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N/OFQ and the NOP receptor: a target with broad potential in Parkinson's Disease

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N/OFQ and the NOP receptor: a target with broad potential in Parkinson's Disease

By Ludovico Arcuri

Whenever, wherever…

[Shakira]

Abstract

The neuropeptide Nociceptin/Orphanin FQ and its receptor play a pathogenic role in Parkinson's Disease. This thesis work tried to address two different and unresolved questions related to the role of the N/OFQ-NOP system in PD. The first, and perhaps more relevant, is whether endogenous N/OFQ contributes to the death of dopamine neurons of substantia nigra compacta, the hallmark of PD. Using pathogenic and etiologic models of PD, we were able to demonstrate that genetic removal or pharmacological blockade of the NOP receptor had a beneficial impact on the parkinsonian neurodegeneration, causing a significant sparing of nigral dopamine neurons and striatal dopamine terminals. The second aspect investigated is whether NOP receptor stimulation prevents the sensitization of striatal neurons to levodopa, a process that underlies the development of dyskinesia, a severe motor complication associated with long term levodopa pharmacotherapy of PD. Using the novel, potent and selective NOP receptor full agonist AT-403, we were able to show that NOP receptor stimulation attenuates the development of abnormal involuntary movements induced by chronic levodopa administration in 6-OHDA hemilesioned rats (a model of PD), and also attenuates the expression of dyskinetic movements induced by acute levodopa challenge in already dyskinetic rats. Nonetheless, this effect was accompanied by a dose-dependent sedation, which narrowed the therapeutic window of the compound. Other two potent NOP agonists tested along the study, the full agonists AT-390 and the partial agonist AT-127, confirmed that the antidyskinetic and the sedative dose-ranges partially overlap. In conclusion, this study confirms the role of N/OFQ in PD, and the NOP receptor as a promising target in PD therapy. Of translational value, it provides the first evidence of a neuroprotective/neurorescue effect of a NOP receptor antagonist, suggesting this class of compounds might prove effective not only as symptomatic but also disease-modifying therapy in PD.

Abbreviations

α-syn - α-synuclein

Ach - acetylcholine

BG - Basal Ganglia

COMT - cathecol-o-methyltransferase

DA – Dopamine

GP - Globus Pallidus

GPe - Globus Pallidus external

GPi - Globus Pallidus internal

L-DOPA - L-3,4-dihydroxyphenylalanine

LID - L-DOPA-induced dyskinesia

MAO - Monoamine Oxidase

MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MSN - Medium Spiny Neuron

N/OFQ - Nociceptin/Orphanin FQ

NOP - Nociceptin/orphanin FQ Peptide Receptor

ORL-1 - Opioid Receptor Like-1

p-α-syn - phosphorylated α-synuclein

PD - Parkinson's Disease

ppN/OFQ - prepro Nociceptin/Orphanin FQ

SNc - Substantia Nigra pars Compacta

SNr - Substantia Nigra pars Reticulata

TH - tyrosine hydroxylase

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Introduction

Parkinson's Disease

Parkinson's Disease (PD) is the second most common neurodegenerative disorder, affecting about 2% of the world population aged over 65 years. The disease is named after the physician James Parkinson, who first described the symptoms in 1817. It has been defined as a movement disorder accompanied by loss of dopaminergic neurons in the nigrostriatal system, in particular in substantia nigra pars compacta (SNc). (Dauer and Przedborski, 2003; Forno, 1996). PD was originally classified as a non-genetic disease, since the majority of cases occur sporadically, and mostly for unknown reasons. However, recent strong evidence pointed out that about 10% of cases co-segregate with mutations in a number of genes.

Parkinson's Disease Symptoms

The neuropathological hallmark of PD is the degeneration of the dopaminergic neurons of SNc and the development of Lewy Bodies (LBs) in surviving dopaminergic neurons. Pathological changes may precede obvious symptoms by two decades or more. This preferential loss of dopamine (DA) producing neurons results in marked impairment of motor control. LBs, i.e. abnormal intracellular aggregates containing various proteins including alpha-synuclein (α-syn) and ubiquitin, impair optimal neuron functioning. Therefore, PD is commonly diagnosed when motor symptoms become explicit, i.e. when at least 50% of DA neurons in SNc have already died (Chinta et al., 2013), and it is at this stage that DA receptor agonists have beneficial effects. Although PD has been described mainly as a movement illness, it features both motor and non-motor symptoms, which have a serious impact on the quality of everyday life of patients. The cardinal symptoms are (i) resting tremor, that disappears when a voluntary movement is performed (Elble, 2000), (ii) hypokinesia (poverty of involuntary movements) resulting from a difficulty in initiating movements (akinesia) combined with slowness in movement execution (bradykinesia) (Marsden et al., 1981), (iii) rigidity and (iv) postural instability, which appear in the late stages of the disease and are associated with loss of equilibrium and falling (Marsden et al., 1981). Other clinical features include secondary motor symptoms such as hypomimia, dysarthria, dysphagia, sialorrhea, micrographia, shuffling gait,

festination, freezing, dystonia and glabellar reflexes. Furthermore, some non-motor symptoms of PD are autonomic dysfunction, neurobehavioral abnormalities, sleep disorders, and sensory abnormalities like anosmia, paresthesias and pain.

Etiology of PD

For the vast majority of patients, PD only relates to age and there is yet no clear explanation of its etiology. Epidemiological studies suggest an involvement of environmental risk factors, which may play a role in the development of the sporadic form of the disease (Adami et al., 2011; Tanner, 2003). Sporadic cases (i.e., not inherited) of the disease are commonly referred to as idiopathic PD (IPD). Nonetheless, some late onset PD cases seem to have an association with genetic changes (Gilks et al., 2005), or with a combination of genetic changes and environmental risk factors (Maraganore et al., 2003).

Although the etiology of the disease remains still partially unknown, approximately 10% of the cases are believed to be monogenic. The identification of the genetic loci responsible for the development of the disease was of staggering importance to further understand the mechanisms behind the onset of the disease. These findings led to the description of familial PD, i.e., cases of the dsisease with mendelian inheritance or with the presence of the disease amongst familial relatives. Several gene loci have been identified and implicated with the development of the disease, and in the current PD genes nomenclature there are 18 genes termed PARK, which are numbered in chronological order of their identification. However, this list is incomplete and carries numerous inconsistencies (i.e., loci were the link between the genetic modification and the disease is missing). Out of the six genetic loci unequivocally linked to familial PD mutations in the α-syn (SNCA) (PARK1-4) (Polymeropoulos et al., 1997) and LRRK2 (PARK8) (Zimprich et al., 2004) genes are responsible for autosomal-dominant forms of the disease, whereas mutations in parkin (PARK2) (Kitada et al., 1998), PINK1 (PARK6) (Bonifati et al., 2003), DJ-1 (PARK7) and ATP13A2 (PARK9) are accountable for autosomal recessive forms of the disease. As for the environmental factors, it is known that exposure to iron or manganese generates reactive oxygen species (ROS), and therefore these metals have also been used to induce parkinsonism in experimental animal models (Jenner, 1998). Moreover, two important chemicals have been classified as parkinsonian toxins, i.e. 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine (MPTP) and the herbicide paraquat; they both

concentrate in the DA neurons of the SNc and eventually kill them (Javitch et al., 1985; Langston and Ballard, 1983; McCormack et al., 2002). MPTP was first found in poorly purified desmethylprodine (MPPP), an opioid analgesic drug. The MPTP metabolite MPP+ is selectively uptaken by the dopamine transporter (DAT) in DA neurons (Javitch et al., 1985; Langston and Ballard, 1983) where it inhibits the mitochondrial respiratory chain, thus depleting the neuron of ATP. Because of its selective uptake in nigral DA neurons it causes parkinsonian symptoms. Nowadays, MPTP is one of the most used and reliable toxin used to induce experimental parkinsonism. Other established risk factors are: use of well water, milk consumption, excess body weight, exposure to hydrocarbon solvents, living in rural areas, farming or agricultural work, living in urban areas or industrialized areas with exposure to copper, manganese and lead, high dietary intake of iron and history of anemia.

Basal ganglia

The basal ganglia are a subcortical interconnected group of nuclei, comprised of the striatum, the globus pallidus (GP), the subthalamic nucleus (STN) and the substantia nigra (SN). Basal ganglia are involved in motor, cognitive and limbic functions (Albin et al., 1989; Alexander et al., 1986; DeLong, 1990; Obeso et al., 2000). The SN is divided into two closely linked parts, the SNc and the substantia nigra pars reticulata (SNr). When the communication between these two areas is malfunctioning or interrupted, the output is altered and motor activity is disturbed. In primates, the GP is divided into an external (GPe) and internal (GPi) portion, whereas in rodents these structures are represented by GP and entopenducular nucleus (EN), respectively. To simplify the functions of the BG, two different pathways that originate in the striatum, have been described: the direct and indirect pathways (Albin et al., 1989; DeLong, 1990). These two pathways oppositely modulate the activity of the BG output nuclei (SNr and GPi). Both nuclei send inhibitory GABA projections to the thalamus in order to control of the level of activity of thalamo-cortical projections, the main goal being the coordinated execution of movement. Conversely, the striatum is the major input target for excitatory glutamatergic projections arriving from the cortex and the thalamus (Smeal et al., 2008) or dopaminergic inputs originating from SNc. The vast majority (95%) of the striatal neuronal population is composed by GABAergic medium-sized spiny neurons (MSNs) (Chang et al., 1982). The remaining 5% are interneurons containing either GABA or acetylcholine (Ach),

which act to modulate the activity of the striatofugal pathways (Pisani et al., 2007).

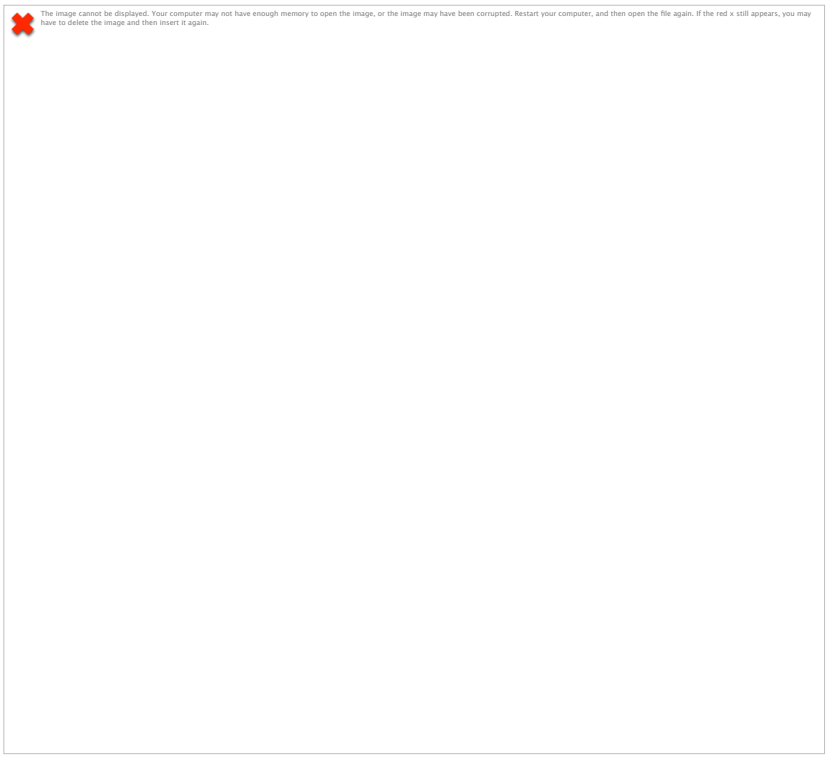

Figure 1. *Schematic representation of basal ganglia connections in the physiological state. DA projections exert a modulation of glutamatergic synapses coming from cortex ad thalamus. GABAergic efferents rising from striatum modulate the activity of GPe and GPi/SNr that in turn modify the firing pattern of STN and thalamus, respectively.*

Direct pathway

The direct pathway originates from a population of striatal GABAergic MSNs and it projects directly (monosynaptically) to the GPi/SNr. The main dopaminergic receptor expressed by these neurons is the D1 receptor, and D1 receptor stimulation potentiates AMPA or NMDA glutamate (GLU) receptor mediated transmission, thereby facilitating firing activity and causing release of GABA in GPi/SNr (Albin et al., 1989). Then, GPi/SNr send inhibitory GABAergic projections to the ventromedial and ventrolateral regions of the thalamus (Kha et al., 2001), modulating re-entral thalamo-cortical excitatory glutamatergic projections. Nigro/pallido-thalamic GABAergic neurons fire at high frequency and tonically inhibit thalamic activity. Thus, when the GPi/SNr is inhibited, the thalamus is relieved and excites the cortex, initiating and reinforcing the desired movement (Deniau and Chevalier, 1985).

Indirect pathway

The indirect pathway is a trisynaptic pathway originating from a different population of striatal MSNs. These neurons project to GP, use enkephalins (ENK) as a co-transmitter and predominantly express the D2 receptor, which is responsible for suppressing neuronal activity (Albin et al., 1989). The GP then sends GABAergic projections to the STN, while the STN sends excitatory glutamatergic projections to GPi/SNr. Therefore, when the indirect pathway is activated, the GP is inhibited, the STN is dishinibited causing stimulation of GPi/SNr neurons, and, as a consequence, the thalamus and movement are inhibited.

This model shows that the two pathways are functionally-opposing and work in a well-organized balance, such that increased activity in the direct pathway causes decreased GPi/SNr output, and increased activity in the indirect pathway does the opposite. This model also suggests that an abnormally decreased BG output results in excessive motor stimulation (chorea, levodopa-induced dyskinesia) and that an abnormally increased BG output results in decreased movement, as seen in Parkinson's Disease (PD).

Pathophysiology of PD

In order to manifest the classical motor symptoms, PD patients have to show at least 50% loss of DA neurons in the SNc (Zarow et al., 2003). As mentioned above, this results in hyperactivation of the STN, which then excessively excites the GPi/SNr. The strong inhibition of thalamo-cortical system is most probably correlated with akinesia and bradykinesia, which are amongst the pivotal features of PD.

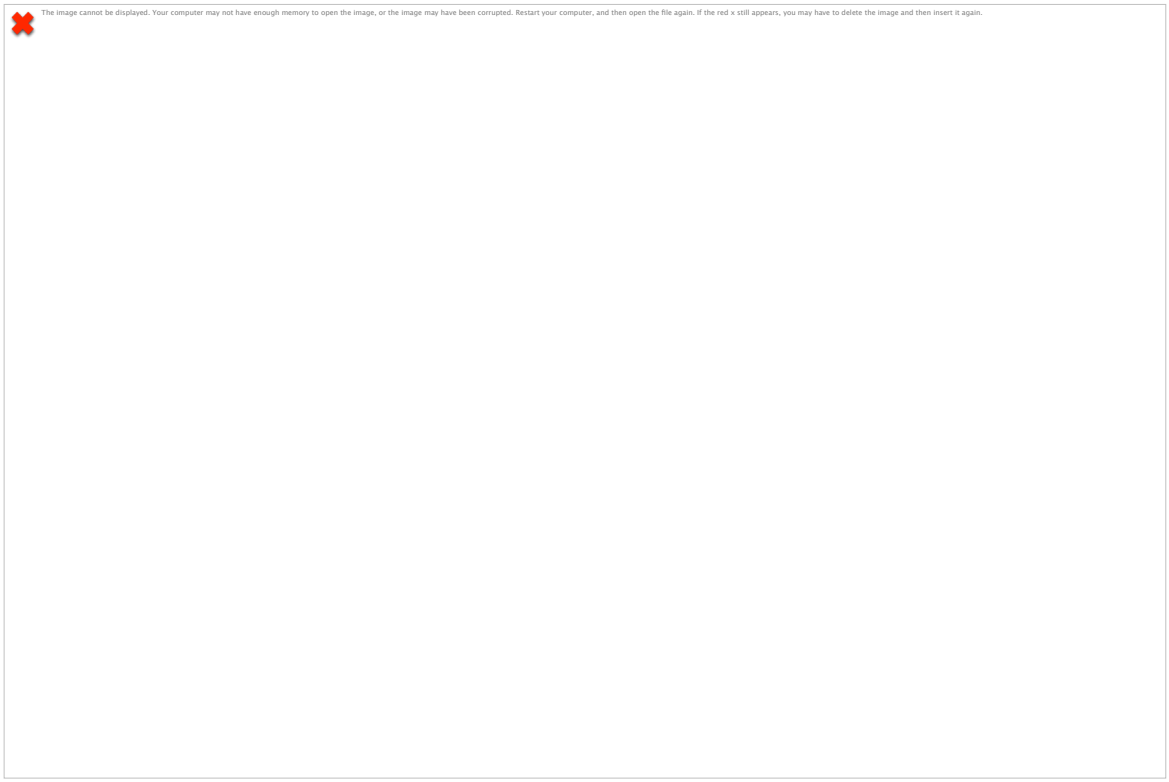

Figure 2. *Schematic representation of the principal pathways affected by the loss of dopaminergic neurons in Parkinson's disease. Two types of dopamine receptors (D1 and D2) are presented in different sets of outputs neurons in the striatum. The striatal neurons output define what are known as direct and indirect pathways (referring to the way they reach the thalamus). Excitatory pathways are shown in blue, and inhibitory pathways in red. The dashed arrows represent an impaired projection and the sizes of the arrows suggest the strength of the signal. The excessive inhibition of the thalamus caused by the loss of SNc neurons result in a poor excitation of the motor areas in the cortex. GPe= external segment of the globus pallidus; GPi= internal segment of the globus pallidus; STN= subthalamic nucleus; SNc= substantia nigra pars compacta; SNr = substantia nigra pars reticulata; PPN= pedunculopontine nucleus.*

Therapeutic approaches

The current therapy for PD is mainly based on L-DOPA (3,4-dihydroxy-L-phenylalanine), which in early stages of disease is beneficial for almost every patient. However, chronic treatment with L-DOPA is associated with the development of side effects such as wearing-off, motor fluctuations and dyskinesia in the majority of patients. Additional drugs have been developed to substitute or coadiuvate L-DOPA, but none of these treatments provide effects superior to L-DOPA. Thus, there remains a need for a therapy that provides (i) symptomatic benefits without carrying motor complications, (ii) treat non-dopaminergic symptoms and (iii) slow, stop or reverse the course of the disease (disease modifying).

L-DOPA

Chronic therapy with the dopamine precursor is still the gold standard of PD therapy (Carlsson, 2002). L-DOPA crosses the blood-brain barrier, and once it has reached the brain, is converted to DA in dopaminergic neurons, which express the conversion enzyme, L-aromatic amino acid decarboxylase (AADC). Moreover, in order to avoid peripheral decarboxylation, L-DOPA is usually administered in combination with a peripherally-restricted AADC inhibitor such as carbidopa or benserazide (Hornykiewicz, 2002). At early stages of disease, L-DOPA is processed mainly in DA neurons and synaptic terminals which retain the ability to store and release newly formed DA in a physiological way (Obeso et al., 2004). It is now clear, however, that the benefits of L-DOPA slowly wear off with the progression of the disease (Lee et al., 2008). In fact, once the disease progresses and DA neurons die, L-DOPA is metabolized to DA in other neural cells that possess AADC but lack D2 autoreceptors (mostly serotonergic neurons) (George et al., 2009). Consequently, DA release becomes pulsatile rather than continuous, and this leads to changes in postsynaptic receptor signaling and development of motor complications (Agid et al., 1985). Therefore, the more the disease progresses, the higher the dose of L-DOPA has to be. As a result of the long-term use of high doses of L-DOPA and the increased frequency of administration, the majority of PD patients develop L-DOPA-induced dyskinesia, which is the main side effect of L-DOPA chronic treatment and which will be discussed in details at page 20.

Dopamine receptor agonists

Despite being less effective than L-DOPA, DA receptor agonists became an alternative to L-DOPA treatment after it was discovered that L-DOPA promotes motor complications due to the pulsatile stimulation of DA postsynaptic receptors (Obeso et al., 2000). DA receptor agonists such as pramipexole, ropinirole and rotigotine bypass the degenerating neurons and target directly the upregulated postsynaptic D2 receptors (Cenci, 2007), providing a long lasting stimulation. Albeit their beneficial effects are not comparable of those of L-DOPA, DA receptor agonists given in de novo patients as mono-therapy can retard the use of L-DOPA, and in dyskinetic patients in combination therapy can reduce the dose of L-DOPA (Calne, 1993). Preclinical and phase II studies suggested a neuroprotective potential for DA receptor agonists (George et al., 2009) not confirmed in larger clinical trials.

Other strategies

Several alternative treatments have been used with different degrees of success: GLU receptor antagonists (Kornhuber et al., 1991), monoamine oxidase (MAO-B) inhibitors (Birkmayer et al., 1975), catechol-O-methytransferase (COMT) inhibitors (Ruottinen and Rinne, 1998), A_{2A} receptor antagonists (Jenner et al., 2009) and cholinergic antagonists (Duvoisin, 1967). In combination with pharmacological therapies, surgical procedures have also been performed in PD patients, amongst which there is ablative surgery (i.e., pallidotomy or thalamotomy), or deep brain stimulation (DBS) of the thalamus, GPi or STN. The aim of pallidotomy and DBS is to reduce the excessive inhibitory output from GPi and SNr (Ashkan et al., 2004).

From this brief summary, it is evident that PD therapy is lacking of treatment options in some important aspects, such as neuroprotection and side effects modulation. Therefore, scientists are focused on finding alternative strategies and orient their efforts towards finding neuroprotective agents that can slow down or stop the neuronal degeneration. Novel restorative therapies for PD under investigation are transplantation of fetal DA neurons or stem cells, and gene therapy based on viral-mediated delivery of enzymes critical for DA metabolism or neurotrophic factors.

Parkinson's disease and Lewy Bodies

The most typical neuropathological feature of PD is the loss of dopaminergic neurons in the SNc. The neuronal loss comes with the presence of cytoplasmatic intraneuronal protein inclusions known as LBs and Lewy Neurites. LBs, which are one of the pathological hallmarks of both familial and sporadic PD cases, are mainly constituted of -syn, (Goedert and Spillantini, 1998). Other neurodegenerative diseases have also been found to have the characteristic α-syn positive inclusions in neuronal or glial cells, and are therefore collectively referred to as synucleinopathies. This group of diseases includes Multiple System Atrophy (Duda et al., 2000; Goedert and Spillantini, 1998), dementia with Lewy bodies (Spillantini et al., 1997) and LB variant of Alzheimer's Disease (Goedert, 2001). According to the Braak staging (Braak et al., 2003), the neurodegenerative process is progressive, and spreads from low brainstem nuclei to the neocortex. It starts in the olfactory bulb, dorsal motor vagal nucleus and locus coeruleus, progressively reaching the midbrain, low forebrain and, finally, the cerebral cortex (Braak et al., 2003).

