio of Gi/Gq is not the normal physiological one. For example, if the normal physiological ratio of Gi/Gq is 60/40 and that in the dyskinetic state it is 20/80, maybe the addition of LY-487,379 and EMD-281,014 balances the ratio back to 60/40 but by putting LY-354,740 on top the ratio goes above it to 80/20 leading to adverse effects and no amelioration of the dyskinetic phenotype. Further studies are needed to determine whether an optimal BI can be computed for drugs interacting with the 5-HT_{2A}/$mGlu_2$ complex to alleviate LID.

That only the combination of EMD-281,014 and LY-487,379 reduced dyskinesia suggests that the anti-dyskinetic effect of the treatment might have been provided through 5-HT_{2A} antagonism and $mGlu_2$ activation. However, as pointed out earlier, it is also possible that $mGlu_2$ positive allosteric modulation alone mediated this effect. Accordingly, we have previously demonstrated that LY-487,379 diminishes AIMs severity in the 6-OHDA-lesioned rat (Kwan et al., 2017). Nevertheless, if indeed an interaction between the two receptors occurred, our experiments do not allow us to conclude whether this interaction was mediated through the 5-HT_{2A}/$mGlu_2$ hetero-complex or through receptor cross-talk. Clarifying the mechanism by which

the anti-dyskinetic action was achieved here could help refine the search for future anti-dyskinetic drugs.

Using a PAM over an OA presents some advantages. First, since PAMs bind to a site other than the active site, no tolerance is usually observed (Galici et al., 2005) meaning that the benefit is likely to be sustained over time. PAMs have 3 ways by which they can exert their effect. By affinity-modulation, which induces conformational changes that increase the affinity of the endogenous ligand, by efficacy-modulation, which induces changes in the intracellular responses that lead to increased signalling capacity of the endogenous ligand and finally, PAMs can elicit a direct agonist effect (Aghajanian & Marek, 1999). LY-487,379 showed PAM activity in rat hippocampal slice in electrophysiology assays (Kew, 2004); it would be interesting to see if the same type of activity could be recorded in the basal ganglia. Locating the precise synapse(s) where the anti-dyskinetic action is provided could help better the search on anti-dyskinetic drugs. It could also help elucidating the mechanisms by which LID occur in the first place.

Unlike EMD-281,014 and LY0-354,740, LY-487,379 pharmacokinetic profile does not make it a clinical candidate, for instance it has short duration of action and low efficacy of low doses of LY-487,379 (Cid, Trabanco, & Lavreysen, 2015). LY-487,379 is in fact the first $mGlu_2$ PAM created (Johnson et al., 2003), nevertheless, several other PAM have been derived from this molecule since all of which could have better drug properties than the original molecule and could potentially be use in the clinic (Cid et al., 2010; Cid et al., 2012; Cube et al., 2005; D'Alessandro et al., 2010; Duplantier et al., 2009; Govek et al., 2005; Pinkerton, Cube, Hutchinson, James, et al., 2004; Pinkerton, Cube, Hutchinson, Rowe, et al., 2004; Pinkerton, Vernier, et al., 2004; Rodriguez, 2004; Sheffler, Pinkerton, Dahl, Markou, & Cosford, 2011; Zhang et al., 2011; Zhang

et al., 2008). As stated above, we selected LY-487,379 because of its selectivity, as it reportedly does not interact with any other target (Johnson et al., 2003).

Whereas a reduction of 10% of ALO AIMs over the entire session could be considered small at first glance, it compares to the reduction of AIMs obtained with amantadine in the same animal model. Thus, in one study, amantadine did not reduce ALO AIMs (Paquette et al., 2012), while it led to an approximate 20% reduction of AIMs in another study (Dekundy, Lundblad, Danysz, & Cenci, 2007).

4.2 Effect of the drugs on L-DOPA anti-parkinsonian action

By testing the effect of the different combinations of the drugs with a low dose of L-DOPA, it was possible to determine whether the drugs, or combination thereof, interfered with L-DOPA anti-parkinsonian effect, which would limit their potential use. A low dose of L-DOPA was used for this test to prevent the occurrence of LID during the test, which would hamper the animals' ability to rear.