PD and a-synuclein

Several studies confirm the involvement of the gene that codes for α-syn, SNCA, in familial forms of PD. As a matter of fact, point mutations (Kruger et al., 1998; Polymeropoulos et al., 1997), and gene multiplications (Pals et al., 2004; Singleton et al., 2003), have been associated with familial forms of the disease. However, even the majority of sporadic PD cases show α-syn accumulation in LBs and yet they do not carry any mutations in the SNCA gene (Mezey et al., 1998). It could be therefore speculated that α-syn has multiple toxic mechanisms contributing to development of PD. Indeed, studies have shown that over-expression of WT α-syn is sufficient for the manifestation of PD, which appear to be dose-dependent (Pals et al., 2004; Singleton et al., 2003). This was confirmed by the finding that carriers of three SNCA copies present a 1.5-fold increase of α-syn mRNA expression, whereas in those presenting four SNCA copies (gene triplications) the increase is 2-fold (Pals et al., 2004).

Also, several models have been studied in order to better illustrate the role of α-syn in PD progression. As a matter of fact, transgenic mice over-expressing α-syn were found to have neuronal inclusions similar to LBs, increased degradation of nigral dopaminergic neurons and motor deficits, when compared to wild-type littermates (Masliah et al., 2000). Additionally, overexpression of WT, A53T and A30P α-syn in

flies gave the same results (Feany and Bender, 2000), suggesting that the over-expression of α-syn might play a role in the etiology of PD, and that understanding how the expression of this protein is regulated might provide novel insights into PD pathogenesis.

α-synuclein

α-syn is a 14 kDa presynaptic protein that belongs to a protein family known as the Synuclein family, which also includes β-synuclein and γ-synuclein. α-syn is encoded by the SNCA gene which maps to chromosome 4q21.3-q22 and is expressed primarily in the CNS, in particular in the presynaptic nerves of the neocortex, hippocampus, striatum, thalamus, cerebellum, cerebral cortex, SN and brainstem (Irizarry et al., 1996; Iwai et al., 1995; Jakes et al., 1994). The primary sequence of α-syn can be divided in three distinct regions: (i) an amino-terminal region, which is responsible for lipid binding (Perrin et al., 2000), (ii) a hydrophobic centre domain, which is thought to be responsible for the ability of α-syn to form b-sheets rich fibrils and (iii) a highly-negative carboxy-terminal region.

Figure 3. *Schematic representation of a-synuclein. α-syn contains three main domains, namely the amphipathic N-terminal domain, the hydrophobic NAC central domain and the acidic C-terminal domain. Arrows a, b and c represent the three disease-related mutations in α-syn, Ala30Pro, Glu46Lys and Ala53Thr, respectively.*

α-syn functions are still not well known. Several studies suggest a role in membrane-associated processes at the presynaptic terminal, such as regulation of synaptic transmission and DA release (Abeliovich et al., 2000). Furthermore, α-syn seems to play a role in the inhibition of lipid oxidation when bound to lipidic membranes, which was correlated to its ability to oxidate through the formation of methionine sulfoxide (Zhu et al., 2006). In addition, α-syn has also been shown to inhibit tyrosine hydroxylase (TH), which is the rate limiting enzyme in DA synthesis, thus supporting the hypothesis that α-syn is involved in the regulation of synaptic neurotransmission

(Perez et al., 2002). Notably, the native state of α-syn is still extensively under investigation. Although some studies have reported that α-syn purified from human cells is a helically folded tetramer, (Bartels et al., 2011; Fauvet et al., 2012; Gould et al., 2014) others have shown that α-syn exists predominantly as an unfolded monomer (Fauvet et al., 2012; Ueda et al., 1993). Taken together, these studies (Conway et al., 1998; Conway et al., 2000; Karpinar et al., 2009) suggest that α-syn exists in a dynamic equilibrium between various conformations and oligomeric states, modulated by factors that either accelerate or inhibit fibrillation.

α-synuclein aggregation

The presence of insoluble aggregates of α-syn is one the hallmark of synucleopathies, including PD, although it still has to be clarified whether α-syn aggregates are directly responsible for neuronal death. However, it is of critical importance to investigate the mechanisms that lead to α-syn aggregation and fibrillation. It has been established that recombinant α-syn and its pathogenic mutants are able to assemble into filaments with morphologies and staining properties similar to those extracted from brains affected by the disease (El-Agnaf et al., 1998; Fredenburg et al., 2007; Uversky et al., 2001). However, α-syn very strongly tends to aggregate even in physiological conditions, and often spontaneously (Hashimoto et al., 1998).

A possible pathway for aggregation has been proposed. The physiological monomeric unfolded protein might undergo conformational changes and become an aggregation-prone intermediate, which can lead to any of the stable end-products of aggregation (fibrils, amorphous aggregates and soluble oligomers) (Fink, 2006; Uversky et al., 2001). Therefore, factors that facilitate the formation of partially folded formations would facilitate the transition to aggregates and fibrils. This is important because it has been proposed that α-syn presents itself as a partially-folded protein, even in physiological conditions (Uversky, 2007), and in vitro experiments have reported that high concentrations of α-syn result in an increased fibrillation rate, which is likely to occur because of a high intermediates concentration (Uversky et al., 2001). These findings are crucial in PD, especially in cases where the α-syn locus is triplicated, thus leading to higher α-syn formation (Pals et al., 2004; Singleton et al., 2003) and, consequently, to a higher rate of concentration and partial folding.

α-syn toxicity

The mechanisms underlying α-syn toxicity, and which forms of the protein are responsible for neuronal death in PD, are still unclear. Surviving neurons contain aggregates in the form of fibrils, thus indicating that other forms of aggregation might be involved. Indeed, studies have shown how pre-fibrillar oligomers and proto-fibrils might be the toxic species (Danzer et al., 2007; Lashuel et al., 2002; Wright et al., 2009). This hypothesis was confirmed by an *in vitro* study using human dopaminergic neurons, which reported that toxicity was observed with non-fibrillar, small α-syn aggregates, suggesting that also soluble species mediate toxicity (Xu et al., 2002). It is still unknown how oligomers damage neurons, but it is thought that they might integrate in the membrane and form pore-like structures which would increase the permeability of the membrane (Volles and Lansbury, 2002), thus leading to an altered ion balance and increased intracellular calcium levels (Danzer et al., 2007). This is relevant because disruption of intracellular calcium homeostasis was a mechanism proposed to underlie the toxicity induced by other amyloidogenic peptides (amyloid β peptide, prion) (Demuro et al., 2005).

L-DOPA-induced dyskinesia

In the 1960s, a novel therapy for the treatment of PD came to light (Dery et al., 1962), based on the substitution of DA with the precursor L-DOPA. L-DOPA became the standard therapy for the disease, but, soon after the enthusiasm that accompanied this discovery, it became clear that the treatment with L-DOPA had several important limitations. Indeed, although in the early stages of treatment L-DOPA brings numerous benefits (the "honeymoon phase"), with the progression of the disease the beneficial effects of L-DOPA start to wear off in terms of duration and intensity, and patients start to fluctuate from "on periods" when the drug works and "off periods" when the symptoms reappear. Therefore, in order to re-establish the beneficial effects of L-DOPA, the dose has to be increased, which causes the development of involuntary movements, or dyskinesia, probably the most severe side effect of L-DOPA therapy. After 10 years of L-DOPA therapy, ~70-80% of patients overall develop dyskinesia, and the percentage goes up to 100% if we consider sporadic cases only (Fahn, 1982; Quinn et al., 1987). L-DOPA-induced dyskinesia (LID) is characterized by three different kinds of involuntary movements: dystonia, chorea and myoclonus. Furthermore, LID is clinically classified in three different domains, depending on the onset after L-DOPA administration: (i) "peak dose" dyskinesia which occurs when the plasmatic levels of L-DOPA reach the peak; (ii) biphasic dyskinesia (also known as "on off") that appears during the rise and fall of L-DOPA plasmatic levels; (iii) "off period dystonia" that causes prolonged muscle spasm affecting the feet, the arms and the face (Luquin et al., 1992), usually in the "off" phase. The main targets of L-DOPA are the striatal MSNs, which receive glutamatergic and dopaminergic inputs. In 1998, Cenci and collaborators, validated an animal model of LID, reporting that chronic L-DOPA treatment in 6-OHDA hemilesioned rats induces long lasting changes of striatal neuronal plasticity and gene expression in striatofugal pathways that correlate with LID (Cenci et al., 1998).

Strategies to reduce L-DOPA-induced dyskinesia

LID is mainly caused by the pulsatile receptor stimulation which follows the intermittent dopamine release caused by oral administration of L-DOPA (Chase, 1998). Several years ago the oral therapy with Madopar©, a controlled-release combination of L-DOPA with carbidopa or benserazide, was introduced which was designed to reduce to a minimum L-DOPA fluctuations. However, the clinical trials performed with this formulation did not show any significant reductions in LID in PD patients (Koller et al., 1999). More recently a gelified version of L-DOPA was developed for intrajejunal administration obtaining constant plasma concentrations of L-DOPA over several days (Nyholm et al., 2005). The efficacy of this formulation was recently confirmed in USA/Europe and New Zealand through a randomized and controlled study (Olanow et al., 2014). However, since the administration route carries some practical limitations and discomfort, a numerous quantity of capsules, prodrugs, or gastric resistant formulation have either successfully completed phase III clinical trial or are still in phase II.

Furthermore, D1 and D2 agonists proved effective in preventing LID development (Rascol, 1999) but failed in attenuating pre-existing LID. Despite these efforts, a potent agent with long duration able to replace L-DOPA and to attenuate or prevent the development of LID is still lacking. Therefore, different pharmacological tools able to alleviate already established LID are being developed, which can be accomplished by targeting any of the neurotransmitter systems that appear to be disregulated in LID.

Serotonergic System

Mounting evidence suggests that the serotonergic system might play a key role in the induction and expression of LID (Chase, 2014) in both patients (Bonifati et al., 1994; Politis et al., 2014) and experimental animal models (Carta et al., 2007; Dupre et al., 2008; Munoz et al., 2008; Navailles et al., 2011). First, the 5-HT_{1A} receptor was targeted. Indeed, buspirone (5-HT_{1A} agonist) attenuated LID in a small-scale investigational study (Politis et al., 2014). Moreover, saritozan reduced AIMs by 40% without modifying the parkinsonian score (UPDRS score). Unfortunately these promising results were not confirmed on a large-scale trial. In a pivotal study in a rat model of LID, Carta and collaborators (Carta et al., 2007) demonstrated that administration of $5HT_{1A}$ and $5HT_{1b}$ receptor agonists abolished established dyskinesia

through the inhibition of ectopic DA release from serotonergic fibers in striatum. Furthermore, the mixed 5-$HT_{1A/1B}$ agonist eltoprazine and anpirtoline (Bezard et al., 2013a; Paolone et al., 2015) proved effective in reducing LID in rat and nonhuman primate models of PD, even though they caused partial attenuation of the therapeutic effects of L-DOPA. Svenningsson and colleagues then tested eltoprazine in PD patients (Svenningsson et al., 2015); the reduction of LID was not nearly as strong as the one in animal models but the serotonergic system remains a popular target in LID.

Glutamatergic system

NMDARs

NMDA receptors (NMDARs) are abundantly expressed in the basal ganglia. They have been for many years the most exploited targets against LID. In animal models of LID and motor fluctuations, pharmacological blockade of NR2B has however produced inconsistent results. For example CP- 101.606 reduced LID in macaques (Blanchet et al., 1999) but exacerbated LID in marmosets (Nash et al., 2004). Moreover, in the same animal models the NR2B antagonists Ro256981 and Ro631908 failed to ameliorate LID (Rylander et al., 2009). Notably, L-DOPA treatment normalized the NR1 and NR2B abundance, while strongly increasing NR2A (Hallett et al., 2005). These results, obtained both in rodents and nonhuman primates models of LID, led to suggesting that the increase in NR2A expression plays a major role in LID development and that the blockade of this NMDA receptor subtype might represent a suitable target for LID (Gardoni et al., 2012).

Currently, amantadine, a weak and non-competitive NMDA receptor antagonist, is the only recommended anti-dyskinetic agent available (Del Dotto et al., 2001). Consistently, amantadine improves motor fluctuation and dyskinesia in MPTP lesioned nonhuman primates and in 6-OHDA-lesioned rodents (Bibbiani et al., 2005; Bido et al., 2011; Dekundy et al., 2007). Metman and collaborators (Metman et al., 1999) first assessed amantadine antidyskinetic efficacy during an acute intravenous L-DOPA infusion in PD patients with motor fluctuations. Interestingly, amantadine reduced peak-dose dyskinesia by 50-60%, when administered for a few weeks (Rajput et al., 1998) or up to one year (Wolf et al., 2010) without affecting PD motor symptoms. Numerous trials have been performed (da Silva-Junior et al., 2005; Del Dotto et al., 2001; Metman et al., 1999; Ory-Magne et al., 2014; Thomas et al., 2004; Wolf et al., 2010, but the question whether amantadine has a long-term antidyskinetic

effect still remains. Notably, a controlled-release formulation will be soon available {Pahwa, 2015 #731). The mechanism underlying the effect of amantadine still remains unclear. The antiparkinsonian effect could be accomplished in part by enhancing L-DOPA decarboxylase activity and DA synthesis (Deep et al., 1999). Moreover the non-competitive inhibition of the NMDA-evoked release of acetylcholine in rat striatal tissue could account for its clinical efficacy as anticholinergic treatment (Stoof et al., 1992).

Beyond amantadine, various NMDA antagonists such as dextromethorphan, remacemide, milacemide, CP-101,606 and memantine were tested for the treatment of PD (Clarke et al., 2001; Giuffra et al., 1993; Merello et al., 1999; Nutt et al., 2008). Notably, none of these drugs achieved similar results to amantadine.

mGLUR5

mGlu5 receptor negative allosteric modulators have been shown to be a potentially effective class of pharmacological agents for the treatment of LID. Indeed, The inhibition of metabotropic GLU receptors, and in particular of mGlu5, has been shown to be effective in attenuating both the priming to L-DOPA and the acute expression of LID (Dekundy et al., 2011; Mela et al., 2007). Numerous trials testing mGlu5 antagonists are undergoing, and validated data are pending.

Other strategies

Pharmacological studies suggest the presence of an enhanced μ-mediated opioid transmission in dyskinetic conditions. Accordingly, the μ receptor antagonists cyprodine and ADL5510 alleviated LID in a nonhuman primate model of LID without affecting the therapeutic activity of L-DOPA (Henry et al., 2001; Koprich et al., 2011).

Safinamide, a reversible and selective MAO-B inhibitor, which also reduces DA degradation and inhibits GLU release (Caccia et al., 2006), has been proposed as an anti-dyskinetic agent (Gregoire et al., 2013). Safinamide showed no substantial results in a Phase III clinical trial as an add-on to L-DOPA, although it slightly attenuated LID over an 18 month period in patients that were already dyskinetic from the beginning. Notably, it presented some benefits in PD patients with mild motor fluctuations as well as prolonging the ON time period without affecting dyskinesia (Borgohain et al., 2014).

Opioids and their receptors

The word *opium* derives from ancient greek and it means "juice". Indeed, the drug is obtained by the juice of the poppy plant, *Papaverum somniferum*. Cultivation and use of opium dates back to the Neolithic Age, and historical evidence shows how the Greeks and the Romans, as well as the Persians were well aware of the potential of opium, which was mainly used as a pain reliever, and as an anesthetic during surgical procedures. Opium contains two main groups of alkaloids: phenanthrens and isoquinolines. The first include morphine, codeine and thebaine, which are the most important narcotic constituents of the plant. The latter contain elements such as papaverine, but they have no particular effect on the CNS. The main overall component of opium is morphine, which counts for 10-16% of the total alkaloid amount and causes pain relief, euphoria, addiction, edema and respiratory difficulties.

The existence of a specific class of receptors for opioids was proposed in 1954 by Beckett and Casy, based on a series of studies on structure-activity for antinociceptive activity in synthetic opiates (Beckett and Casy, 1954). Only 11 years later, structure-activity analysis studies revealed the existence of more than one opioid receptor, or at least the possibility of different ways of interaction between the ligand and the opioid receptors (Portoghese, 1965). The first clear evidence that opioid receptors were more than one was published in 1976, by Martin and colleagues (Martin et al., 1976). Since then, extensive pharmacological studies led to the discovery of 4 different opioid receptor types, based on the different pharmacological actions they carry.

At first, the different receptor types were named after the drugs used for their identification; i.e., μ (mu for morphine) and κ (kappa for ketocyclazocine) (Martin et al., 1976). Furthermore, other pharmacological analysis of the effects of the opioid peptides in the guinea pig ileum and mouse vas deferens led to the proposal and discovery of a third opioid receptor named δ (delta for vas deferens) (Lord et al., 1977). Since their discovery, the nomenclature used to refer to these receptors changed several times. According to the International Union of Basic and Clinical Pharmacology (IUPHAR), μ receptor has been renamed mu opioid peptide (MOP) receptor, κ receptor kappa opioid peptide (Koprich et al.) receptor and δ delta opioid peptide (DOP) receptor. The opioid receptors were successfully cloned in the 1990s. Indeed the cloning of the DOP receptor (Evans et al., 1992; Kieffer et al., 1992), was

quickly followed by the cloning of the KOP and MOP receptors (Chen et al., 1993; Yasuda et al., 1993).

Opioid receptors are part of the G protein-coupled receptor superfamily and are composed of 7 transmembrane domains coupling with specific GTP binding proteins. These G proteins are heterodimeric proteins consisting of three distinctive subunits: α, β and γ. Opioid receptors are known to bind G_i (Hawes et al., 2000b). After activation, the beta and gamma subunits dissociate from one another and subsequently act on various extracellular effector pathways (Childers et al., 1979; Childers and Snyder, 1978). It was later established that opioid agonists and endogenous opioid peptides stimulate GTPase activity (Barchfeld and Medzihradsky, 1984) and inhibits cAMP production (Minneman and Iversen, 1976). Opioid receptors are also known to reduce neurotransmitter release and neuronal excitability through their intracellular actions, although disinhibition may also occur due to indirect modulation. Moreover, evidence links opioid receptors and overall GPCRs to the activation of mitogen-activated protein kinase pathways (MAPK), which are diverse signaling cassettes that govern cellular responses, including cell proliferation, differentiation, apoptosis, transcription factor regulation, channel phosphorylation, and protein scaffolding (Raman et al., 2007).

The ORL-1 receptor

After cloning of the three classical opioid receptors, further attempts to clone additional receptors types and/or subtypes were made, leading to the isolation of a cDNA encoding a homologous protein with a high degree of sequence similarity to the opioid receptors (Bunzow et al., 1994; Chen et al., 1994; Fukuda et al., 1994; Lachowicz et al., 1995; Mollereau et al., 1994; Wang et al., 1994).

The novel protein, named Opioid Receptor Like-1 (ORL-1) receptor, is a typical GPCR with 7 transmembrane domains, showing approximately 50% identity with the classic opioid receptors overall, with the transmembrane region reaching 80% identity. Despite these similarities, opioid ligands displayed very low affinity for the ORL-1 receptor, which was therefore considered an orphan receptor.

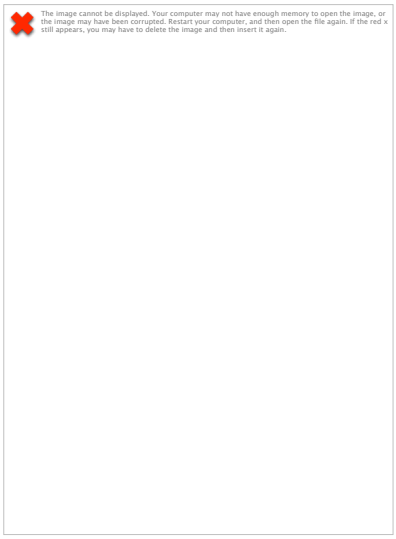

Figure 4. *Schematic representation of the ORL-1 receptor (Topham et al., 1998). TM helices are numbered 1 to 7. E/IL: Extracellular/Intracellular Loop. Visible at the C-terminal of TM 6 is the Gln 286 (human receptor numbering) side chain.*

The ORL-1 receptor was isolated in various species, namely the human (hNOP (Mollereau et al., 1994)), rat (XOR1 (Wang et al., 1994), ROR-C (Fukuda et al., 1994), LC132 (Bunzow et al., 1994), C3 (Lachowicz et al., 1995)), mouse (MOR-C (Nishi et al., 1994)) and pig (NOP (Osinski et al., 1999b)) species. The human ORL-1 receptor protein consists of 370 amino acids (Mollereau et al., 1994) and contains seven transmembrane (TM) domains. The N-terminal 44 amino acids contain 3 consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr). There are also sites for potential phosphorylation by protein kinase A (in the third intracellular loop) and protein kinase C (in the second intracellular loop and the C-terminal).

The NOP receptor sequence has a 57-58% aa (amino acid) identity to each of the rat MOP (Chen et al., 1994), DOP (Fukuda et al., 1993) and KOP (Minami et al., 1993) receptors. The identity among the four receptors varies between the different transmembrane domains. It is indeed highest (>70%) in the I, III and VII domains, approximately 50% in the II, V, and VI domains, but significantly lower (24%) in the IV domain. This high level of sequence conservation within the transmembrane domains suggests that the NOP receptor contains a transmembrane domain pocket that is the structural equivalent of the alkaloid-binding pocket of the other opioid receptors. Indeed, the NOP receptor retains the ability to bind, even if with very low affinity, the opioid ligands, receptor agonists or antagonists, such as diprenorphine (Mollereau et al., 1994), buprenorphine (Wnendt et al., 1999), lofentanil (Butour et al., 1997) and naloxone benzoylhydrazone (Noda et al., 1998).

In terms of exon-intron organization, the NOP receptor gene carries a striking similarity with the MOP, KOP and DOP receptors, suggesting that the four genes evolved from a common ancestor and therefore belong to the same family (Mollereau et al., 1997). So far, pharmacological studies failed to discover ORL-1 receptor subtypes (Calo et al., 2000), but its heterogeneity remains a question that needs to be answered. Indeed, five splice variants have been identified so far, although with no distinct pharmacological profiles. One, identified in rat (Wang et al., 1994), encodes a ORL-1 variant with an insertion (intron 5) in the second extracellular loop. The second variant, exhibiting an in frame deletion of 15 nucleotides at the 3' end of the transmembrane domain 1 coding region (Halford et al., 1995; Wick et al., 1994), encodes a functional receptor and has already been isolated from human tissue (Peluso et al., 1998). Furthermore, insertions of exons 3 and 4 (Curro et al., 2001) after the first coding exon (exon 2) in rats result in three additional splice variants (Pan et al., 1998).

Nociceptin/Orphanin FQ (N/OFQ)

In 1995, i.e. a year after the cloning of the ORL-1 receptor, two different research groups, through a process of reverse pharmacology, simultaneously identified the heptadecapeptide Nociceptin/Orphanin FQ (N/OFQ) as its endogenous ligand (Meunier et al., 1995; Reinscheid et al., 1995). The ORL-1 receptor was then renamed N/OFQ peptide (NOP) receptor. Based on the structural analogies with the classic opioid receptors, and the same consequences of receptor activation (i.e., inhibition of cyclic AMP), N/OFQ was assumed to be very similar to the classical opioid ligands. The two research teams screened extracts from rat (Meunier et al., 1995) and pig (Reinscheid et al., 1995). Fractions that were able to inhibit the cAMP activity (NOP receptor being a $G_{i/o}$-coupled orphan receptor) were further fractionated through reverse-phase high-performance liquid chromatography. Subsequent analysis revealed the presence of a heptadecapeptide, and its sequence was determined. This new synthetic peptide strongly inhibited the forskolin-induced accumulation of cAMP in CHO cells (Chinese Hamster Ovary cells) expressing the NOP receptor (Ec_{50} about 1 nM). Furthermore, when tested *in vivo* by intracerebroventricular (i.c.v.) injection in mice, the peptide induced hyperalgesia in two different tests, namely the hot plate test (Meunier et al., 1995) and the tail flick test (Reinscheid et al., 1995).

N/OFQ is cleaved from the polypeptide precursor preproN/OFQ (ppN/OFQ), which consists of 176 aa in humans, 181 aa in the rat, and 187 aa in the mouse (Mollereau et al., 1996; Nothacker et al., 1996). Further evidence of a common ancestor for N/OFQ and the opioid peptides was brought to light by analysis of the ppN/OFQ nucleotidic sequence, which revealed structural and organizational similarities with those of the classical opioid peptide precursors (Mollereau et al., 1996; Nothacker et al., 1996). Despite the fact that N/OFQ has strong structural homologies with the classical opioid ligands, it has no significant affinities with the other opioid receptors (Reinscheid et al., 1998).

In the ppN/OFQ nucleotidic sequence there are different pairs of basic amino acids that could be sites of various cleavages for precursors maturation, differentiation and transcriptional regulation (Zaveri et al., 2000). Therefore, it is highly possible that several biologically relevant peptides derive from ppN/OFQ. Indeed, two other peptides with biological activity have been found to be generated from the same precursor: nocistatin and N/OFQ II (Okuda-Ashitaka et al., 2012). None of them bind

to the NOP receptor (Mollereau et al., 1996; Nothacker et al., 1996), and so far no specific receptor has been discovered, although they both exert an independent and relevant biological activity.

NOP receptor and N/OFQ localization

The widespread distribution of N/OFQ and the NOP receptor has been well described in the literature over the years (Bunzow et al., 1994; Fukuda et al., 1994; Houtani et al., 2000; Lachowicz et al., 1995; Letchworth et al., 2000; Mollereau et al., 1994; Neal et al., 1999a; Neal et al., 1999b; Nothacker et al., 1996; O'Donnell et al., 2001; Riedl et al., 1996; Wick et al., 1995). The NOP receptor is highly expressed in the CNS, especially in the forebrain (cortical areas, olfactory regions, limbic structures such as hippocampus and amygdala, thalamus), throughout the brainstem (central periaqueductal gray, SN, several sensory and motor nuclei), and in both dorsal and ventral horns of the spinal cord (Mollereau and Mouledous, 2000; Neal et al., 1999a). Due to this pattern of distribution, N/OFQ and the NOP receptor are involved in motor and balance control, reinforcement and reward, nociception, stress response, sexual behavior, aggression, mood, food intake (Neal et al., 1999a).

Notably, in some areas like the dorsal horn of the spinal cord, the hippocampal formation and the caudate putamen, the midbrain periqueductal area and the nucleus raphe magnus, the expression patterns of the NOP receptor overlap with those of the MOP receptor (Anton et al., 1996) (Houtani et al., 2000). However, in other areas, the distribution patterns do not overlap, providing an explanation for the different in vivo actions of N/OFQ and classical opioid ligands (Ikeda et al., 1998) (Monteillet-Agius et al., 1998; Sim and Childers, 1997).

The NOP receptor mRNA has also been identified in the peripheral nervous system and several other organs, in the peripheral ganglia and in the immune system. Notably, Peluso and colleagues were the first to characterize the distribution of the NOP receptor system in humans (Peluso et al., 1998), by employing RT-PCR techniques. The highest amplification was found in cortical areas, hypothalamus, mammillary bodies, SN and thalamus. Limbic areas, brainstem and the pituitary gland also express the NOP receptor, albeit at a lower level. The distribution of the NOP receptor in humans highly resembles that seen in rodents, strongly suggesting its important role in numerous physiological functions, such as emotive and cognitive processes, neuroendocrine and sensory regulation.

The expression pattern of N/OFQ and/or the localization of ppN/OFQ often overlap with that of the NOP receptor (Berthele et al., 2003; Peluso et al., 1998). Indeed N/OFQ is highly expressed in limbic areas, i.e., the bed nucleus of stria terminals and amygdala nuclei (Boom et al., 1999; Neal et al., 1999b), lateral septum, hypothalamus, ventral forebrain, claustrum, mammillary bodies, amygdala, hippocampus, thalamus, medial habenula, ventral tegmentum, SN, central gray, interpeduncular nucleus, locus coeruleus, raphe complex, solitary nucleus, nucleus ambiguous, caudal spinal trigeminal nucleus, and reticular formation, as well as in the ventral and dorsal horns of the spinal cord (Neal et al., 1999b). N/OFQ is also detected in the periphery. Indeed, the mRNA is found in rat ovary, in the human spleen, lymphocytes, fetal kidneys (Mollereau et al., 1996; Nothacker et al., 1996) and, under physiological conditions (Brooks et al., 1998). Moreover, plasma N/OFQ levels were found to be elevated in pathological conditions such as postpartum depression (Gu et al., 2003), Wilson's Disease (Hantos et al., 2002b), hepatocellular carcinoma (Horvath et al., 2004) and acute and chronic pain states (Ko et al., 2002).

Cellular effects of N/OFQ

The classic opioid receptors (MOP/KOP/DOP) and the NOP receptor are coupled with inhibitory G-proteins, i.e., G-proteins endowed with $G_{i/o}$ alpha subunits (Reinscheid et al., 1996). Activation of the MOP, KOP, DOP or NOP receptor leads to same consequences: (i) closing of voltage-sensitive Ca^{2+} channels, belonging to the L, N and P/Q types (Connor et al., 1996b; Knoflach et al., 1996), (ii) opening of inwardly rectifying K^+ channels, leading to membrane hyperpolarization, specifically in dorsal raphe nucleus (Vaughan and Christie, 1996), locus coruleus (Connor et al., 1996a), periaqueductal grey (Vaughan et al., 1997) and, likely, hippocampus (Amano et al., 2000), and (iii) reduction of cAMP production via inhibition of adenylyl cyclase. These effects lead to general reduced neuronal excitability and, therefore, to a reduction in transmission of nerve impulses together with a diminished neurotransmitter release (Hawes et al., 2000a).

Figure 5. *Schematic representation of the cellular consequences of NOP receptor activation*

These findings suggest that N/OFQ primarily acts to reduce neuronal synaptic transmission and firing in the central nervous system. Further evidence pointing towards this general inhibitory role on neurotransmission was found using synaptosomes. This technique allowed to show that N/OFQ inhibits the release of noradrenaline (NA), serotonin (5-HT), dopamine (DA), acetylcholine (Ach), γ-aminobutiric acid (GABA) and glutamate (GLU) (Schlicker and Morari, 2000). Moreover, N/OFQ and other opioids, act not only through the Gi pathway, but also through the MAP-kinase pathway, quite possibly summoned by the beta and gamma subunits (Kirik et al., 2002), which is interesting because the same pathway is involved in the degeneration of DA neurons (Crotty et al., 2008). Studies have also shown a general inhibitory role of N/OFQ in the peripheral nervous system, regarding its role in neurotransmitter release from sympathetic, parasympathetic and noradrenergic non-cholinergic sensory endings. Indeed, N/OFQ has shown to play an inhibitory role in the cardiovascular, respiratory, gastrointestinal ad genitourinary system (Lohith et al., 2012).

Biological effects of N/OFQ

As already said, the N/OFQ-NOP receptor system plays an important role in a vast number of physiological responses with effects that target the central and peripheral nervous system, the cardiovascular system, the airways, the gastrointestinal tract and the immune system. The pharmacological and biological tools available nowadays allow to investigate this system using (i) antisense oligonucleotides targeting ppN/OFQ or the NOP receptor, (ii) antibodies directed against N/OFQ, (iii) transgenic mouse models where the precursor or the receptor genes have been eliminated, (iv) potent and selective agonists and antagonists.

Pain

N/OFQ and the NOP receptor have very peculiar effects on pain. Indeed, N/OFQ can have both pronociceptive and antinociceptive effects, depending on the administration route, the injection site, the drug dose and the test performed. Low doses (fmol) of N/OFQ produce pronociceptive responses (Sakurada et al., 1999), whereas higher doses (nmol) of N/OFQ are antinociceptive (Micheli et al., 2015). When injected i.c.v., N/OFQ causes hyperalgesia, measured in the hot plate test (Meunier et al., 1995; Suaudeau et al., 1998) and in the tail-flick test (Reinscheid et al., 1995; Suaudeau et al., 1998). These effects were likely mediated by the NOP receptor based on the results of a study reporting that the pronociceptive action of N/OFQ is absent in NOP knockout mice, and that antisense oligonucleotides targeting the NOP receptor prevent the N/OFQ effect (Calo et al., 2000).

On the contrary, i.c.v. N/OFQ administration in the pmol range was ineffective in altering the response to noxious stimuli (Vanderah et al., 1998; Zhu et al., 1998). Interestingly, it has also been suggested that the pronociceptive effects produced by i.c.v. N/OFQ may provide an explanation for the anti-opioid action of N/OFQ (Grisel et al., 1996). This phenomenon was confirmed by the finding that intratecal (i.t.) injection of N/OFQ was not able to induce analgesic effects in morphine-tolerant rats, whereas i.t. injection of morphine in animals tolerant to N/OFQ evoked analgesic effects (Micheli et al., 2015). On the other hand, when administered at the spinal level, N/OFQ has generally antinociceptive effects, which are in fact similar to those of classic opioid agonists, without inducing signs of sedation or motor impairment. Notably, i.t. N/OFQ shows these antinoceptive effects at doses lower than those causing hyperalgesia, allodynia and motor impairment. Thus, N/OFQ at low doses in

the pmol and fmol ranges displayed pronociceptive action resulting from the activation of NOP receptors at the spinal level, and exhibited antinociceptive potential at higher, nmol doses. Furthermore, strong evidence that the induction of chronic pain states is associated with the upregulation of N/OFQ synthesishas been provided (Dubner and Ruda, 1992; Kajander et al., 1990; Malan et al., 2000; Mika et al., 2010).

Anxiety, stress and depression

Preliminary evidence that N/OFQ might be implicated in the modulation of stress and anxiety came from the finding that N/OFQ is able to reverse stress-induced analgesia (Mogil et al., 1996) and that the NOP receptor is expressed in brain areas known to play a role in anxiety, such as the hypothalamus and the brainstem. Indeed, central administration of N/OFQ was shown to have strong anxyolytic effects, even comparable to those of diazepam (Jenck et al., 1997). In this study, investigators reported that low doses of N/OFQ decreased measurements of anxiety and that, like diazepam, conferred motor impairment at higher doses. These findings were later confirmed with mice in the elevated plus maze paradigm (Gavioli et al., 2002) the hole-board exploration test (Kamei et al., 2004) and in the defense test battery (Griebel et al., 1999), although in the latter paradigm, the effects were observed only after very high stress. Although it is clear that N/OFQ has a role in regulating anxiety and stress related behaviors, the mechanism by which the N/OFQ system modulates stress is not fully understood but implicates regulation of the hypothalamic–pituitary–adrenal (HPA) axis. The first indication that the N/OFQ system may be involved in modulating depressive states of behavior came from the observation that rat pups that were separated from their mothers exhibit elevated levels of N/OFQ expression in adolescence (Ploj et al., 2002). Consistently, N/OFQ levels are elevated in postpartum depressive women (Berthele et al., 2003), and, more importantly, N/OFQ antagonists produce antidepressant effects, such as reduced immobility time in the mouse forced-swim test, which reflects behavioral despair (Gavioli and Calo, 2013).

Food intake

The distribution of N/OFQ and the NOP receptor in areas such as the hypothalamus suggest their involvement in the regulation of appetite and metabolism. Indeed, after the discovery and isolation of N/OFQ, Pomonis and co-workers showed that supraspinal N/OFQ increased food intake in satiated rats (Pomonis et al., 1996). This effect is short lasting, specific to food intake (i.e., not to water intake) and accompanied by mild hypolocomotion (Polidori et al., 2000). In the following years investigators reported the strong orexigenic effect of administration of exogenous N/OFQ and NOP receptor agonists (Civelli, 2008; Economidou et al., 2006). Moreover, chronic 12-day long infusion of N/OFQ in mice produced sustained augmentation in food intake {Matsushita, 2009 #79}. The orexigenic actions of N/OFQ seem to occur through the inhibition of the anorexigenic pathway, rather than through the activation of the orexigenic one. Indeed, Indeed, N/OFQ inhibits cfos expression in arcuate nucleus POMC neurons (precursor for α-melanocyte-stimulating hormone) associated with meal termination (Bomberg et al., 2006). Overall, N/OFQ might have anti-hyperphagic and anti-anorectic effects. Thus, and NOP receptor agonists might have a potential as orexigenic drugs (Ciccocioppo et al., 2004; Economidou et al., 2006), and could be used to fight obesity, or eating disorders such as binge eating.

Reward and addiction

Numerous studies performed with N/OFQ and NOP receptor knockout mice highlighted that the N/OFQ-NOP receptor system plays a pivotal role in reward and drug abuse processes. Indeed, the NOP receptor is highly expressed in the brain areas that regulate such behaviors: nucleus accumbens, ventral tegmental area, medial prefrontal cortex, lateral hypothalamus, amygdala, and the bed nucleus of stria terminalis [22, 23]. For instance, i.c.v. N/OFQ administration suppresses basal and drug-stimulated dopamine release from the nucleus accumbens (Di Giannuario and Pieretti, 2000; Di Giannuario et al., 1999; Murphy et al., 1996; Murphy and Maidment, 1999), which is consistent with the suggestion that N/OFQ blocks the rewarding properties of several common drugs of abuse. This was proven using the conditioned place preference (CPP), which is an animal model to evaluate the modulation of drug-induced reward; N/OFQ blocked the reward induced by morphine (Ciccocioppo et al., 2000; Murphy et al., 1999), cocaine (Kotlinska et al., 2002;

Sakoori and Murphy, 2004), amphetamines (Kotlinska et al., 2003; Zhao et al., 2003) and alcohol (Ciccocioppo et al., 1999; Kuzmin et al., 2004). Of all the most popular substances of abuse, alcohol is certainly the most thoroughly examined in studies investigating the anti-reward properties of N/OFQ. As a matter of fact, not only does N/OFQ block acquisition and expression of ethanol-induced CPP in mice, but, when administered i.c.v., it also blocks reinstatement of extinguished alcohol CPP [35], a model of drug-induced relapse. Moreover, Ciccocioppo and colleagues (Economidou et al., 2008) showed that i.c.v. N/OFQ reduced alcohol self-administration in genetically selected alcohol preferring rats. The reward-blocking potential of N/OFQ might be sustained by its inhibitory effect on neurotransmitter release. Indeed, NOP receptor activation by exogenously administered N/OFQ decreases basal [41] as well as morphine- [24, 25] and cocaine-induced [42] dopamine release from mesolimbic areas in rodents.

Although most studies show the ability of exogenous N/OFQ to regulate the reward pathways, it also became clear the endogenous N/OFQ could play a role in the acquisition of addiction. Indeed, it has been reported that NOP receptor knockout mice show a stronger methamphetamine-, ethanol- and cocaine-induced CPP [47, 48]. Altogether, these data shed light on the involvement of the N/OFQ-NOP receptor system in the regulation of drug abuse and reward, suggesting the use of NOP receptor agonists as drug abuse medications.

Learning and memory

The presence of N/OFQ in the anterior cingulate, frontal cortex, basolateral complex of the amygdala and hippocampus suggests an involvement in learning and memory. Indeed, N/OFQ injected in the hippocampus impaired spatial learning in mice (Sandin et al., 1997). Moreover, mice presenting the genetic deletion of the NOP receptor ($NOP^{-/-}$) mice show better learning ability and have better memory retention than their wild-type littermates (Manabe et al., 1998). Therefore, it has been suggested that decreased activation of the NOP receptor leads to enhanced memory, whereas its activation should impair memory. Initial studies suggested a dose-dependent biphasic effect of an intrahippocampal infusion of N/OFQ, in which low doses (0.33 to 1 nmol) enhanced memory in the water maze and high doses (5 nmol) impaired it (Kuzmin et al., 2004; Redrobe et al., 2000; Sandin et al., 1997). Of note, the effect was reverted by NOP receptor antagonists (Hiramatsu et al., 2008; Kuzmin et al.,

2004; Redrobe et al., 2000). The impairment of learning and memory by a single dose of N/OFQ or NOP receptor agonists has been consistently reported in different tasks that are highly dependent on hippocampal and amygdala functioning, when N/OFQ or NOP receptor agonists are administered through different routes. Consistently, NOP receptor stimulation with N/OFQ or the NOP receptor agonist 8-[(1*S*,3a*S*)-2,3,3a,4,5,6-hexahydro-1*H*-phenalen-1-yl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one, Ro64-6198, has been shown to impair memory consolidation in contextual fear and passive avoidance paradigms (Fornari et al., 2008; Goeldner et al., 2009; Higgins et al., 2002; Hiramatsu and Inoue, 1999; Mamiya et al., 2003; Roozendaal et al., 2007), tasks highly dependent on hippocampal functioning. These findings suggest that N/OFQ may play a negative role in learning and memory, and that NOP receptor antagonist could be an efficient tool in memory disorders.

Gastrointestinal tract

Studies conducted both *in vitro* and *in vivo* suggest that N/OFQ has a pharmacologically distinct role from other opioids, although they are functionally similar. Indeed, N/OFQ inhibits *in vitro* neurogenic contractions of the stomach and the small intestine, as do morphine and other opioid receptor agonists in numerous species (Calo et al., 1996; Zhang et al., 1997). On the other hand, when tested *in vivo*, N/OFQ has contradictory effects on the gastrointestinal tract. Indeed, Osinski and colleagues found that central administration of N/OFQ inhibits *in vivo* colon transit in the mouse (Osinski et al., 1999a), but in 1998 Taniguchi and co-workers reported that, when given subcutaneously, N/OFQ accelerates transit rate in the large intestine in rat, which is the opposite effect of morphine and other opioids (Taniguchi et al., 1998). Despite these differences, N/OFQ effects on the gastrointestinal tract were found to be antagonized by the NOP receptor antagonist [Nphe$_1$]N/OFQ(1-13)-NH$_2$, thus proving the direct involvement of the NOP receptor in the mechanism (Broccardo et al., 2004).

Airways

Studies show that N/OFQ has a negative effect in the contraction of the bronchus in guinea pigs, (Fischer et al., 1998; Rizzi et al., 1999), rats (Wu et al., 2000) and humans (Basso et al., 2005). Moreover N/OFQ inhibits cough responses in guinea pigs when induced by capsaicin, and in cats when induced by mechanical stimulation of intrathoracic airways (Bolser et al., 2001; McLeod et al., 2001). A study by McLeod and colleagues in 2004 reported that this anti-tussive effects is mimicked by non-peptidic NOP receptor agonist Ro64-6198 and that this effect is sensitive to the non-peptidic NOP receptor antagonist 1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H benzimidazol-2-one, J-113397 (McLeod et al., 2004), which suggests a potential use of NOP receptor agonists for the treatment of cough (Lambert, 2008).

Immune System

The N/OFQ-NOP receptor system is widely spread in the immune system. Indeed, the mRNA for the NOP receptor has been found in mouse lymphocytes (Halford et al., 1995), human peripheral mononuclear cells (Wick et al., 1995) and human circulating granulocytes, lymphocytes and monocytes (Fiset et al., 2003; Peluso et al., 1998). Neutrophils are thought to be a source of N/OFQ in inflammatory tissues (Fiset et al., 2003). N/OFQ can function as an immunosuppressant by suppressing antibody production in mouse lymphocytes, by decreasing proliferation of phytohemagglutinin-stimulated PBMCs, and by inhibiting mast cell function (Civelli, 2008).