All of the different combinations of molecules, when added to L-DOPA, led to use of the right forepaw when rearing that was significantly different from the lesion condition, indicating that L-DOPA anti-parkinsonian action was maintained when the drug combinations were added. Unexpectedly, the combinations of EMD-281,014 0.03 mg/kg with LY-354,740 or LY-487,379 and EMD-281,014 0.1 mg/kg with LY-354,740 were significantly different from the L-DOPA alone condition. This might indicate that these combinations enhanced the anti-parkinsonian action of L-DOPA, suggesting that they might have potential as adjunct therapy to L-DOPA. The mechanism by which this phenomenon occurs is unclear, but a possible explanation might be an action at the corticostriatal synapse. Indeed, new views on PD centre the attention on the synapses

instead of the entire neuron to explain the disease. Indeed, recent *in vivo* and *in vitro* evidence on human samples show nigrostriatal synapses being affected in early stages of the disease, which is accompanied by a loss of corticostriatal plasticity (Schirinzi et al., 2016). Experiments on animal models showed a link between dopaminergic deficiency and corticostriatal synaptopathy (Schirinzi et al., 2016). Alteration of glutamatergic synapses leads to increased glutamatergic transmission in the striatum, which could underlie cardinal features of PD and is also thought to be implicated in LID (Picconi, Piccoli, & Calabresi, 2012). It has also been experimentally shown that short- and long-term changes in corticostriatal synaptic plasticity could play a role in parkinsonism (Gubellini et al., 2002; Picconi et al., 2003).

In summary, a possible mechanism to explain both the anti-dyskinetic and anti-parkinsonian effects encountered here might involve targeting 5-HT_{2A} and $mGlu_2$ receptors located at the cortico-striatal synapse.

4.3 Limitations

The extent of the findings of this study are restricted to methodology limits. Indeed, all experiments can only be as good as the method used. Despite carefully selecting the best experiments and optimise the methodology to answer our hypotheses, the discoveries and their interpretation are limited by the animal model used, the AIMs and the cylinder test.

First the animal model, the brain of a rat is not the same as a human brain, with 66-82% of the brain regions being conserved between rat and human (Nilsson et al., 2001). This could be a reason why 5-HT_{2A} antagonists reduce LID in the MPTP-lesioned marmoset but not in the 6-OHDA-lesioned rat (Frouni et al., 2018; Hamadjida et al., 2018).

The toxin-based, 6-OHDA-lesioned, rat model also has limitations. Since it consists of a single 6-OHDA injection, and the lesion takes only 3 weeks to develop, it is not progressive as human PD and mimics better latter stages of the disease. Furthermore, injection into the medial forebrain bundle leads to close to complete depletion of the dopaminergic neurons which once again is closer to advance PD phenotype. It is noteworthy however that, as LID tends to occur with disease progression, a model of severe dopamine depletion is well suited to induce this complication. Another difference between the 6-OHDA-lesioned rat and the human disease is that the injection is limited to one hemisphere while in PD, the lesion extends to both hemispheres. The 6-OHDA-lesioned rat is also lacking Lewy bodies which are present in some form of PD (Bezard & Przedborski, 2011).

A discussion of the AIMs rating process is also important. Thus, the test is scored by an observer, and therefore cannot be completely impartial; blinding to treatment administered is a way to mitigate this bias. Moreover, both duration and amplitude scales only have 5 levels of severity, which does not allow the observer much variation in the rating process. In order to give more accuracy to the test, we added an extra duration, the duration of the highest amplitude, which gave us a maximum score of 8 for the duration. Furthermore, in the initial AIMs test the locomotor activity is considered, however, in our test we did not evaluate it , as the interpretation of locomotor and rotational behaviour in the rat is difficult, as it can reflect each of an anti-parkinsonian action, a LID correlate and an equivalent of motor fluctuations (Hamadjida, Frouni, Kwan, & Huot, 2019).

Lastly, the cylinder test also has limitations. Thus, when the cylinder test is repeated too many times, the rats get used to being in the cylinder and do not rear as much as they initially did, which may induce a bias in the results (Schallert et al., 2000). Furthermore, the cylinder test may

be compromised when rats exhibit AIMs, as these may interfere with the rats' ability to rear (Dekundy et al., 2007).