Locomotor activity

Endogenous N/OFQ exerts a general inhibitory control over motor functions (Marti et al., 2004b; Marti et al., 2008). Indeed NOP receptor antagonists such as the peptide [Nphe1,Arg14,Lys15]N/OFQ-NH2 (UFP-101; (Calo et al., 2002)) and nonpeptide J-113397 or Compound B; (Kawamoto et al., 1999)) and its achiral analogue 1-[1-(cyclooctylmethyl)-1,2,3,6-tetrahydro-5-(hydroxymethyl)-4-pyridinyl]-3- ethyl-1,3-dihydro-2H-benzimidazol-2-one (Trap-101; (Trapella et al., 2006)) increased, stepping activity, speed and rotarod performance in naïve rats (Marti et al., 2004b; Marti et al., 2008; Marti et al., 2009). Consistently, J-113397 and Trap-101 also ameliorated the motor performance of naïve mice (Viaro et al., 2008), and J-113397 increased arm movement speed and in nonhuman primates (Viaro et al., 2008). The view of N/OFQ as an endogenous motor constraint is corroborated by the finding that

$NOP^{-/-}$ mice had a better motor performance than their wild-type ($NOP^{+/+}$) littermates (Marti et al., 2005; Marti et al., 2004b; Viaro et al., 2008). Nonetheless, recent data suggested that the modulation of motor behavior by N/OFQ might be more complex than expected. Indeed, it was shown that J-113397 and Trap-101, through NOP receptor blockade in the SNr, facilitated motor activity at lower doses, but impaired it at higher ones (Marti et al., 2008; Viaro et al., 2008). A similar dual response was detected after N/OFQ i.c.v. injection, with low doses facilitating (Florin et al., 1996; Higgins et al., 2001; Jenck et al., 1997; Kuzmin et al., 2004; Marti et al., 2009), and high doses impairing (Devine et al., 1996; Higgins et al., 2001; Kuzmin et al., 2004; Marti et al., 2009; Reinscheid et al., 1995; Rizzi et al., 2001) spontaneous locomotion. Importantly, motor improvements given by low doses of N/OFQ, either given i.c.v., or given into the SNr, was associated with augmented motor cortex excitability and motor output, whereas motor impairment induced by higher doses was accompanied by opposite electrophysiological changes (Marti et al., 2009). Moreover, NOP receptor antagonists replicated the electrophysiological and behavioral changes induced by low doses of N/OFQ, suggesting that dual responses to NOP receptor ligands are mediated by NOP receptors in the SNr, and that NOP receptor antagonists and N/OFQ at low doses activate common pathways. Evidence that mesencephalic DA neurons transduce motor actions of NOP receptor ligands has been presented. Indeed, N/OFQ and NOP receptor antagonists, given systemically or into SNr, inhibited and facilitated DA release in dorsal striatum, respectively (Marti et al., 2004b). Furthermore, N/OFQ inhibited DA release in the striatum (Murphy and Maidment, 1999; Narayanan et al., 2004) while J-113397 elevated it, although via NOP-unrelated mechanisms (Koizumi et al., 2004). Finally, even the hyperlocomotive response to N/OFQ was reported to be DA dependent (Florin et al., 1996; Kuzmin et al., 2004). Based on such evidence, Viaro and coworkers suggested the motor effects of N/OFQ and NOP receptor antagonists might be caused by the stimulation of different subpopulations of DA receptors (Viaro et al., 2013). Indeed, it was reported that the modulation exerted by endogenous N/OFQ relies on the modulation of DA release, which in turn acts at the D2 receptors. For instance, postsynaptic D2L receptors mediate N/OFQ-induced motor facilitation, whereas N/OFQ-induced hypolocomotion occurs also in the absence of D2 receptors, possibly through direct inhibition of mesencephalic DA neurons.

N/OFQ and PD

NOP receptor antagonists and PD

We already discussed the effects of N/OFQ on locomotor activity in physiological conditions, but a cumulating amount of evidence suggests a role for N/OFQ in the regulation of locomotor activity also in pathological conditions. Indeed, studies reported that endogenous N/OFQ sustains the motor deficits and the neurodegenerative process of PD in animal models of the disease (Marti et al., 2005). For instance, NOP receptor antagonists attenuated parkinsonian-like motor deficits in 6-OHDA rats (Marti et al., 2013; Marti et al., 2005; Marti et al., 2008; Marti et al., 2007), haloperidol-treated rats (Marti et al., 2004a), reserpinized mice (Volta et al., 2010b) as well as MPTP-treated mice and nonhuman primates (Viaro et al., 2010; Viaro et al., 2008; Visanji et al., 2008). NOP receptor antagonists likely act via blockade of the NOP receptor in the SNr, thus causing a decrease in GLU and an increase in GABA release in the same area (Marti et al., 2004a). These changes lead to an overinhibition of the nigro-thalamic pathway and motor restoration. These pharmacological findings were confirmed in $NOP^{-/-}$ mice; indeed, these mice were found to be partially resistant to haloperidol-induced (Marti et al., 2005), and reserpine-induced akinesia (Volta et al., 2010a), a phenomenon linked to the diminished ability of haloperidol and reserpine to elevate GLU in SNr (Mabrouk et al., 2010). Remarkably, DA depletion upregulated N/OFQ synthesis (Brown et al., 2006; Di Benedetto et al., 2009; Marti et al., 2005) and release (Marti et al., 2005) in SNr, thus exacerbating its detrimental role on DA neurons and motor functions.

In the mid 2000s, two different studies suggested that N/OFQ role in parkinsonism might go beyond the modulation of the motor phenotype. Indeed, mice with genetic deletion of ppN/OFQ ($ppN/OFQ^{-/-}$mice) were found to be partially resistant to MPTP treatment, as seen by a milder loss of nigral DA neurons and striatal DA terminals, compared to their wild-type littermates ($ppN/OFQ^{+/+}$) after acute toxin injection (Brown et al., 2006; Marti et al., 2005). However, two other neuropeptides are generated by the cleavage of ppN/OFQ, namely nocistatin and N/OFQ II (Okuda-Ashitaka and Ito, 2000). Both neuropeptides exert their biological functions independent of the NOP receptor, thus questioning the view that N/OFQ is the culprit for MPTP-induced neurotoxicity.

N/OFQ and PD in humans

Despite mounting evidence linking N/OFQ and PD, no clear relationship between N/OFQ and PD in humans had yet been established (Lambert, 2008). However, changes in the expression of N/OFQ has been implicated in different neurological diseases. Indeed, lower ppN/OFQ and NOP expression was found in the hippocampus and central amygdala, respectively, of alcoholics, (Kuzmin et al., 2009) and lower N/OFQ binding was observed in patients with temporal lobe epilepsy. Moreover, N/OFQ plasma levels are found elevated in patients affected by Wilson's Disease (Hantos et al., 2002a), which is an autosomal recessive disorder related to copper metabolism in the liver associated with basal ganglia degeneration (Madsen and Gitlin, 2007). Converserly, Collins and coworkers (Collins et al., 2015) found a statistically significant down-regulation of N/OFQ but no change in the NOP receptor in the SNc of PD patients, compared to controls. In light of these findings, the reduction of N/OFQ in the human SNc might be viewed as a compensatory mechanism to prevent excessive NOP receptor stimulation and protect residual DA neurons. However, these results go against previous findings showing an increase in increase in N/OFQ expression in 6-OHDA hemilesioned rats (Di Benedetto et al., 2009; Marti et al., 2005; Marti et al., 2010) or MPTP-treated mice (Di Benedetto et al., 2009; Gouty et al., 2010), which correlate with the increase of N/OFQ levels monitored in the rat SNr (Marti et al., 2005) and in the CSF of PD patients (Marti et al., 2010). It is possible that the impact of acute loss of SNc DA neurons on N/OFQ transmission caused by neurotoxins in rodent models is different from the slow and progressive degeneration occurring in human PD. Moreover, Marti and colleagues in 2010 took their samples from a different age group than the ones in the Zhang et al., study or in the Moran et al., study, thus suggesting biphasic adaptive changes in N/OFQ expression in the SNc, reflecting plastic modifications inside and outside the SN (Collins et al., 2015).

NOP receptor agonists and L-DOPA-induced dyskinesia

When DA depletion occurs, N/OFQ levels in the SNr rise, and the NOP receptor is down-regulated (Brown et al., 2006; Di Benedetto et al., 2009; Gouty et al., 2010; Marti et al., 2005; Norton et al., 2002). In parallel, striatal levels of N/OFQ are diminished and the NOP receptor is up-regulated (Marti et al., 2010), suggesting a different adaptive change of N/OFQ transmission in parkinsonian conditions. Based on these findings, NOP receptor antagonists are expected to act where N/OFQ tone is elevated. Indeed, UFP-101 (peptidic NOP receptor antagonist) worsened dyskinetic behaviours in hemilesioned animals (Marti et al., 2012) when administered intranigrally but not intrastriatally. The general prodyskinetic effect of NOP receptor antagonists was first reported by Visanji and collaborators in 2008, when they showed that a high dose of J-113397 potentiated the therapeutic effect of L-DOPA at the cost of causing dyskinesia appearance. (Visanji et al., 2008). Since NOP receptor antagonists worsen dyskinesia, it was suggested that endogenous N/OFQ might act as a natural restrain against LID. In fact, acute i.c.v. injection of N/OFQ or systemic administration of a NOP receptor agonist (Ro 65-6570) attenuated LID expression in hemilesioned rats primed with L-DOPA (Marti et al., 2012).

Remarkably, the anti-dyskinetic effect was detected at doses that did not cause hypolocomotion, which is one the main side-effects of N/OFQ and NOP agonists (Devine et al., 1996; Jenck et al., 1997; Marti et al., 2004b; Marti et al., 2009; Reinscheid et al., 1995), thus suggesting different mechanisms underlying the two effects. This view was supported by biochemical evidence that NOP receptor stimulation opposes D1 receptor mediated signalling in striatum. Indeed, N/OFQ prevented the D1-mediated ERK phosphorylation and loss of striatal depotentiation of striatal plasticity in striatal MSNs (Marti et al., 2012). These data add to the previous finding that N/OFQ inhibited the D1 receptor stimulated cAMP accumulation in striatal slices (Olianas et al., 2008) and suggest the existence of a negative interaction between the NOP and D1 receptor on the membranes of striatal MSNs. The action of N/OFQ at the striatal postsynaptic site might be privileged in dyskinetic conditions, since in the striatum of 6-OHDA rats the NOP receptor is upregulated, probably as compensation after DA denervation (Marti et al., 2010).

In this scenario, NOP receptor agonists would normalize the inhibitory control mediated by endogenous N/OFQ over striatal D1 signaling (Marti et al., 2012). In vivo microdialysis studies reported that NOP receptor agonists attenuated LID by

interfering with the activity of striatal GABAergic MSNs projecting to the SNr. Indeed, i.c.v. injection of N/OFQ strongly attenuated the rise of nigral GABA and the reduction of thalamic GABA that correlate with the expression of LID (Mela et al., 2007). Remarkably, the LID-associated increase of nigral GABA is dependent on striatal D1 receptor application, since it is blocked by striatal perfusion of a D1 receptor antagonist (Mela et al., 2012). The anti-dyskinetic potential of NOP receptor antagonists such as Ro 65-6570 was also confirmed in MPTP-treated marmosets; in fact, the small molecule was able to attenuate LID without affecting L-DOPA therapeutic effects (Marti et al., 2012). The effect was mild (30%) and affected the dystonic but not the choreiform component of dyskinesia. Still, it provides a proof-of-concept of the efficacy of NOP receptor agonists in the treatment of LID.

Aim of the Study

The overall purpose of this study is to provide novel insights into the role of N/OFQ in PD, and particularly the contribution of the NOP receptor to the neurodegeneration associated with PD and to the development of LID.

In *part 1* of thesis, we employed immunohistochemical methods to investigate the in vivo neuroprotective potential of N/OFQ transmission blockade in PD models, either achieved through genetic deletion or pharmacological inhibition of the NOP receptor. We first discovered that the genetic deletion and the pharmacological blockade of the receptor successfully rescued a significant amount of DA neurons in animals treated acutely and subacutely with MPTP, respectively. Because of these promising data we used the same approach in a more predictive, and recently validated, animal model: rat overexpressing A53T α-syn in SNc. The NOP receptor antagonist administered, SB-612111, successfully spared nigral DA neurons, once again suggesting an important role for N/OFQ in the neurodegeneration associated with PD.

In *part 2* of the thesis, we used the highly potent and selective non-peptidic NOP receptor agonists AT-403 and AT-390, and the NOP receptor partial agonist AT-127 to test whether pharmacological stimulation at the NOP receptor, in parkinsonian conditions, prevents the sensitization of striatal neurons to levodopa, a process that underlies the development of dyskinesia. We found that AT-403 attenuates both the development and the expression of LID in 6-OHDA rats. However, AT-403, along with the positive effect, carried a dose-dependent sedation effect that dramatically narrowed its therapeutic window. As this effect was shared by AT-390 and AT-127, we conclude that pharmacological strategies should be implemented to selectively increase the antidyskinetic potency and widen the therapeutic windows of this class of compounds.

Materials and Method

Animal Subjects

Experiments were performed in accordance with the EU directive of September 22, 2010 (2010/63/EU) and the Italian directive of March 4, 2014 (26/2014) on the protection of animals used for scientific purposes. Male $NOP^{-/-}$ and $NOP^{+/+}$ mice (Nishi et al., 1997), bred on a CD-1 background for 9 generations (Gavioli et al., 2007), and male C57BL/6J mice (10 weeks old, Harlan Italy, S. Pietro al Natisone, Italy) were housed with free access to food and water and kept under regular lighting conditions (12 hr dark/light cycle), after approval of the experimental protocols by the Italian Ministry of Health (license 171/2010-B e 170/2013-B).

Male Sprague-Dawley rats (150g; Harlan Italy, S. Pietro al Natisone, Italy) were kept under regular lighting conditions (12 hr light/dark cycle) and given food and water ad libitum. Adequate measures were taken to minimize animal pain and discomfort. When experiments were performed at the University of Bordeaux (Bordeaux, France), male Sprague-Dawley rats (180 gr; Charles River Laboratories, France) were housed in the same conditions at the IMN (Bordeaux). The Institutional Animal Care and Use Committee of Bordeaux (CE50) approved these experiments under the license number 5012099-A.

Behavioural Studies

A battery of three validated behavioural tests to evaluate different motor functions was employed (Marti et al., 2005). The three tests, i.e., the bar, the drag and the rotarod test were repeated in a fixed sequence (bar, drag and rotarod). Motor activity was then expressed either as absolute values, i.e. immobility time (in sec) in the bar test, number of steps in drag test and time on rod (in sec) in the rotarod test.

Bar Test

The bar test, also known as catalepsy test (Marti et al., 2005; Sanberg et al., 1988; Viaro et al., 2008), measures the ability of the animal to react to an externally imposed position. The right and left forepaws were alternatively placed on blocks of increasing heights (1.5, 3 and 6 cm for mice; 3, 6, 9 cm for rats). The immobility time (in sec) of each forepaw on the blocks was recorded (the cut-off was set at 20 sec).

When values between right and left forepaws did not differ significantly, data were pooled together.

Drag Test

The drag test, modification of the "wheelbarrow test", (Schallert et al., 1979) measures the ability of the animal to balance its body posture using the forelimbs, in response to an external imposed dynamic stimulus (backward dragging) (Marti et al., 2005; Marti et al., 2004b; Viaro et al., 2008). Each animal was gently lifted from the tail (mice) or from the abdomen (rats) leaving the forepaws on the table and dragged backwards at a constant speed of 20cm/s for a fixed distance of 1m. The number of touches made by each forepaws was counted by two different observers. Since values between the right and left forepaws did not differ significantly, data were pooled together.

Rotarod Test

This test measures the ability of the animal to run on a rotating cylinder and provides information on different motor parameters such as coordination, balance, muscle tone, gait and motivation to run (Rozas and Labandeira Garcia, 1997). The fixed-speed rotarod test was employed using a previously validated protocol (Marti et al., 2005; Marti et al., 2004b). Animals were tested starting from 5rpm, speed was increased every 180sec and total time spent on the rod was calculated.

Stepping Test

This test evaluates forelimb akinesia (Sanberg et al., 1988). Rats were gently held sideways and dragged at a constant speed of 20cm/s for a fixed distance of 1m forth and back to allow forehand and backhand steps count. Stepping activity was measured once a week, three times over two consecutive days. Since values between paws did not differ significantly, they were pooled together, and the mean number of left/right backhand steps was averaged over the 3 sessions.

Figure 6. *The bar test: this test measures akinesia after an imposed static posture. Mice are placed on 3 separate block heights of 1.5, 3 and 6 cm respectively, whereas in rats paws forepaws were placed alternatively on separate blocks of 3, 6 and 9 cm respectively.*

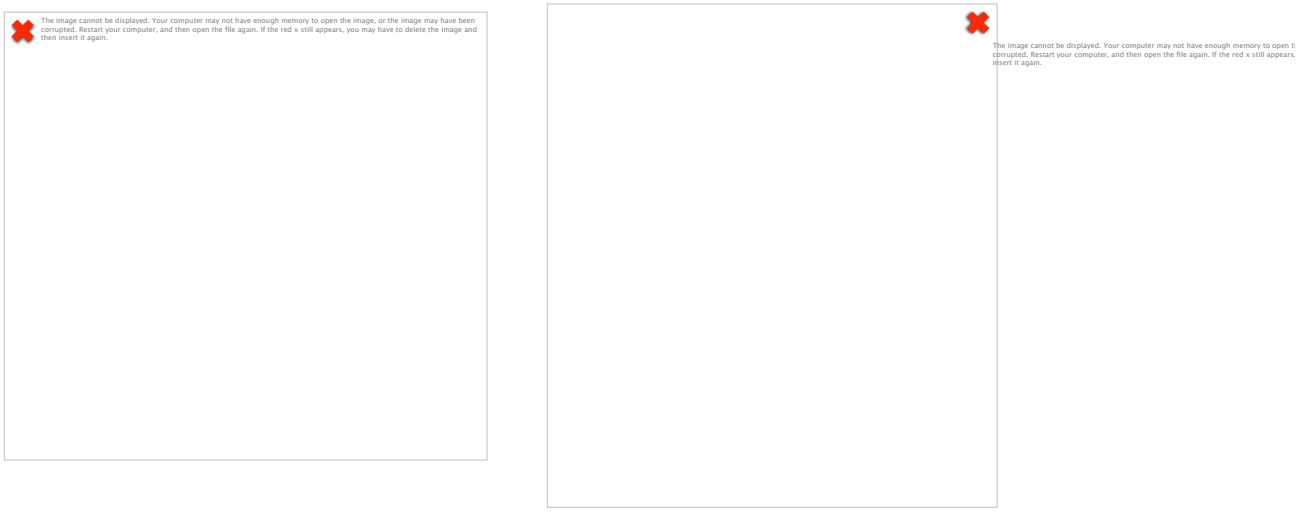

Figure 7. *The drag test: this test measures the animal ability to balance body posture using the forelimbs in response to an externally imposed dynamic stimulus (i.e. backward dragging)*

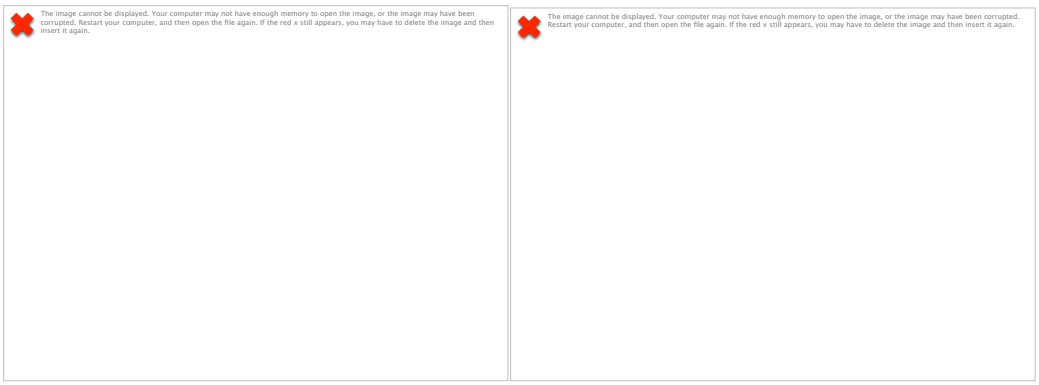

Figure 8. *The rotarod test: this test measures overall motor performance as an integration of coordination, gait, balance, muscle ton and motivation to run.*

Figure 9. *The stepping test: this test measures forelimbs akinesia and the ability of the animal to adjust the forelimbs to a lateral externally-imposed movement.*

L-DOPA treatment and Abnormal Involuntary Movements Rating

6-OHDA hemilesioned rats were treated for 21 days with L-DOPA (6 mg/Kg + Benserazide 12 mg/Kg, s.c., once daily). Quantification of Abnormal Involuntary Movements (AIMs), a correlate of L-DOPA-induced dyskinesia (Cenci, 2014), was performed as previously described (Berthet and Bezard, 2009; Lundblad et al., 2002; Mela et al., 2010; Munoz et al., 2008). Rats were observed for 1 minute, every 20 minutes, during the 3 hours that followed L-DOPA injection or until dyskinetic movements ceased. Dyskinetic movements were classified based in their topographical distribution into three subtypes: (1) Axial AIMs, i.e., twisted posture or turning of the neck and upper body toward the side contralateral to the lesion; forelimb AIMs, i.e., jerky and dystonic movements and/or purposeless grabbing of the forelimb contralateral to the lesion; (3) orolingual AIMs, i.e., orofacial muscle twitching, purposeless masticatory movement and contralateral tongue protrusion. Each AIM subtype was rated on a frequency scale from 0 to 4 (1, occasional; 2, frequent; 3, continuous but interrupted by an external distraction; 4, continuous and not interrupted by an external distraction). In addition, the amplitude of these AIMs was measured on a rating scale from 0 to 4 (Cenci and Lundblad, 2007), as reported by Cenci and Lundblad in 2007 (Cenci and Lundblad, 2007):

Limb Amplitude:

Score 1: Tiny movements of the paw around a fixed position.

Score 2: Movements resulting in a visible displacement of the whole limb either sideways or up-and-down (e.g., the paw loses contact with the snout, and reaches halfway to the floor).

Score 3: Large displacement of the whole limb with visible contraction of shoulder muscles.

Score 4: Vigorous limb displacement of maximal possible amplitude, with conspicuous contraction of both shoulder muscle groups and extensor muscles.

Axial amplitude:

Score 1: Sustained deviation of the head and neck, at ~30◦ angle.

Score 2: Sustained deviation of the head and neck, angle ≤60◦.

Score 3: Sustained twisting of the head, neck, and upper trunk at an angle >60◦ but ≤90◦.

Score 4: Sustained twisting of the head, neck, and trunk at maximal amplitude (angle >90◦), causing the rat to lose balance (from a bipedal position).

Orolingual amplitude:

Score 1: Twitching of facial muscles accompanied by small masticatory movements without jaw opening.

Score 2: Twitching of facial muscles, accompanied by noticeable masticatory movements, occasionally leading to jaw opening.

Score 3: Movements with broad involvement of facial muscles and masticatory muscles.

Jaw opening is frequent, tongue protrusion occasional.

Score 4: All the above muscle categories are involved to the maximal possible degree.

Axial, Limb and Orololingual (ALO) AIMs total value were obtained as the sum of the product between amplitude and frequency of each observation. (Cenci and Lundblad, 2007). Therefore the theoretical maximum ALO AIMs score is 432; to be considered fully dyskinetic an animal has to score ≥100.

Surgical Procedures

AAV2/9-hα-syn vector preparation and injection

Recombinant AAV2/9 vectors were prepared as already described (Bourdenx et al., 2015; Engeln et al., 2013; Zolotukhin et al., 1999). Briefly, vectors were transfected into HEK-293 T/17 cells (ATCC, Teddington, UK) for three times using a polyethylenimine solution. Seventy-two hours after transfection, cells were re-suspended in lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 8.5), and then lysed using a freeze-thaw cycle (-80°C/ +37°C). The obtained supernatant was purified by iodixanol gradient step centrifugation, and finally aliquoted and kept in stock at -80°C.