5. Conclusion

Despite constant progress in the field of PD and LID, full alleviation of LID symptoms without adverse effects remains unattained. Here, using LY-487,379 as an $mGlu_2$ PAM, LY-354,740 as an $mGlu_2$ OA and EMD-281,014 as a 5-HT_{2A} antagonist, we demonstrated that targeting the 5-HT_{2A}/$mGlu_2$ complex might be an effective way to alleviate, but not to eradicate, LID. We also showed that no further anti-dyskinetic benefit was obtained by adding an $mGlu_2$ OA to an $mGlu_2$ PAM, combined with a 5-HT_{2A} antagonist. Interestingly, combining the 5-HT_{2A} antagonist with either the $mGlu_2$ PAM or OA increased the anti-parkinsonian effect of L-DOPA, making the drug combination a possible option for adjunct therapy. It would be interesting to conduct a study with these drug combinations in a *de novo* design to determine whether these drugs could delay the onset of LID or perhaps suppress the development of LID, in the 6-OHDA rat.

Despite negative results for the combination of the 3 drugs in the rat model, it would be interesting to perform a similar study in the parkinsonian NHP to determine whether similar results would be obtained. Indeed, we know that selective 5-HT_{2A} antagonists do not alleviate LID in the 6-OHDA-lesioned rat but does alleviate LID in MPTP-lesioned NHPs (Frouni et al., 2018; Hamadjida et al., 2018). Because of their selectivity, the molecules that we tested here allow us an insight on the role of targets in LID and PD and open an avenue for the development of new drugs.

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7. Appendix

Supplementary Figure 1: AIMs rating scales (Winkler et al., 2002)

Phenomenology of Rat AIMs and Rating Criteria

Definition
- (A) Abnormal involuntary movements
- (B) Primarily affect the side of the body contralateral to the lesion
- (C) Are purposeless, and cannot be ascribed to enhanced manifestations of normal motor activities, such as grooming, gnawing, rearing, and sniffing
- (D) Normal motor behavior is disturbed or totally replaced
- (E) Are repetitive, but variable in frequency and amplitude

Subtypes of AIMs
- Locomotive: Circular locomotion with contralateral side bias.
- Axial: Lateral deviation or torsion of the head, neck and trunk towards the side contralateral to the lesion. This position is often sustained (dystonic-like), but may also have the character of a choreiform twisting movement
- Limb: Movements of the forelimb (or the paw) contralateral to the lesion, which may occur in the sagittal plane (flexion-extension), in the frontal plane (abduction-adduction), or in both (circumduction). Include opening/closing of the digits and pronation/supination of the wrist. May have a jerky, choreiform, or dystonic character.
- Orolingual: Jaw movements, facial grimacing, tongue protrusion. These movements are bilateral but more pronounced on the side contralateral to the lesion. Usually accompanied by biting (either injurious or noninjurious) of the fur and the skin on the forelimb contralateral to the lesion.

Severity rating
- 0: Absent
- 1: Occasional, i.e., present during less than 50% of the observation time
- 2: Frequent, i.e., present during more than 50% of the observation time
- 3: Continuous but interrupted by strong sensory stimuli (i.e., sudden, noisy opening of the cage lid)
- 4: Continuous, not interrupted by strong sensory stimuli, nor by threatening manoeuvres (i.e., coarse movements of the experimenter's hand inside the rat cage)

Amplitude scores

Axial AIMs

Angle is estimated with respect to the deviation from (or torsion around) the longitudinal axis of the body
- 1: Consistent lateral deviation of head and neck at ~30° angle
- 2: Lateral deviation of head and neck, $30° < \text{angle} \leq 60°$
- 3: Lateral deviation and/or torsion of head, neck and upper trunk, $60° < \text{angle} \leq 90°$
- 4: Torsion of head, neck and trunk at $> 90°$ angle, causing the rat to lose balance

Forelimb AIMs
- 1: Tiny oscillatory movements of the paw and the distal forelimb around a fixed position
- 2: Movements of low amplitude but causing visible translocation of both distal and proximal limb
- 3: Translocation of the whole limb with visible contraction of shoulder muscles
- 4: Vigorous limb and shoulder movements of maximal amplitude. May have a ballistic character