Sixteen male Sprague-Dawley rats received a bilateral SNc stereotaxic injection of AAV2/9-hα-syn (6.9 x 10^{13}genome containing particles/ml; 2 µl), under isoflurane anesthesia. Viral vector was injected with a glass pipette at a flow rate of 0.25ul/min and was left in place for 4 minutes to prevent backflush. Coordinates from bregma were: AP: -4.9, ML: ±2.2 and ±2.0, DV: -7.8 from dura(Paxinos and Watson).

Unilateral 6-OHDA lesion

Unilateral lesion of dopaminergic neurons was induced under isoflurane anesthesia to male rats as previously described (Marti et al., 2005). Eight micrograms of 6-OHDA (dissolved in 0.9% saline solution containing 0.02% ascorbic acid) were stereotaxically injected in the Medial Forebrain Bundle (MFB) according to following coordinates from bregma: AP= -4.4 mm, ML= -1.2, DV=-7.8 mm below dura (Paxinos and Watson). In order to select rats that were successfully lesioned the amphetamine challenge was employed (Ungerstedt and Arbuthnott, 1970). Briefly, two weeks after 6-OHDA injection, dopaminergic denervation was evaluated with a test dose of amphetamine (5 mg/Kg, i.p., dissolved in saline). Rats performing >7 turns per minute in the direction ipsilateral to the lesion were enrolled in the study. This behaviour has been associated with >95% loss of striatal dopaminergic terminals (Marti et al., 2007) and extracellular dopaminergic levels (Marti et al., 2002).

In vitro assays

Receptor Binding

The binding affinities of AT-127 and AT-390 for the opioid receptors were determined in the laboratories of Astraea Therapeutics (Mountain View, CA, USA) by radioligand competition experiments on receptor-transfected CHO cells, using [^{3}H]N/OFQ for the NOP receptor, [^{3}H]U69,593 for the KOP receptor, [^{3}H]DAMGO for the MOP receptor, and [^{3}H]Cl-DPDPE for the DOP receptor, as previously reported (Adapa and Toll, 1997; Zaveri et al., 2004).

The intrinsic activity of the compounds alone was determined by their ability to stimulate [35S]GTPγS binding to cell membranes and compared to the standard agonists N/OFQ (NOP), DAMGO (MOP), U69,593 (Koprich et al.), and DPDPE (DOP), as previously reported. (Adapa and Toll, 1997; Spagnolo et al., 2008; Zaveri et al., 2004)

Experimental design: part I

$NOP^{+/+}$ and $NOP^{-/-}$ mice (Experiment #1)

Mice were trained for a week to perform three different tests (bar, drag and rotarod test) and then treated with MPTP (20 mg/Kg, i.p., 4 administration, 90 min apart) or saline (Marti et al., 2005). Motor activity was assessed for 6 days after toxin administration. On day 7 animals were transcardially perfused with 4% PFA and brains removed for post-mortem analysis.

MPTP sub acute treatment (Experiment #2)

Naïve C57BL/6J mice were trained as already described, and then divided in two groups treated with either MPTP (25 mg/Kg, i.p.) or saline, once daily for 7 days (175 mg/Kg cumulative), according to a modified version of the subacute protocol described by Tatton&Kish (Tatton and Kish, 1997) (30 mg/Kg for 5 days; 150 mg/Kg cumulative). At the 4^{th} day each group was divided in two subgroups, treated with SB-612111 (10 mg/Kg, i.p.) or its vehicle twice daily for 10 days. This protocol was employed in order to mimic clinical conditions, i.e., pharmacological treatment starting after neurodegeneration onset (Bezard, 2003). SB-612111 was administered at 9:00 and at 18:00 hr, whereas MPTP, when coadministered, was given 5 hr after the first daily administration of SB-612111. Motor activity was evaluated before (baseline) and at days 1, 5 and 10 after toxin administration using the bar, drag and rotarod tests.

α-syn overexpression (Experiment #3)

Rats were bilaterally injected with AAV2/9-ha-syn in SNc as previously described (Bourdenx et al., 2015). Seven days after surgery rats were divided in two groups and treated twice daily for 8 weeks with SB-612111 (1 mg/Kg, s.c.) or its vehicle, again in order to have clinical relevance (Bezard, 2003). SB-612111 was administered at 9:00 and at 18:00 hr. Motor activity was monitored with the stepping test before (baseline) and once a week for 9 weeks after surgery.

Post-mortem processing

At the end of each experiment, animals were deeply anesthetized (ketamine 85mg/ml, or chloral 15% in saline 0.9%), and transcardially perfused with 0.9% NaCl and after with 4% paraformaldehyde in PBS (0.1M, pH 7.4). Brains were then removed, cryoprotected in 20% sucrose in PBS and then stored at -80°C.

Immunohistochemistry

Experiment #1. Coronal sections (40 μM) encompassing the whole SNc (AP from 3.16 to -3.52 from bregma) (Paxinos and Franklin, 2001) were cut using a cryostat (Leica Microsystems, Wetzlar, Germany), and then collected free floating for immunohistochemistry. Serum containing TH antibody (polyclonal rabbit primary TH antibody, J. Boy, Reims, France) was diluted 1:2000 in PBS containing 0.3% Triton x100 and 1% bovine serum albumin (BSA). Sections were incubated overnight at 4°C with primary antibody, then for 1 hr with biotinylated horse anti-rabbit antibody (universal secondary antibody, AbCys SA, Paris, France) diluted 1:200 in PBS containing 1% BSA and 0.3% Triton Å~100, and finally revealed with 3,3′-diaminobenzidine tetrahydrochloride (DAB kit, Vector Laboratories). Sections were then mounted on gelatine-coated slides, counterstained with cresyl violet, dried with ethanol and xylene and coverslipped with mounting medium.

Experiment #2. Coronal sections (35 μm) of striatum (AP from +1.42 to +0.14 from bregma) and SNc (AP from -3.16 to -3.52 from bregma) (Paxinos and Franklin, 2001) were cut with a cryostat and then collected free floating in five different series for immunohistochemistry. After being rinsed in TBS (Tris Buffered Saline), serial SNc sections were incubated for 1 hr with blocking solution

(Bovin Serum Albumin; 3% in TBS), then incubated overnight with TH primary antibody (Purified rabbit polyclonal antibody; 1:500 in 1% BSA in TBS; Merck Millipore, Darmstadt, Germany) and with a fluorescent marker of Nissl Bodies (Neurotrace; 1:150 in 1% BSA in TBS; Life Technologies, Grand Island, NY, USA). Sections were then incubated with a secondary antibody (AlexaFluor 488, Goat anti-rabbit IgG; 1:500 in TBS; Life Technologies) for 40 min, mounted on slides and coverslipped with mounting medium. Three representative striatal sections were incubated for 1 hr with blocking solution (Goat Serum 5% in TBS), then incubated overnight with TH primary antibody (Purified rabbit polyclonal antibody; 1:500 in 1% Goat Serum in TBS; Merck Millipore, Darmstadt, Germany). Finally, sections

were incubated with a secondary antibody (AlexaFluor 488, Goat anti-rabbit IgG; 1:500 in TBS; Life Technologies) for 40 min, mounted on slides and coverslipped with mounting medium.

Experiment #3. Coronal sections (50 µm) of rat striatum (AP from +2.28 to -1.08 from bregma) and SNc (AP from -4.36 to -6.72 from bregma) (Paxinos and Franklin, 2001) were obtained using a cryostat. Serial SNc sections were incubated for 1 hr with blocking solution, then overnight at 4°C with mouse monoclonal antibody raised against TH. TH staining was then revealed with a specific mouse EnVision™ System (mouse HRP EnVision™ kit DAB+ DAKO). Sections were then mounted on gelatine coated slides, counterstained with 0.1% cresyl violet, dried with xylene and coverslipped with mounting medium. In these brains, expression of hα-syn and p-hα-syn was also quantified in SNc. Selected sections were incubated overnight at room temperature with mouse monoclonal antibody against hα-syn or against p-α-syn (clone syn211 Thermo Scientific, 1:1000; mouse monoclonal [p-syn 81/A], Abcam, 1:1000), and then revealed with an anti-mouse peroxidase EnVision™ system (DAKO) followed by DAB substrate.

Stereological cell count in SNc

Experiment #1 and #3. To count TH-positive (TH+) neurons (phenotypic marker) and cresyl violet stained cells (structural marker) in SNc, an unbiased stereological sampling method (Larsen et al., 1998) (West and Gundersen, 1990), based on optical fractionator stereological probe was used, as previously described (Bezard, 2003; Fernagut et al., 2014; Gross et al., 2003). TH+ neurons were counted using a Leica DM6000B motorized microscope coupled with the Mercator Pro Software (Mercator Digital Imaging System, Explora Nova, La Rochelle, France). Five sections were used for each brain. For each animal, SNc boundaries were delimited at low magnification (2.5x) by examining the size and shape of the different groups of TH+ neurons and their axonal projections, as well as nearby fibre bundles (Paxinos and Franklin, 2001), and probes for stereology were applied. Any TH+ cell within the probe, or intersecting an acceptance line delimiting the probe (green line) that came into focus at 40x magnification was counted. The optical fractionator method was then used to estimate the total number of TH+ cells in the SNc of each animal. Mean estimated number of neurons and SEM were then calculated for each group.

Experiment #2. Images were taken with a fluorescence microscope (Leica

Microsystems, Wetzlar, Germany) equipped with a motorized Z and X-Y stage. Five serial SNc slices were used in order to analyze the entire SNc volume, and the area was identified with 10x magnification according to a mouse brain atlas (Paxinos and Franklin, 2001). The area was then divided into 3 rectangles (350 x 260 μm) and each rectangle was then amplified (40x). To count all TH+ positive neurons, 8 images of the same rectangle were taken on different levels of the Z axis. The images were then mounted and analyzed off-line using ImageJ (NIH, USA). Every neuron that appeared on focus was counted and the number obtained for each brain multiplied by five to approximate the total number of SNc TH+ neurons.

TH quantification in striatum

Experiments #2 and #3. Images were taken at 10x magnification with a fluorescence microscope (Experiment #2; see above) or at 20x magnification with NanoZoomer 2.0 HT (BIC facility, Bordeaux) (Experiment #3), and optical densitometry analyzed off-line as grey level with ImageJ using the cerebral cortex as background.

Quantification of hα-syn and p-α-syn expression

Images were obtained using the NanoZoomer 2.0 HT, and later analyzed with the Mercator Pro Software using a colour threshold method. SNc limits were delineated to obtain a representative surface of hα-syn and p-α-syn expression. Finally, the immunopositive surface as percentage of total SNc area was calculated, in order to compare the different treatments.

Experimental design: part II

Motor behaviour

Successfully hemilesioned rats were challenged to perform different motor behaviour tasks. The bar, drag and rotarod test were employed in a fixed sequence to test the effect of the different compounds on parkinsonian-like symptoms. Animals were randomized to test AT-403 at 0.03 and 0.1 mg/Kg, AT-390 at the doses of 0.3, 0.1 and 1 mg/Kg, AT-127 at the doses of 0.1 and 1 mg/Kg and motor behaviour was evaluated before, 20 or 30 minutes and 90 minutes after drug administration.

LID expression

Successfully hemilesioned rats were treated daily for 3 weeks with L-DOPA and their ALO AIMs score evaluated 5 times. Only animals that reached a score ≥100 were enrolled in the study. Animals were then randomized to test AT-403 on the expression of LID at 0.03 and 0.1 mg/Kg, AT-390 at the doses of 0.3, 0.1 and 1 mg/Kg, AT-127 at the doses of 0.1 and 1 mg/Kg. Moreover motor performance was measured using the rotarod test, both before ("off") L-DOPA and 60 minutes after drug administration, to evaluate whether the anti-dyskinetic effect correlated with an improvement in general motor activity

LID induction

Animals enrolled in the study were divided in two groups and treated daily for 3 weeks with L-DOPA in combination with a NOP agonist or saline. ALO AIMs score was evaluated throughout the study. At the end of the three weeks period animals were allowed a washout day and then tested once again only with L-DOPA (challenge) in order to verify whether the anti-dyskinetic effect was preventive (causal) rather than symptomatic. During the 3 weeks, motor activity was assessed with the rotarod test performed both off and on L-DOPA.

Data presentation and statistical analysis

Motor performance in the bar, drag and rotarod test was presented as percentage of baseline (calculated as time on bar or on rod in sec, number of steps) and analyzed by one-way ANOVA followed by the Newman-Keuls test or by two-way ANOVA followed by the Bonferroni Test (Fig. 10, 12, 17, 18, 23), whereas motor performance in the stepping test was presented as absolute data and analyzed by two-way ANOVA

followed by the Bonferroni test (Fig. 14). Data obtained from stereological counting were expressed as absolute values (number of cells) and analyzed by one-way ANOVA followed by the Newman-Keuls test (Fig. 11 and 13), or by the Student t-test when only two groups were compared (Fig. 15). Striatal TH density was expressed as absolute data (mean absolute value of grey scale between the two striata), and analyzed by one-way ANOVA followed by the Newman-Keuls test (Fig. 13), or by the Student t-test (Fig. 15). Density of α-syn aggregates was analyzed using the Student t-test (Fig. 16). The cumulative ALO AIMs score of AT-403 LID induction was expressed as absolute values and statistical analysis was performed with Student t-test (Fig. 20), whereas, the time-course of LID was expressed as absolute values (ALO AIMs score) and statistical analysis was performed with two-way ANOVA followed by the Bonferroni test (Fig. 19, 20, 22, 24, 25). On the contrary, cumulative ALO AIMs score in LID expression was expressed as percentage of the challenge with L-DOPA of the day before and statistical analysis was performed with one-way ANOVA followed by the Newman-Keuls test (Fig. 19, 22, 24). Rotarod performance of dyskinetic animals was expressed as percentage of the performance off L-DOPA (LID expression) or as absolute values in seconds (LID induction) and statistical analysis was performed with one-way ANOVA followed by the Newman-Keuls test (Fig. 19, 21, 26).

Substances

MPTP was purchased from Sigma Aldrich (St. Louis, MO, USA) and dissolved in 0.9% NaCl saline solution. SB-612111 used in Experiment #2, AT-403, AT-390 and AT-127 were synthetized by Dr Zaveri (Astraea Therapeutics, Mountain View, CA, USA), whereas SB-612111 used in Experiment #3 was purchased from Sequoia Research Product Ltd (Pangbourne, UK). SB-612111 was dissolved in 3% DMSO saline solution, AT-403 and AT-390 in 1% HCl 1M, 3% DMSO saline solution, whereas AT-127 was dissolved in 2% Tween 20, 2% DMSO saline solution.

Results

Part I

NOP$^{-/-}$ mice are partially resistant to MPTP-induced hypolocomotion and neurodegeneration

Effect of MPTP acute treatment on motor activity in NOP$^{+/+}$ and NOP$^{-/-}$ mice

As an initial screening to prove the role of endogenous N/OFQ and the NOP receptor in contributing to parkinsonian-like neurodegeneration, NOP$^{-/-}$ mice were acutely treated with MPTP, in parallel with NOP$^{+/+}$ controls. Basal immobility time in the bar test was similar between NOP$_{+/+}$ and NOP$_{-/-}$ mice (0.3±0.1 vs 0.1± 0.1 sec, respectively). Conversely, NOP$_{-/-}$ mice outperformed NOP$_{+/+}$ mice in the drag test (13.2±0.7 vs 10.8±0.2 steps, respectively; p=0.0012) and rotarod test (1926.6±50.6 vs 1517.6±54.6 sec, respectively; p<0.0001). MPTP treatment caused motor impairment in both genotypes, although NOP$_{-/-}$ mice were less affected (Fig. 10). ANOVA on the average performance of the last three testing days (i.e. when mice motor activity was stable), revealed a significant treatment effect on bar test (Fig. 10A; $F_{3,25}$=6.74, p=0.0017), drag test (Fig. 10B; $F_{3,25}$=8.24, p=0.0006), and rotarod test (Fig. 10C; $F_{3,25}$=5.50, p=0.0048) values. Post hoc analysis revealed that MPTP significantly enhanced immobility time in NOP$_{+/+}$ but not NOP$_{-/-}$ mice (Fig. 10A), and reduced stepping activity in both genotypes, although NOP$_{-/-}$ mice were less affected than NOP$_{+/+}$ mice (p<0.05; Fig. 10B). Conversely, MPTP equally reduced rotarod performance in both genotypes (Fig. 10C).

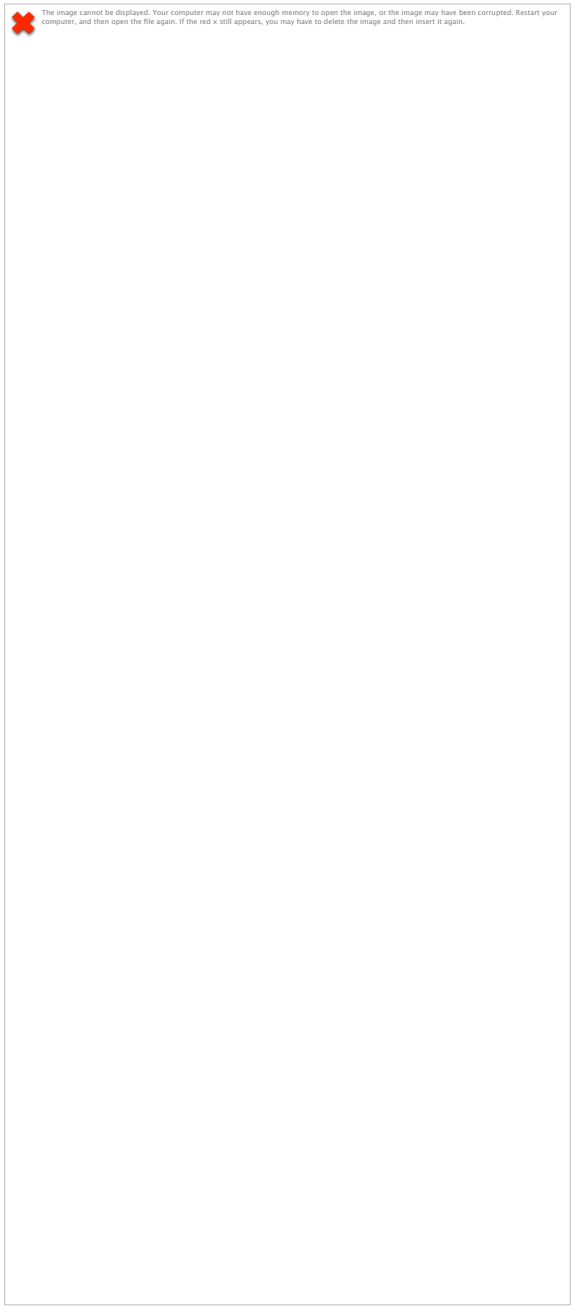

Figure 10. *NOP-/- mice are less susceptible than NOP+/+ mice to develop hypokinesia in response to MPTP. Motor activity was measured using the bar (A), drag (B), and rotarod (C) tests, before (day 0) and for up to 6 days after systemic administration of MPTP (20 X 4 mg/Kg, i.p.). Data are mean ± SEM of 6-9 mice per group. Statistical analysis was performed on the average performance of the last three days of observation (D4-D6) *p<0.05, **p<0.01 different from saline-treated mice of the same genotype; #p<0.05, different from MPTP-treated NOP-/- mice (one-way ANOVA followed by Newman-Keuls test for multiple comparisons).*

Effect of MPTP acute treatment on TH-positive neurons in NOP$^{+/+}$ and NOP$^{-/-}$ mice

To investigate the different impact of MPTP-induced neurotoxicity in NOP$^{+/+}$ and NOP$^{-/-}$ mice stereological count of TH-positive neurons in the SNc of the animals was performed. Stereology revealed that under control conditions (saline treatment) both NOP$_{-/-}$ and NOP$_{+/+}$ mice showed similar estimated numbers of tyrosine hydroxylase (TH) positive (TH+) neurons in SNc (Fig. 11). ANOVA showed that total number of cells significantly differed between genotypes and treatments ($F_{3,26}$=16.84, $p<0.0001$). Post hoc analysis revealed that MPTP caused a marked loss of TH+ cells in both genotypes. However, the number of TH+ cells surviving MPTP treatment was 50% higher in NOP$_{-/-}$ than NOP$_{+/+}$ mice ($p<0.01$; Fig. 2).

Figure 11. *NOP$^{-/-}$ mice are more resistant than NOP$^{+/+}$ mice to MPTP-induced dopamine cell loss. Representative microphotographs (left) of tyrosine hydroxylase (TH) positive neurons in substantia nigra compacta (SNc) of NOP+/+ and NOP-/- mice, at 7 days after saline or MPTP (20 X 4 mg/Kg, i.p.) administration. Stereological quantification of dopamine neurons in SNc (Wright et al.). Data are mean ± SEM of 6-9 mice per group. **p<0.01, different from saline-treated mice of the same genotype;##p<0.01, different from MPTP-treated NOP+/+ mice (one-way ANOVA followed by the Newman-Keuls test for multiple comparisons).*

Pharmacological blockade of the NOP receptor protects from MPTP-induced neurodegeneration

Effect of the pharmacological blockade of the NOP receptor on motor behavior in subacutely MPTP-treated mice

Data obtained in NOP$^{-/-}$ mice indicate that endogenous N/OFQ contributes to parkinsonian-like neurodegeneration via the NOP receptor, further suggesting that pharmacological blockade of the NOP receptor could provide some degree of neuroprotection/neurorescue to DA cells. To prove this concept, we subacutely treated mice with MPTP in combination with 10 mg/Kg SB-612111, a dose found effective in producing NOP-specific central effects in the mouse (Khroyan et al., 2009) (Rizzi et al., 2007). SB-612111 was administered in a clinically-driven experimental design (Bezard et al., 2013b), i.e. starting from the 4th day of MPTP administration. Motor behavior using the bar, the drag and the rotarod test was evaluated at day 1, 5 and 10 (Fig. 12) to monitor whether the MPTP protocol employed had a worsening effect on motor activity, and to possibly analyze whether animals treated with MPTP and SB-612111 showed improvements compared to animals treated with MPTP alone. Finally, no differences in motor performance were detected among groups before, during and after treatments. Subacute MPTP either alone or in the presence of SB-612111 did not affect motor behavior.

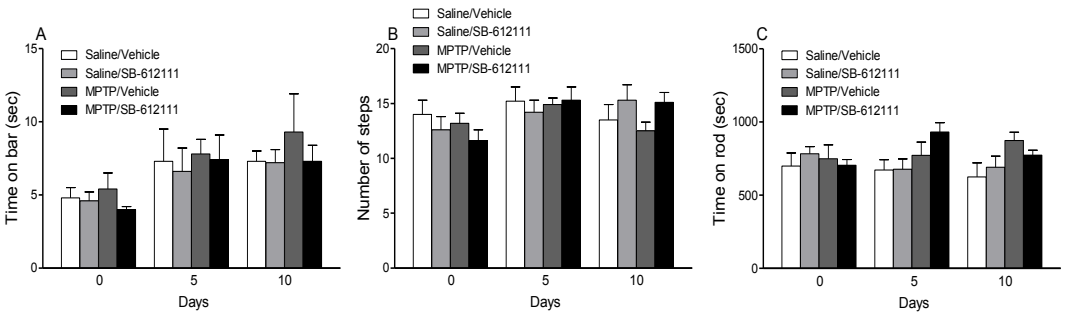

Figure 12. *Subacute MPTP administration does not cause motor changes in mice. MPTP (25 mg/kg, i.p.) or saline were administered once daily for 7 days. Four days after treatment onset, MPTP-treated and saline-treated mice were divided into two groups and administered either SB-612111 (10 mg/kg, twice daily, i.p.) or vehicle (twice daily) for 10 days. Motor activity was evaluated using the bar (**A**), drag (**B**) and rotarod (**C**) tests before (baseline, day 0) and at days 5 and 10 along drug treatment. Data are expressed as immobility time (sec; **A**), number of steps (**B**) and time on rod (sec, **C**), and are means ± SEM of n=7 mice per group. Statistical analysis was performed with RM ANOVA followed by the Bonferroni test for multiple comparisons.*

Effect of MPTP sub acute treatment on TH-positive neurons and DA striatal fibers intensity in NOP$^{+/+}$ and NOP$^{-/-}$ mice

Stereology revealed that mice treated with SB-612111/vehicle and saline/vehicle had a similar estimated number of TH+ neurons in SNc (Fig. 13). ANOVA showed that subacute MPTP treatment caused significant DA neuron loss when given alone (treatment: $F_{3,24}$=8.050; p=0.0007). However, delayed administration of SB-612111 prevented MPTP-induced dopaminergic neurodegeneration (Fig. 13A; p<0.05). Accordingly, MPTP induced a significant loss of TH staining in striatum (treatment: $F_{3,24}$=14.42; p<0.0001; Fig. 13B) and this effect was prevented by combined administration of SB-612111 (p<0.01).

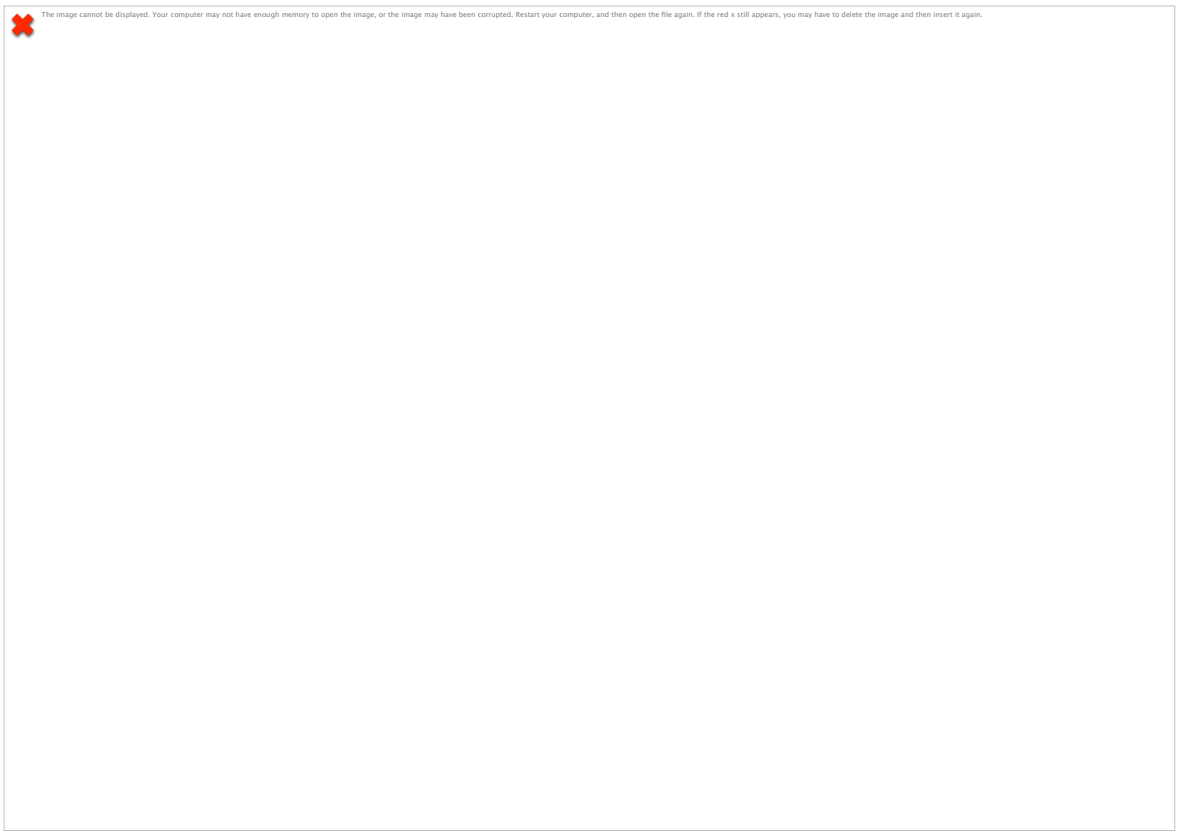

Figure 13. *SB-612111 attenuates subacute MPTP-induced neurotoxicity. Representative microphotographs (left) of tyrosine hydroxylase (TH) positive fibers in striatum. Stereological count of TH-positive neurons in substantia nigra compacta (A) and quantification of TH-positive terminals in striatum (B) of mice treated with MPTP (25 mg/kg, once daily for 7 days, i.p.) and/or SB-612111 (10 mg/kg, twice daily for 10 days, i.p.). Immunohistochemistry was performed 8 days after the last MPTP injection. Data are expressed as number of positive neurons (A) or mean grey level of TH striatal immunoreactivity (B), and are mean ± SEM of n=7 mice per group. p**<0.01 different from Saline/vehicle; #p<0.05, ##p<0.01 different from MPTP/vehicle (one-way ANOVA followed by the Newman-Keuls test for multiple comparisons).*

Pharmacological blockade of the NOP receptor protects from hα-syn induced neurodegeneration

Effect of pharmacological blockade of the NOP receptor on stepping activity after hα-syn nigral overexpression in rats.

The AAV2/9-hα-syn rat model was used to test the ability of the NOP antagonist SB-612111 to also counteract a-synuclein-induced degeneration, in order to confirm the neuroprotective potential of this class of compounds in PD. The stepping test revealed that rats chronically treated with SB-612111 displayed a better motor performance, i.e. a higher number of adjusting steps, over the course of the experiment compared to vehicle-treated animals (Fig. 14) (Bourdenx et al., 2015). ANOVA showed a significant effect of treatment ($F_{1,117}$=52.00, p<0.0001), time ($F_{8,117}$=20.68, p<0.0001) and time x treatment interaction ($F_{8,117}$=3.055, p=0.0037). Post hoc analysis revealed that SB-612111 significantly attenuated motor impairment from week 6 onward, the maximal difference between groups being observed at week 8 (6.9±0.3 vs 10.0±0.4 steps; p<0.01).

Figure 14. *SB-612111 attenuates motor deficits induced by AAV2/9 A53T human a-syn (ha-syn) injection in rats. SB-612111 (1 mg/Kg, s.c., twice daily) or its vehicle, were administered for 8 weeks starting one week after AAV2/9-ha-syn injection, and motor activity assessed using the stepping test once a week. Data are expressed as number of backhand steps and are mean ± SEM of n=7 (Vehicle) and n=8 (SB-612111) rats per group. ##p<0.01 (two-way ANOVA followed by Bonferroni test for multiple comparisons).*

Effect of pharmacological blockade of the NOP receptor from hα-syn induced neurodegeneration in rats

In line with behavioral data, the number of TH+ cells was about two times higher in the SNc of SB-612111-treated than in vehicle-treated rats ($t=3.66$, $df=13$, $p=0.0029$; Fig 15A), demonstrating strong neuroprotection in this etiologic model of PD. This effect was paralleled by a significantly higher mean grey level value in the striatum of SB-612111-treated rats ($t=5.61$, $df=13$, $p<0.001$; Fig 15B), suggesting a significant sparing of striatal DA terminals. For instance, the neuroprotection provided by SB-612111 was partial since in the group of control animals injected with AAV-GFP the number of nigral TH+ neurons was 12,614 ± 960 (n= 6).

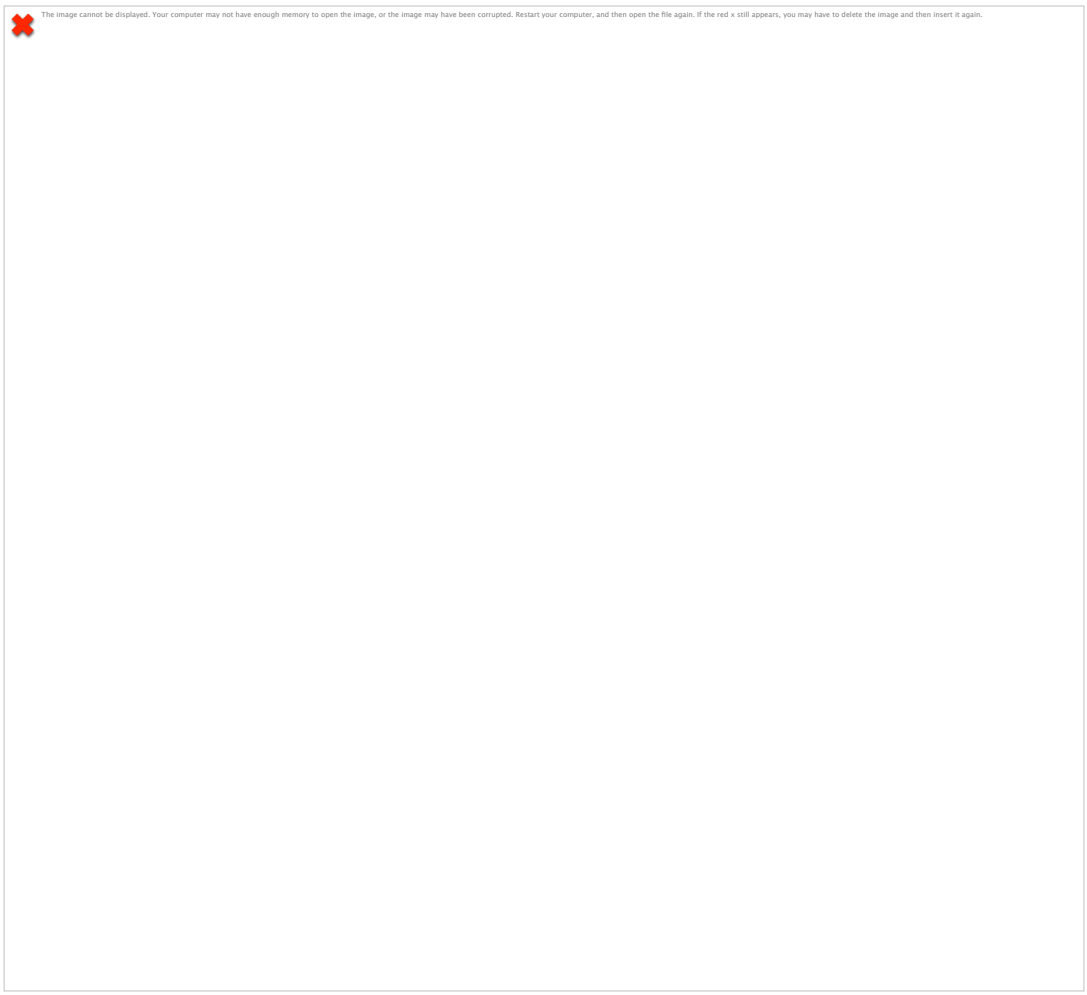

Figure 15. *SB-612111 attenuates neurodegeneration induced by AAV2/9 A53T human a-syn (ha- syn) injection in rats. Stereological count of TH-positive DA neurons in the substantia nigra compacta (A) and quantification of TH-positive terminals in the striatum (B) of rats injected with AAV2/9-ha-syn and treated with SB-612111 (1 mg/kg, twice daily for 8 weeks, s.c.) or vehicle. Data are expressed as number of TH-positive neurons (A) or mean grey level of TH immunoreactivity (B), and are mean ± SEM of n=7 (Vehicle) and n=8 (SB-612111) rats per group. ##p<0.01 (Student t-test).*

Effect of the pharmacological blockade of the NOP receptor on ha-syn and p-a-syn load in the SNc.

To check for the efficiency of AAV2/9 injections and to investigate whether SB-612111 interferes with a-syn aggregation, the expression patterns of ha-syn and Ser129 phosphorylated a-syn (p-a-syn) were finally evaluated in the two groups. SB-612111 treatment did not affect the load of ha-syn (Fig. 16A) or p-a-syn (Fig. 16B) in SNc.

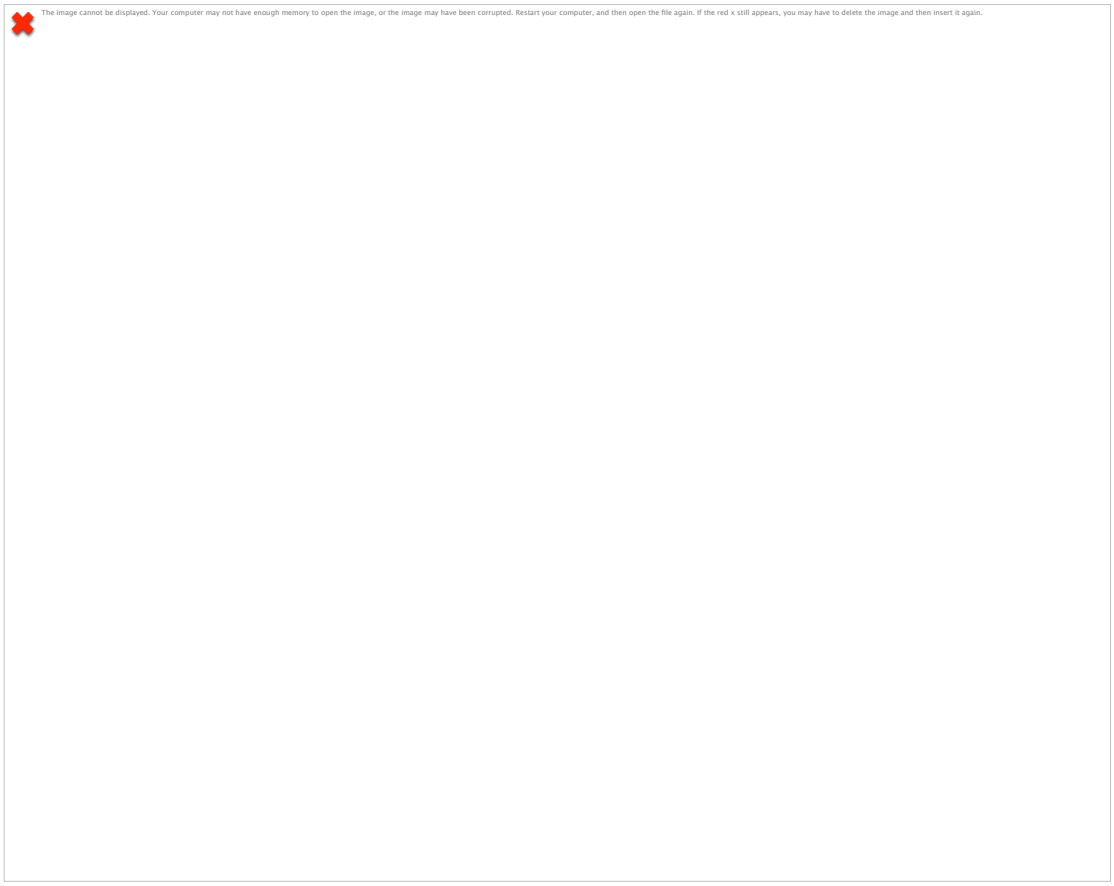

Figure 16. *SB-612111 does not change the load of ha-syn (A) and phospho-a-syn (p-a-syn) (B) in the SNc of AAV A53T human a-syn injected rats. Data are expressed as immunopositive surface percentage of total nigral surface, and are mean ± SEM of n=7 (Vehicle) and n=8 (SB-612111) rats per group.*

Part II

Receptor Binding

AT-403, AT-390 and AT-127 were tested for binding affinity at the four opioid receptors. AT-403, AT-390 and AT-127 showed a potent, nanomolar affinity for the NOP receptor (K_i of 1.13 nM, 0.90 nM and 1.18 nM respectively) and lower affinity for the other opioid receptors (Tab. 1). Moreover, the compounds were then tested for their intrinsic activity in the [35S]GTPγS binding assay up to 10 μM. In this assay, AT-403, AT-390 and AT-127 mimicked the stimulatory effects of N/OFQ at the NOP receptor with similar maximal effects and comparable potency (EC_{50} 6.3 nM, 15.20 nM, respectively and, vs 3.6 nM), indicating that both compounds are highly potent and selective NOP receptor agonists. On the other hand, AT-127 showed a high potency (EC_{50} 15.5 nM), comparable to AT-403 and AT-390, but showed a stimulatory effect of 61 ± 1.4% compared to N/OFQ at the NOP receptor, thus revealing its nature of a NOP receptor partial agonist.

	Binding Data K_i (nM)			
	ORL ± SEM	MU ± SEM	DELTA ± SEM	KAPPA ± SEM
Nociceptin	0.12 ± 0.01			
DAMGO		2.96 ± 0.54		
DPDPE			1.11 ± 0.07	
U69,593				1.05 ± 0.02
SCH221510	13.7 ± 2.30	65.42 ± 11.30	403.69 ± 109.7	49.68 ± 11.3
AT-390	0.9 ± 0.32	53.13 ± 16.5	113.15 ± 9.8	85.28 ± 19.8
AT-403	1.13 ± 0.13	97.94 ± 15.0	4074.3 ± 17.3	1563.74 ± 203.9
AT-127	1.18 ± 0.2	71.65 ± 27.8	47.21 ± 10.22	149.21 ± 18.26

[^{35}S]GTPgS FUNCTIONAL ASSAY								
	NOP		MOP		DOP		KOP	
Compound	EC_{50}	% Stim	EC_{50}	% Stim	EC_{50}	% Stim	EC_{50}	% Stim
Nociceptin	3.6 ± 0.7	100	-----	-----	-----	-----	-----	-----
DAMGO	----	-----	32.6 ± 4.1	100	-----	-----	-----	-----
DPDPE					8.98 ± 2.31	100	----	-----
U69,593					-----	-----	60.1 ± 7.5	100
SCH221510	18.9 ± 5.9	95.1 ± 7.8	139.3 ± 4.6	76.8 ± 13.1	-----	-----	142.2 ± 15.7	82.72 ± 0.2
AT-390	15.20 ± 0.4	110.1 ± 11.4	143.8 ± 0.6	54.3 ± 9.4	3847.0 ± 73	61.3 ± 6	534.3 ± 147.2	19.25 ± 1.3
AT-403	6.3 ± 1.42	104.6 ± 1.2	206.4 ± 79	33.5 ± 14.6	------	------	------	------
AT-127	15.5 ± 3.1	61.1 ± 1.4	59.2 ± 3.0	37.0 ± 2.0	573.8 ± 151.3	65.4 ± 13.0	FLAT	0

Table 1. *Receptor binding and [^{35}S]GTPgS functional activity. Experiments were performed as previously described (references in text).* Values shown represent average + deviation from the mean for two experiments conducted in triplicate. *Ki values were derived from the equation $K_i=IC_{50}/1+[L]/K_d)$ where [L] is the concentration of the radioligand.*

AT-403

Effect of AT-403 on motor behaviour

Previous studies showed that i.c.v. injection of N/OFQ has a biphasic effect on motor behaviour in naïve animals. To investigate whether a highly selective NOP receptor agonist could affect parkinsonian-like symptoms, AT-403 was administered systemically at 0.03 and 0.1 mg/Kg (s.c.) and motor activity evaluated by the bar, drag and rotarod tests 30 and 90 minutes after injection.

In the bar test (Fig. 17A), two-way ANOVA showed a significant effect of treatment ($F_{2,36}$=46.37; p<0.0001), time ($F_{1,36}$=6.964; p=0.0122) and time x treatment interaction ($F_{2,36}$=4.967; p=0.0125) at the contralateral paw. Post hoc analysis revealed that AT-403 significantly reduced the time on bar at the contralateral paw at both time points. Converserly, ANOVA showed a significant effect of treatment ($F_{2,36}$=7.308; p<0.0022), but no effect of time or time x treatment interaction (time: $F_{1,36}$=0.4381, p=0.5122; interaction: $F_{2,36}$=2.291, p=0.1157) at the in the ipsilateral paw. Post hoc analysis revealed that AT-403 0.03 mg/Kg significantly reduced the time on bar at the ipsilateral paw 30 minutes after compound administration.

In the drag test, two-way ANOVA showed an overall lack of effect both at the contralateral paw (treatment $F_{2,36}$=1.165, p=0.3233; time $F_{1,36}$=0.4474, p=0.5078; time x treatment interaction $F_{2,36}$=0.6944, p=0.5059) and at the ipsilateral paw (treatment $F_{2,36}$=0.1592, p=0.8534; time $F_{1,36}$=0.008243, p=0.9282; time x treatment interaction $F_{2,36}$=1.506, p=0.2355) (Fig. 17B). Nonetheless, a trend for an increase in stepping activity at the contralateral paw was clearly seen at both doses 90 minutes after administration.

In the rotarod test, two-way ANOVA showed an overall effect of AT-403 treatment (Fig. 17C) ($F_{2,36}$=4.955; p=0.0126). However no effect of time ($F_{1,36}$=3.808; p=0.0588), or time x treatment interaction ($F_{2,36}$=2.237; p=0.1214) was detected. Post hoc analysis at 30 minutes revealed a significant decrease with AT-403 0.1 mg/Kg (~28%). This effect did not persist at 90 minutes.

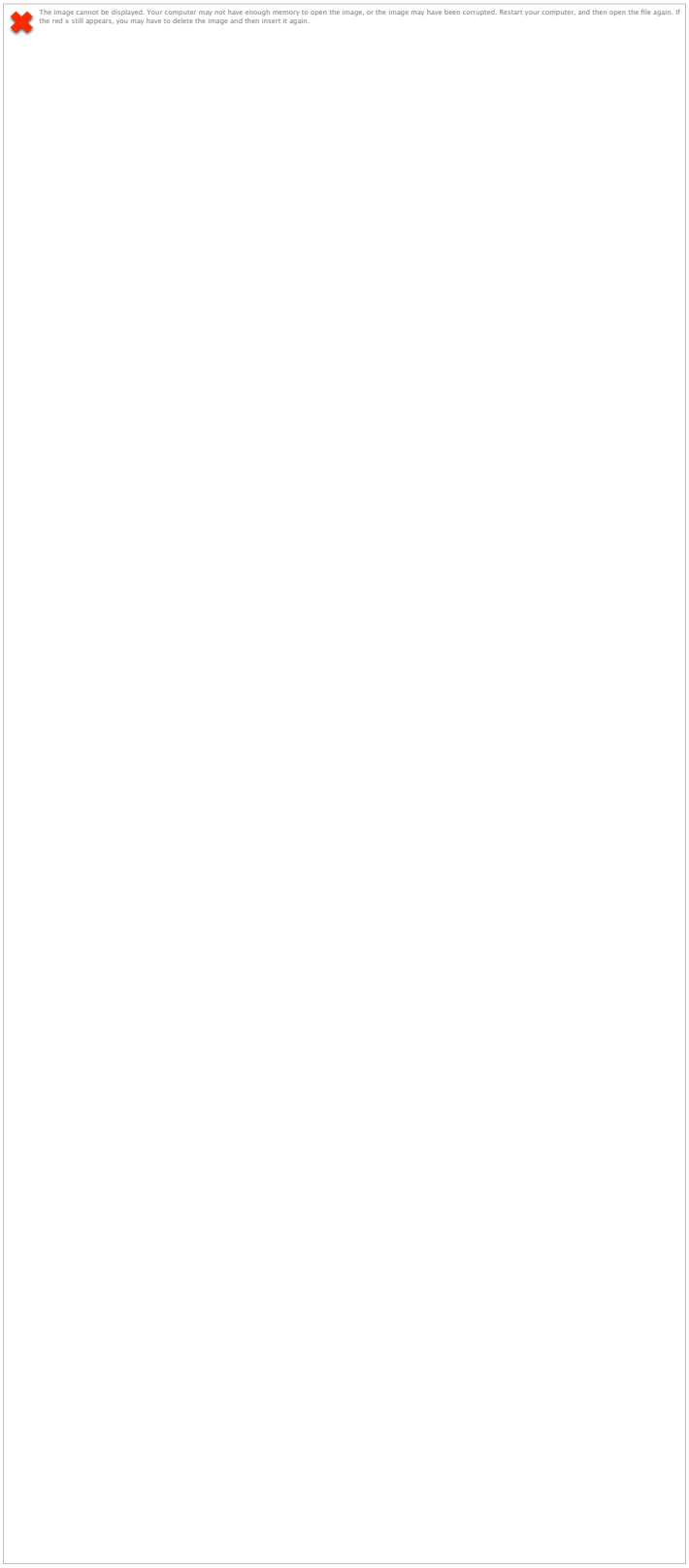

Figure 17. *Effect of systemic administration of AT-403 (0.03 and 0.1 mg/Kg; s.c.) in the bar (A), drag (B) and rotarod (C) tests. Data are expressed as percentages of basal motor activity in the control session and are means ± SEM of n=7 determinations per group. *p<0.05, **p<0.01, significantly different from vehicle. Statistical analysis was performed by RM ANOVA followed by Newman-Keuls test for multiple comparisons.*

Effect of AT-403 and SB-6121111 on motor behaviour

To investigate the selectivity of AT-403, the effects of systemic administration of AT-403 (0.01 mg/Kg, s.c.) in combination with SB-612111 (0.01 mg/Kg, s.c.) were evaluated in the bar, drag and rotarod test. ANOVA showed a significant effect of treatment ($F_{3,26}$=5.084; p=0.0067) in the bar test at the contralateral paw (Fig. 18A), but no significant effect in the drag (Fig. 18B) and rotarod test (Fig. 18C). Post hoc analysis revealed that AT-403 reduced the time on bar by ~40%. SB-612111 was ineffective alone but counteracted the effect of AT-403. No effect was observed at 90 min after administration in in any of the groups tested.

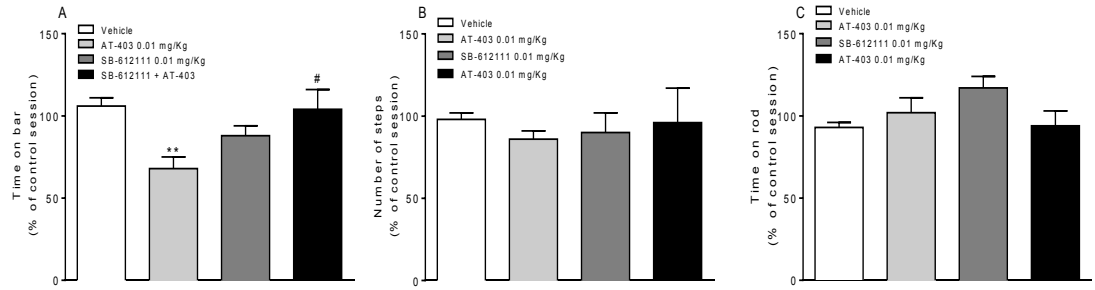

Figure 18. *Effect of systemic administration of AT-403 (0.01 mg/Kg, s.c.), SB-612111 (0.01 mg/Kg, s.c.) and their combination in the bar (A), drag (B) and rotarod test (C). Data are expressed as percentage of basal motor activity in the control session and are means ± SEM of n=7 (vehicle), n=8 (AT-403 0.01 mg/Kg), n=8 (SB-612111 0.01 mg/Kg) and n=7 (AT-403 + SB-612111) determinations. Statistical analysis was performed by one-way ANOVA followed by Newman-Keuls Test.*

Effect of AT-403 on the expression of LID.

ANOVA showed that AT-403, given 15 minutes before L-DOPA, significantly attenuated LID (treatment: $F_{2,44}$=29.88; $p<0.0001$) (Fig.19). The effect was mild at 0.03 mg/kg (87±3% compared to the challenge with L-DOPA performed the day before) and stronger at 0.1 mg/Kg ($p<0.01$), that caused LID regression to 57±5% of the effect of L-DOPA alone (Fig. 19A). At this dose, AIMs appearance was delayed for about 40 min, so it was AIMs peak, which occurred at 100 min (Fig. 19B). However, at 0.1 mg/Kg the beneficial effect was accompanied by sedation and hypolocomotion within the first hour after compound administration. This effect was not detected at the lower (0.03 mg/Kg) dose.

Rotarod activity was monitored 60 minutes after L-DOPA administration (Fig 19C). As expected, animals treated with L-DOPA alone suffered a strong motor impairment due to dyskinesia, and their performance was 24±6% of baseline (OFF L-DOPA). Animals treated with AT-403 (0.03 mg/Kg) performed significantly better on the rotarod (54±16% of their baseline) than controls. Conversely, rats treated with AT-403 (0.01 mg/Kg) showed dramatically impaired performance, with a tendency to perform worse than controls (8±2% of baseline).

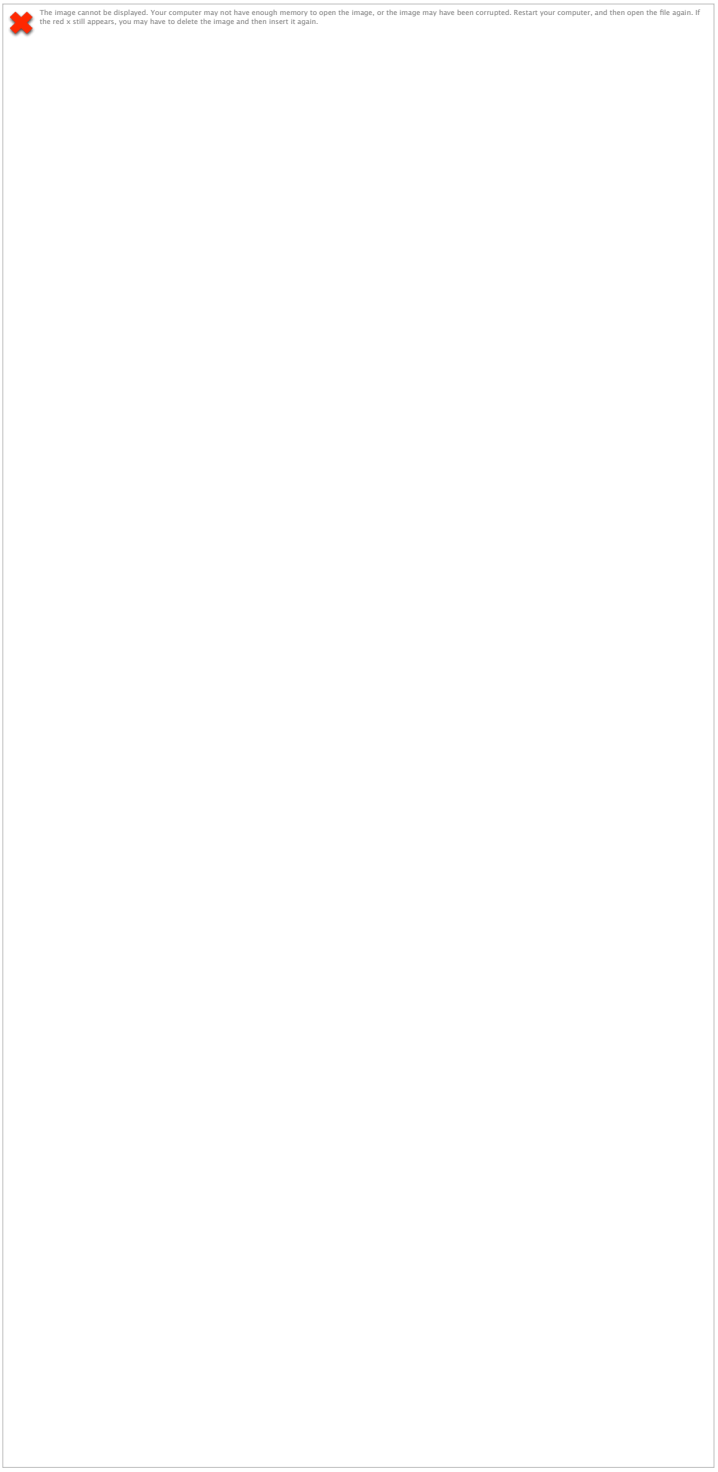

Figure 19. *AT-403 (0.03 and 0.1 mg/Kg, s.c.) attenuates L-DOPA-induced dyskinesia in rats restoring motor functions at 0.03 mg/Kg. Data are expressed as percentage of the L-DOPA challenge performed the day before (A), as sum of the axial, limb and orolingual AIMs score, i.e. ALO AIMs score (B), or as percentage of the basal performance OFF L-DOPA (C) and are means ± SEM of n=15-16 (A-B) or n=10 (C) determinations per group. *p<0.5, **p<0.01, significantly different from vehicle. Statistical analysis was performed by one-way ANOVA followed by Newman-Keuls test for multiple comparisons (A, C) and by two-way ANOVA followed by Bonferroni test for multiple comparisons (B).*

Effect of AT-403 on the induction of L-DOPA-induced dyskinesia

Animals treated with L-DOPA developed severe dyskinesia within the first week of treatment, and their score persisted throughout the entire experiment. Pharmacological treatment with AT-403 0.03 mg/Kg provided only mild, non significant attenuation of LID. Indeed, animals treated with AT-403 0.03 mg/Kg scored an overall 78±21% compared to the animals treated with L-DOPA alone Fig. 20A). Moreover, two-way ANOVA over the 20-day treatment period (treatment $F_{1,96}$=2,677, p=0.1051; time $F_{5,96}$=3.98, p=0.0025, time x treatment interaction $F_{5,96}$=0.03341, p=0.9994; Fig. 20B). Furthermore, the evaluation of the ALO AIMs time-course during the 20-days priming period revealed that AT-403 0.03 mg/Kg attenuated LID appearance only at the first day of administration (treatment $F_{1,144}$=12.09, p=0.0007; time $F_{8,144}$=4.788, p<0.0001; time x treatment $F_{8,144}$=1.367, p=0.2159; Fig. 20 C-E)).

Rotarod activity was evaluated ON L-DOPA at days 2, 8, 14 and 19 (Fig 21). As expected, L-DOPA treatment significantly reduced rotarod performance at day 2 ($F_{2,24}$=4.562; p=0.0209), day 8 ($F_{2,24}$=4.482; p=0.0222), day 14 ($F_{2,24}$=10.74; p=0.0005) and day 19 ($F_{2,24}$=4.393; p=0.0237). On the contrary, animals treated with AT-403 0.03 mg/Kg did not show a significant impairment in rotarod performance in all experimental sessions.

Figure 20. *Chronic treatment with AT-403 0.03 mg/Kg mildly attenuated LID. The degree of dyskinesia was evaluated with the ALO AIMs score (A-B). The attenuation was visible from day 1 (C) until the last day of treatment (D), and the challenge with L-DOPA (E). Data are expressed as absolute values as sum of the axial, limb and orolingual AIMs score, i.e. ALO AIMs score and represents means ± SEM of n=9 rats per group. p*<0.05, p**<0.01, significantly different from vehicle. Statistical analysis was performed by Student T test (A) and by two-way ANOVA followed by Bonferroni test for multiple comparisons (B-E).*

Figure 21. *Chronic treatment with AT-403 0.03 mg/Kg did not compromise motor activity ON L-DOPA in the rotarod test. Motor performance was evaluated before, 60 and 120 minutes after L-DOPA administration. Data are expressed as absolute values as time on the rod (in sec)and represent means ± SEM of n=9 rats per group. p*<0.05, p**<0.01, significantly different from vehicle. Statistical analysis was performed by one-way ANOVA followed by Newman-Keuls test.*

AT-390

Effect of AT-390 on the expression of L-DOPA-induced dyskinesia

AT-390, given 15 minutes before L-DOPA, was administered to fully dyskinetic rats to test the effect on the expression of LID. The AIMs time-course in the presence of AT-390 0.3 mg/Kg was superimposable for intensity and duration with that of controls. Conversely, AT-390 1 mg/Kg caused a dose-dependent delay in AIMs appearance; AIMs peaked at 120 min but were much prolonged with respect to controls, disappearing at 260 min after L-DOPA administration. A similar time-course, with an even more pronounced rightward shift in the time scale, was observed with the 3mg/Kg dose. AIMs quantification over all the time course revealed that AT-390 did not attenuate LID at any tested doses (treatment: $F_{3,23}$=1.362; p=0.2792) (Fig 22A). We should also report that in the 1-3 mg/Kg dose-range, AT-390 caused an initial sedative and hypolocomotive effect that lasted for 40 minutes (1 mg/Kg) and 100 minutes (3 mg/Kg; Fig 22B).

The rotarod test performed 60 and 120 minutes after L-DOPA administration revealed that rats treated with AT-390 (0.03 and 1 mg/Kg) performed similar to controls, i.e. significantly worse compared to their basal performance. Rats treated AT-390 (3 mg/Kg) were not tested on the rotarod due to prolonged sedation and hypolocomotion.

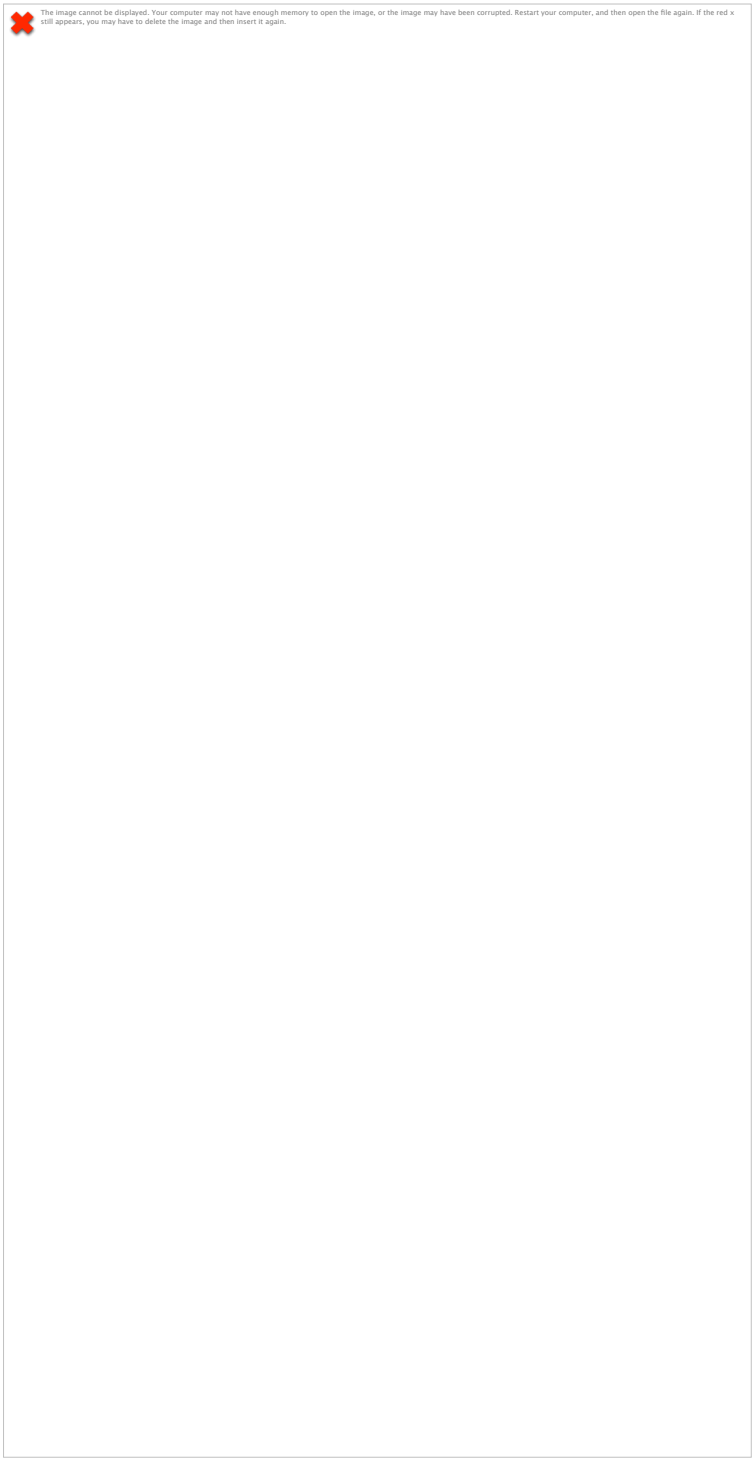

Figure 22. *AT-390 failed to attenuate the expression of L-DOPA-induced dyskinesia in rats. Data are expressed as percentage of the L-DOPA challenge performed the day before (A), as absolute values, as sum of the axial, limb and orolingual AIMs score, i.e. ALO AIMs score (B), or as percentage of the basal performance OFF L-DOPA (C) and are means ± SEM of n=8 (vehicle), n=7 (AT-390 0.3 mg/Kg, s.c.), n=7 (AT-390 1 mg/Kg, s.c.) and n=4 (AT-390 3 mg/Kg, s.c.) determinations per group. *p<0.5, **p<0.01. Statistical analysis was performed by one-way ANOVA followed by Newman-Keuls test for multiple comparisons (A, C) and by two-way ANOVA followed by Bonferroni test for multiple comparisons (B).*

AT-127

Effect of AT-127 on motor behaviour

The NOP receptor partial agonist AT-127 was administered systemically (i.p.) and motor activity evaluated by the bar, drag and rotarod tests 20 and 90 minutes after injection.

In the bar test (Fig. 23A), two-way ANOVA showed a significant effect of treatment ($F_{2,50}$=8.766; p=0.0005), but not time ($F_{1,50}$=3.470; p=0.0684) or time x treatment interaction ($F_{2,50}$=0.6953; p=0.5037) at the ipsilateral paw. Post hoc analysis revealed that AT-127 1 mg/Kg significantly reduced the time on bar at the ipsilateral paw 30 minutes after compound administration (p<0.01).

Similarly, two-way ANOVA showed a significant effect of treatment ($F_{2,50}$=8.792; p=0.0005), but not time ($F_{1,50}$=0.05626; p=0.8135) or time x treatment interaction ($F_{2,50}$=1.034; p=0.3632) at the contralateral paw. Post hoc analysis revealed that AT-127 1 mg/Kg significantly reduced the time on bar at the contralateral paw 30 minutes after compound administration (p<0.01).

Two-way ANOVA showed a lack of effect at the ipsilateral paw when performing the drag test (Fig. 23B) (treatment: $F_{2,50}$=3.181, p=0.0501; time: $F_{1,50}$=0.2460, p=0.6221; interaction: $F_{2,50}$=0.5989, p=0.05533). On the contrary, two-way ANOVA showed a significant effect of treatment ($F_{2,50}$=4.647; p=0.0141), but not time ($F_{1,50}$=0.9131; p=0.3439), or time x treatment interaction ($F_{2,50}$=0.7193; p=0.7193) at the contralateral paw. Post hoc analysis revealed that AT-127 1 mg/Kg significantly increased the number of steps at the contralateral paw 30 minutes after compound administration (p<0.01).

Two-way ANOVA showed no effect of AT-127 on the time spent on the rotarod (Fig. 23C) (treatment: $F_{2,50}$=1.254, p=0.2942; time: $F_{1,50}$=0.03430, p=0.8538; interaction: $F_{2,50}$=0.08885, p=0.9151).

AT-127 caused a dose-dependent sedation and hypolocomotion that lasted for 20 minutes at the highest dose tested. However, the detected sedation was quite mild, and animals were easily woken up by an external stimulus (i.e., sound or manipulation).

Figure 23. *Effect of systemic administration of AT-127 (0.1, 1 mg/Kg; i.p.) in the bar (A), drag (B) and rotarod (C) tests. Data are expressed as percentages of basal motor activity in the control session and are means ± SEM of n=10 (vehicle), n=7 (AT-127 0.1 mg/Kg) and n=11 (AT-127 1 mg/Kg) determinations per group. *p<0.05, **p<0.01, significantly different from vehicle. Statistical analysis was performed by two-way ANOVA followed by Bonferroni test for multiple comparisons.*

Effect of AT-127 on the expression of L-DOPA-induced dyskinesia

The effect of the NOP receptor partial agonist AT-127 on the expression of LID was tested. AT-127, administered simultaneously with L-DOPA, failed to attenuate the ALO AIMs score at both doses tested, i.e. 0.1 and 1 mg/Kg (Fig 24A). One-way ANOVA showed no effect of AT-127 treatment (treatment: $F_{2,17}$=0.5033; p=0.9577). Regardless of treatment, dyskinetic behaviours extinguished 180 minutes after L-DOPA administration.

Figure 24. *AT-127 failed to attenuate the expression of L-DOPA-induced dyskinesia in rats. Data are expressed as absolute values, as sum of the axial, limb and orolingual AIMs score, i.e. ALO AIMs score (A), or as percentage of the L-DOPA challenge performed the day before (B) and are means ± SEM of n=8 (vehicle), n=6 (AT-127 0.1 and 1 mg/Kg, i.p) determinations per group. Statistical analysis was performed with one-way ANOVA followed by Newman-Keuls test (A) and by two-way ANOVA followed by Bonferroni test (B).*

Effect of AT-127 on the induction of LID

Despite the negative results obtained on the expression of LID, we decided to test the efficacy of AT-127 1 mg/Kg on the development of dyskinesia throughout the 21 day-L-DOPA-priming period.

Chronic treatment with AT-127 1 mg/Kg did not affect LID development (Fig 25A). Two-way ANOVA showed a negative effect of time (time: $F_{7,112}$=4.893; p<0.0001), no significant effect of treatment (treatment: $F_{1,112}$=2,112; p=0.1480) and no interaction time x treatment (interaction: $F_{7,112}$=0.3035; p=0.9510). Actually, we observed a detrimental effect of AT-127 1 mg/Kg on AIMs appearance at the end of chronic treatment, particularly at day 21 (treatment $F_{1,126}$=2.929, p=0.0894; time $F_{8,126}$=6.186, p>0.0001; time x treatment interaction $F_{8,126}$=4.405, p=0.0001; Fig 25B) and in the challenge session (Fig 25C), and when the challenge with L-DOPA was performed, a negative effect of treatment was detected (treatment: $F_{1,126}$=17.91; p<0.0001), as well as a negative effect of time (time: $F_{8,126}$=12.78; p<0.0001), but no time x treatment interaction (interaction: $F_{8,126}$=0.6514; p=0.7331) (Fig 25D).

Rotarod performance was evaluated at days 3, 9 and 16 along chronic treatment. (Fig 26). As expected, rats chronically treated with AT-127 1 mg/Kg showed a poor rotarod performance, similar to controls.

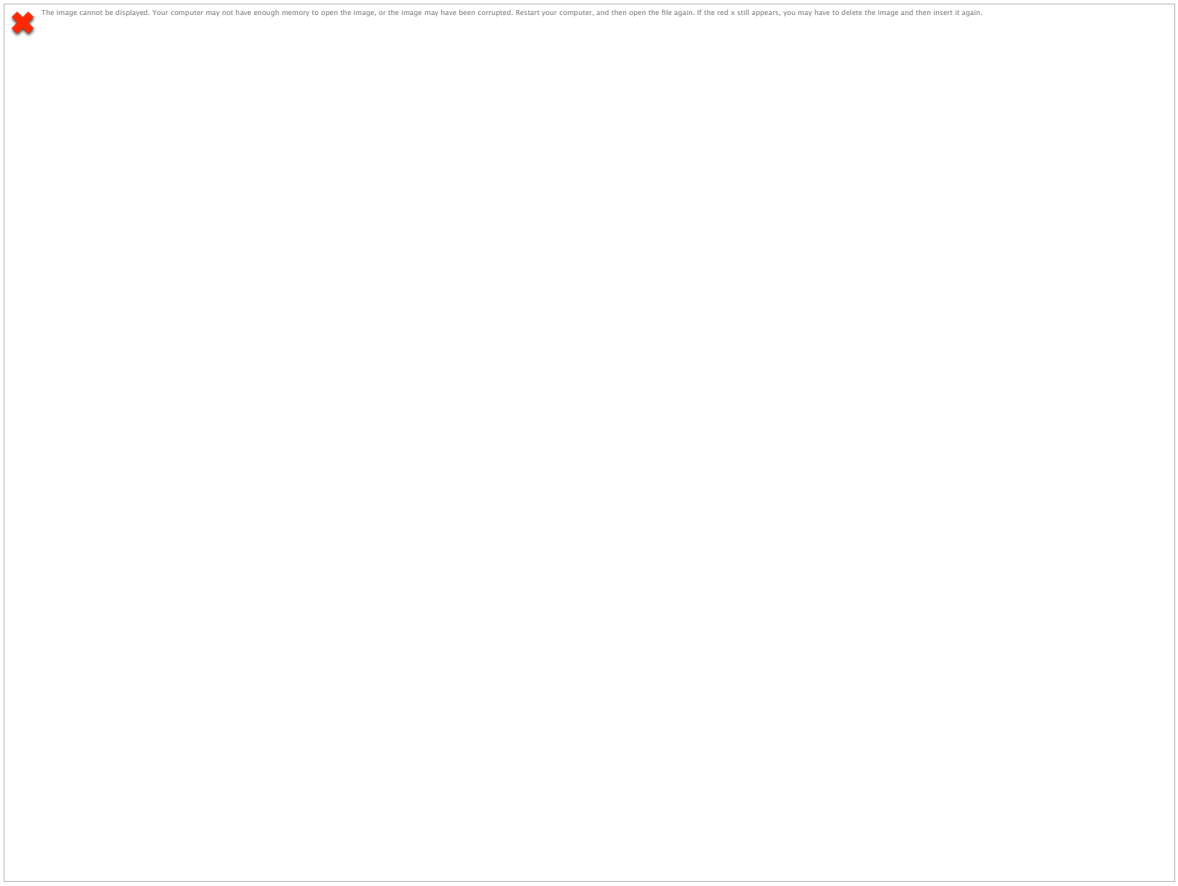

Figure 25. *Chronic treatment with AT-127 1 mg/Kg failed to attenuate LID and motor deficits on L-DOPA. The degree of dyskinesia was evaluated with the ALO AIMs score (A). a mild attenuation in the ALO AIMs score was visible at day 1 (B), when animals were not fully dyskinetic, but disappeared once maximal dyskinesia was reached until the last day of treatment (C) and the challenge with L-DOPA (D). Data are expressed as absolute values as sum of the axial, limb and orolingual AIMs score, i.e. ALO AIMs score and represents means ± SEM of n=9 rats per group. p*<0.05, p**<0.01, significantly different from vehicle. Statistical analysis was performed by two-way ANOVA followed by Bonferroni test for multiple comparisons.*

Figure 26. *Chronic treatment with AT-127 1 mg/Kg did not affect motor activity ON L-DOPA in the rotarod test. Motor performance was evaluated before, 60 and 120 minutes after L-DOPA administration. Data are expressed as absolute values as time on the rod (in sec)and represent means ± SEM of n=9 rats per group. p*<0.05, p**<0.01, significantly different from vehicle. Statistical analysis was performed by one-way ANOVA followed by Newman-Keuls test.*

Discussion

Part I

Genetic deletion and pharmacological blockade of the NOP receptor protected mice from acute and subacute MPTP intoxication. Pharmacological blockade of the NOP receptor provided protection also in a more progressive and recently validated rat model of PD, where DA neurons death is accomplished by nigral overexpression of human A53T α-synuclein.

These data prove that endogenous N/OFQ contributes to neurodegeneration associated with experimental parkinsonism. They extend previous findings that pharmacological blockade of the NOP receptor relieves parkinsonian-like hypokinesia (Marti et al., 2005; Marti et al., 2010; Marti et al., 2007; Viaro et al., 2008; Volta et al., 2010b), and provide strong support to the notion that endogenous N/OFQ plays a key role in the pathogenesis of PD. This study was inspired by previous findings that genetic deletion of the precursor of N/OFQ, ppN/OFQ, provides protection from acute MPTP in mice (Brown et al., 2006; Marti et al., 2005). Since two other peptides are generated by the cleavage of ppN/OFQ, we first aimed at replicating these data using $NOP^{-/-}$ mice, to prove that N/OFQ was involved. MPTP is still considered a reference model to study the mechanisms underlying neurodegeneration in PD, as well as for testing potentially neuroprotective compounds. Indeed, it replicates some of the cardinal features of the disease, such as degeneration of nigral DA neurons, and the hypolocomotion that comes along with it (Jackson-Lewis and Przedborski, 2007; Meredith and Rademacher, 2011; Przedborski et al., 2001). Nevertheless, the mechanism of action and therefore the pathways activated by MPTP, vary depending on the protocol used in the investigation. In fact, in the acute protocol, when MPTP is given at 25 mg/Kg, 4 injections every 90 minutes, DA cells die rapidly within the first 4 days (Jackson-Lewis and Przedborski, 2007; Sundstrom et al., 1988), through a non-apoptotic, possibly a microglia-mediated inflammatory, mechanism (Jackson-Lewis and Przedborski, 2007) (Furuya et al., 2004; Liberatore et al., 1999). On the contrary, in the subacute protocol, MPTP is administered at 25 mg/Kg once daily for 7 days, cell death is delayed, and both apoptosis and astrocyte activation are clearly occurring (Serra et al., 2002; Tatton and Kish, 1997). Consistent with previous reports, we detected a ≈70% loss of striatal DA terminals after acute MPTP and a ≈30% loss of striatal DA terminals after subacute MPTP (Jackson-Lewis and

Przedborski, 2007; Thomas et al., 2007). Genetic deletion of the NOP receptor provided only partial neuroprotection from acute MPTP, whereas pharmacological NOP receptor blockade with SB-612111 fully protected from subacute MPTP. These results suggest that the pharmacological blockade of the NOP receptor might prove more effective when the degeneration is slower and more progressive. It could be argued that MPTP metabolism and uptake in $NOP^{-/-}$ was not measured, nor it was evaluated whether SB-612111 could alter these parameters (Jackson-Lewis and Przedborski, 2007). However, the possibility that the neuroprotection was achieved because of a reduced MPTP metabolism is quite unlikely. In fact, MPTP metabolism is unaltered in $ppN/OFQ^{-/-}$ mice (Marti et al., 2005), and neuroprotection was also observed in a model where MPTP was not employed. It therefore appears that endogenous N/OFQ, acting through the NOP receptor, mediates the events that directly or indirectly lead to neurodegeneration.

Research performed in Morari lab already reported that exogenous N/OFQ administered in the SNr of awake rats increased GLU release (Marti et al., 2002) and that, conversely, NOP receptor antagonists reduce GLU release in the SNr of 6-OHDA rats (Marti et al., 2005; Marti et al., 2004a; Marti et al., 2008; Marti et al., 2007; Volta et al., 2011), and MPTP-treated mice (Mabrouk et al., 2010). Therefore, it can be hypothesized that N/OFQ causes DA neuron death through excitotoxic mechanisms (Brown et al., 2006; Marti et al., 2005). For instance, changes in mitochondrial potential caused by the inhibition of complex I by MPP^{+}, the active metabolite of MPTP, lead to oxidative stress and glutamate-related excitotoxicity (Meredith and Rademacher, 2011; Serra et al., 2002). Moreover, N/OFQ has been shown to exacerbate ibotenate-induced excitotoxic lesions of the periventricular murine white matter in vivo, without affecting the loss of grey matter (Laudenbach et al., 2001), whereas NOP receptor antagonists prevented it. These data indicate that endogenous and exogenous N/OFQ regulate microglia activation, a phenomenon that underlies astrocyte cell death and white matter lesion, rather than potentiating a direct NMDA receptor-mediated toxic insult on cortical neurons (Laudenbach et al., 2001). Since that study, numerous lines of evidence implicated N/OFQ in neuroinflammation and microglial responses, although both anti- and pro-inflammatory effects of N/OFQ, at the peripheral and central level, have been reported (Mallimo and Kusnecov, 2013). Another possible route of neurotoxicity for N/OFQ has recently emerged; indeed, N/OFQ has been shown to exert a direct detrimental effect on DA

neurons, since it inhibits the vitality and growth of DA neurons in cultures, through the NOP receptor-mediated activation of p38-MAPK pathway (Collins et al., 2015).
After we obtained preliminary but promising data in the MPTP model we decided to move further. Indeed, the MPTP model does not fully recapitulate the neurodegeneration associated with PD in humans, because mice treated with the neurotoxin do not present Lewy Bodies, and the neurodegenerative process itself is fast and abrupt, directly targeting cell bodies (Jackson-Lewis and Przedborski, 2007; Meredith and Rademacher, 2011). This pattern does not correlate well with the pattern of neurodegeneration occurring in PD patients, i.e., a slow and progressive process first detected at striatal axon terminals (Burke and O'Malley, 2013; Kordower et al., 2013). Therefore, we employed an experimental model where human A53T α-synuclein is overexpressed in the rat SN. Researchers have reported that this model provides a progressive 65-70% neurodegeneration over the course of 8 weeks (Bourdenx et al., 2015), which is indeed in line with what we found. SB-612111, given chronically twice a day in a clinically driven protocol, spared a significant amount of DA neurons in the SNc and DA fibers in the striatum. Indeed, although providing only partial neuroprotection, SB-612111 increased by about 50% the numbers of neurons surviving A53T α-synuclein overexpression. This finding becomes even more relevant when we consider that SB-612111 treatment started a week after the virus injection. i.e., when neurodegeneration had already affected 50% of nigral DA neurons (Bourdenx et al., 2015). Moreover, SB-612111 treatment resulted to be physiologically relevant, since it also improved the motor performance of the animals. Finally, it is noteworthy to mention that very few strategies have proven effective using a clinically-driven protocol in this model (Bezard et al., 2013b), and we now provide evidence of efficacy in two different models. Regarding the mechanism of action of SB-612111 in AAV2/9-hα-syn injected rats, SB-612111 does not appear to target α-syn in a direct manner, i.e. α-syn clearance or aggregation, since no differences in hα-syn and p-α-syn load in SNc were found between SB-612111-treated and untreated animals. Therefore, the mechanism underlying neuroprotection could lie downstream of α-syn aggregation, and affect other aspects of its toxicity, such as inflammation and a consequent activation of the immune response, or mitochondrial damage (Ulusoy et al., 2010; Yamada et al., 2004).

Part II

In the second part of the study we employed two small and selective NOP receptor full agonists, AT-403 and AT-390, and a small molecule partial agonist AT-127, to further investigate the role of N/OFQ in parkinsonian-like motor deficits and LID. The rational for testing a partial agonist in PD model was inspired by various studies showing that ppN/OFQ expression is oppositely changed in the striatum (reduction) and SNr (increase) after dopamine depletion. Specular changes are observed for the NOP receptor, with reduced expression in SNr (Marti et al., 2005) and increased binding in striatum (Marti et al., 2012) (expression was not measured in that study). For these reasons, we proposed that a NOP partial agonist could provide a simultaneous antiparkinsonian and antidyskinetic effect, blocking the NOP receptor in SNr (where N/OFQ tone is elevated) and stimulating the NOP receptor in striatum (where N/OFQ tone is low).

Parkinsonian-like motor deficits

We report that full agonists exert a biphasic effect on parkinsonian-like motor deficits in 6-OHDA rats, i.e. facilitation (reduction of akinesia) at low doses and inhibition of rotarod performance (and, more in general, sedation/hypolocomotion) at higher ones. Endogenous N/OFQ is considered a natural motor constraint, whereas exogenous N/OFQ causes a biphasic regulation of spontaneous and exercise-induced locomotion (Marti et al., 2002). Motor facilitation is observed when low doses of N/OFQ are administered, quite possibly due to stimulation of D2 receptor-mediated transmission (Viaro et al., 2013), whereas motor inhibition is predominant when intermediate or high doses are given, likely because of direct inhibition of mesencephalic dopaminergic pathways (Marti et al., 2004b). A biphasic effect on motor function was less consistently reported with small molecule NOP receptor agonists. In fact, Ro64-6198 only produced monophasic inhibition (Kuzmin et al., 2003) whereas SCH655842 caused a biphasic effect, low doses stimulating and high doses inhibiting total distance travelled in the open field test in mice (Adami et al., 2011). The finding that AT-403 reduces the immobility time in the bar test at low doses and inhibited rotarod performance at higher ones, point to a biphasic effect on motor activity. The fact that SB-612111 prevented the effect of AT-403 in the bar test, confirms the involvement of the NOP receptor. However, since a stimulation of stepping activity

and rotarod performance was not observed at low doses, it might be questioned whether reduction of immobility time is a true motor, antiakinetic effect, or is secondary to nonmotor effects. For instance, NOP receptor agonists are known to exert anxiolysis at doses devoid of hypolocomotive/sedative effects (Varty et al., 2005).

Different from full agonists, the NOP partial agonist AT-127 markedly reduced the immobility time in the bar test and elevated stepping activity in the drag test, without affecting rotarod performance. This pattern might be justified considering the "partial agonist" nature of AT-127, which is expected to act as an antagonist where N/OFQ tone is high, e.g. in SNr. Thus, we expect AT-127 to counteract hypolocomotory actions of N/OFQ in SNr. Indeed, NOP receptor antagonists have been consistently shown to reduce the immobility time in the bar test and elevate both stepping activity in the drag test and time on rod in the rotarod test in 6-OHDA hemilesioned rats, acting on nigral NOP receptors (Marti et al., 2013; Marti et al., 2005; Marti et al., 2008; Marti et al., 2007; Volta et al., 2010b).

If this is the case, what is the mechanism underlying the motor facilitating action of NOP full agonists? Perhaps NOP stimulation could act in brain dopaminergic areas unaffected by 6-OHDA (e.g. ventral tegmental area) and promote DA release and postsynaptic D2 receptor stimulation (Viaro et al., 2013).

Antidyskinetic activity

The present study confirms that small molecules NOP receptor agonists attenuate the expression of dyskinetic movements upon L-DOPA challenge in dyskinetic rats. In fact, in a previous study we showed that centrally injected N/OFQ or systemically injected Ro65-6570 inhibited AIMs expression in dyskinetic rats (Marti et al., 2012). There was a 100-fold difference between doses causing hypolocomotion and doses attenuating dyskinesia (from 0.01 mg/Kg). Thus, compared to the dose-response curve of Ro65-6570, the therapeutic window of AT-403 is narrower, since only at 0.03 mg/kg the compound exerted a mild antidyskinetic effect without causing sedation/hypolocomotion, whereas at higher doses (0.1-0.3 mg/Kg) both effects were evident. Nonetheless, consistent with this antidyskinetic effect, rotarod performance ON L-DOPA was significantly better in 0.03 mg/Kg treated-rats than controls. The antidyskinetic effect was in the form of a delay in AIMs appearance rather than of a reduction in maximal severity and duration of AIMs. Therefore, it could be speculated

that NOP receptor stimulation provides an initial inhibitory effect, which is overcome when L-DOPA levels rise. Mechanistically, we could speculate that NOP receptor mediated inhibition of D1 signalling induced by AT-403 is too mild to prevent full D1 receptor activation.

The reason why AT-390 delays AIMs appearance and, at the same time, dramatically prolongs AIMs duration is presently unclear. It might be due to NOP-independent effects of the molecule or its metabolites, and interpreted as combination of different and independent mechanisms. The delay in AIMs appearance might be due to the sedative effect, also shared by other NOP agonists, whereas AIMs prolongation to a potentiation of L-DOPA responses, either on a pharmacodynamics or pharmacokinetic basis. This finding deserves further investigation since it suggests that AT-390 can potentially be used in preventing wearing-off of L-DOPA effects over long-term therapy.

The finding that AT-127 also failed to attenuate LID expression, or to alter LID time-course, might again be explained by the pharmacological nature of this molecule. This emerged also from the chronic study. In fact, we noticed that not only AT-127 did not attenuate LID, but also worsened them. In particular, at the end of the 21-day chronic treatment, AT-127 caused a prolongation of the dyskinesiogenic effect of L-DOPA, very much like AT-390 did in the acute studies. We interpret this as a clue of the NOP antagonistic action of AT-127 since NOP receptor antagonists are known to potentiate the action of L-DOPA at the cost of causing dyskinesia appearance (Visanji et al., 2008). This finding suggests that AT-127 could be administered at low doses in combination with L-DOPA to prolong its effects and lower its dose. Therefore, the beneficial effect on LID would be indirect, because a lower dose of L-DOPA, with the same therapeutic effect, would delay the appearance of LID.

The finding that AT-127 exacerbates LID might shed light on the mechanism underlying the effect of AT-390. This could be further interpreted as being due to a NOP antagonistic action, perhaps caused by a metabolite of this compound.

Differently from AT-127, chronic administration with a low dose of AT-403, mildly effective against LID when given acutely, did not significantly affect LID development. Nonetheless, this dose produced a marked and positive effect on motor balance since it preserved motor performance on the rotarod on L-DOPA. The positive effect of NOP receptor agonists on axial symptoms such as gait and balance,

observed in dyskinetic macaques (Marti et al., 2012) might explain why, despite suffering from dyskinesia, animals can perform well on the rotarod.

Concluding remarks

The present study tried to answer two fundamental and unaddressed questions regarding the role of N/OFQ in PD, which can be summarized as follows:

i) Does endogenous N/OFQ contribute to the death of mesencephalic DA neurons in PD?

ii) Does endogenous N/OFQ play a role in the process of brain sensitization underlying dyskinesia development during chronic L-DOPA treatment?

We feel the first answer is clearly positive since we proved that both the genetic deletion and pharmacological blockade of the NOP receptor provide neuroprotection in different animal models of the disease. Notably, here we report the first evidence for a neuroprotective/neurorestorative effect for a NOP receptor antagonist in a PD model; moreover, SB-612111 was administered in a clinically driven protocol, which is highly relevant from a clinical perspective. While these new findings need confirmation using different NOP receptor antagonists, they lead the way for a new therapeutic approach for PD, in which chronic therapy with NOP receptor antagonists with subthreshold doses of L-DOPA may not only provide symptomatic relief and delay the incumbent appearance of dyskinesia but also protect from the neurodegenerative process associated with the disease (Marti et al., 2013; Marti et al., 2005; Marti et al., 2007).

The answer to the second question is disappointingly negative, since AT-403 could not attenuate LID development in a validated model of LID, although it showed a mild beneficial effect on the expression of LID. However, we collected limited evidence that NOP receptors can modulate LID expression through specific antidyskinetic mechanisms, not involving sedation, which was detected with both compounds at the higher doses tested. Notably, the therapeutic window of AT-403 was very narrow, predicting this compound will not be clinically useful. This series of chemical compounds, however, needs to be better characterized, also considering the dramatic prolongation of LID induced by AT-390. Paradoxically, the study on LID seemed to confirm that a partial agonist, through its antagonist portion, might be useful to potentiate the therapeutic action of L-DOPA, when coadministered.

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