



NOP Receptor Ligands and Parkinson's Disease

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Contents

1	Expression of ppN/OFQ and NOP in PD	214
2	N/OFQ Release in PD	217
3	NOP Receptor Ligands in PD Models: Symptomatic Efficacy	218
3.1	Models of Functional Parkinsonism: Genetic and Pharmacological Blockade of NOP Receptor	218
3.2	Models of Neurotoxic Parkinsonism: 6-OHDA Hemilesioned Rats	219
3.3	Models of Neurotoxic Parkinsonism: MPTP-Treated Mice	221
3.4	Models of Neurotoxic Parkinsonism: MPTP-Treated Nonhuman Primates	221
3.5	Models of α -Synucleinopathy	222
4	Mechanisms of Symptomatic Action of NOP Receptor Antagonists	222
4.1	NOP Receptor Antagonists and GLU Release	223
4.2	NOP Receptor Antagonists and GABA Release	224
4.3	NOP Receptor Antagonists and Nigro-Thalamic GABAergic Transmission	225
5	NOP Receptor Ligands in PD Models: Neuroprotective Efficacy	227
6	Conclusions	229
	References	229

Abstract

Nociceptin/Orphanin FQ (N/OFQ) and its NOP receptor are highly expressed in motor areas of the rodent, nonhuman, and human primate brain, such as primary motor cortex, thalamus, globus pallidus, striatum, and substantia nigra. Endogenous N/OFQ negatively regulates motor behavior and dopamine transmission through NOP receptors expressed by dopaminergic neurons of the substantia nigra compacta. Consistent with the existence of an N/OFQ tone over dopaminergic transmission, blockade of NOP receptor antagonists increases striatal

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dopamine release. In this chapter, we will review the evidence linking the N/OFQ-NOP receptor system to Parkinson's disease (PD). We will first discuss data showing that the central N/OFQ-NOP receptor system undergoes plastic changes in different basal ganglia nuclei following dopamine depletion. Then we will show that NOP receptor antagonists relieve motor deficits in different rodent and nonhuman primate models of PD. Mechanistically, NOP receptor blockade in substantia nigra reticulata results in rebalancing of the inhibitory GABAergic and excitatory glutamatergic inputs impinging on nigro-thalamic GABAergic neurons, leading to thalamic disinhibition. We will also present data showing that, in addition to motor symptoms, N/OFQ also plays a role in the parkinsonian neurodegeneration. In fact, NOP receptor antagonists possess neuroprotective/neurorescue properties in *in vitro* and *in vivo* models of PD.

Keywords

Compound 24 · J-113397 · L-DOPA · Nociceptin/Orphanin FQ · NOP · Parkinson's Disease · SB-612111 · Trap-101

1 Expression of ppN/OFQ and NOP in PD

The first evidence of changes of pre-proN/OFQ (ppN/OFQ) and NOP gene expression in models of PD came from the *in situ* hybridization (ISH) study of Watson and collaborators (Norton et al. 2002) who reported a threefold increase of ppN/OFQ mRNA levels and a ~80% reduction of NOP mRNA levels in the substantia nigra compacta (SNc) after unilateral injection of the parkinsonian toxin 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle (mfb) of the rat (Table 1). Considering that also a ~70% reduction of N/OFQ-stimulated [³⁵S]GTPγS binding was simultaneously detected in SNc, it was concluded that dopamine (DA) depletion results in upregulation of ppN/OFQ and downregulation of NOP receptor expression in SNc (Norton et al. 2002). We have later confirmed these findings (Marti et al. 2005), reporting a threefold increase of ppN/OFQ and a ~60% reduction of NOP receptor mRNA in the DA-depleted rat SNc showing, in addition, similar changes in SN reticulata (SNr; twofold increase of ppN/OFQ and ~25% loss of NOP receptor mRNA). Romualdi and collaborators confirmed an increase of ppN/OFQ in SNc after 6-OHDA using RT-PCR (di Benedetto et al. 2009), and also showed a reduction of ppN/OFQ in striatum (–50%), indicating that opposite changes of N/OFQ transmission might occur in these areas. Consistently, we reported an increase and a reduction of N/OFQ autoradiographical binding in striatum and SNc/SNr, respectively, in rats hemilesioned with 6-OHDA (Marti et al. 2012). Interestingly, chronic treatment with L-DOPA and dyskinesia onset did not change this pattern (Marti et al. 2012). These adaptive changes seemed to specifically affect the nigro-striatal pathway since ppN/OFQ levels were unaffected in other brain areas such as cerebral cortex, nucleus accumbens, thalamus, and globus pallidus (Marti et al. 2010). Studies using MPTP, its active metabolite MPP+, or even a combination of the pesticides paraquat and maneb as

Table 1 Summary of the studies on ppN/OFQ and NOP gene expression

Model	Species	Technique	Readout	Area	% change	Extent of lesion	Reference
6-OHDA (mfb)	Rat	ISH	ppN/OFQ mRNA	SNC	+200	>95 ^a	Norton et al. (2002)
				VTA	+60		
6-OHDA (mfb)	Rat	ISH	ppN/OFQ mRNA	SNC	-85	>90 ^a	Marti et al. (2005)
				VTA	-85		
				SNC	+200		
				SNr	+100		
6-OHDA (icv)	Rat	RT PCR	ppN/OFQ mRNA	SNC	-60	50 ^b 60 ^b	di Benedetto et al. (2009)
				SNr	-20		
MPP+ (icv)	Rat	RT PCR	NOP mRNA	SN	+50	75 ^b 65 ^b	di Benedetto et al. (2009)
				Striatum	-50		
				SN	n.e.		
				Striatum	-50		
MPTP (systemic)	Mouse	ISH	ppN/OFQ mRNA	SN	+60	58-80 ^d	Gouty et al. (2010)
				Striatum	-50		
				SN	-30		
				Striatum	-50		
6-OHDA (mfb)	Rat	ISH	ppN/OFQ mRNA	SNC	+150 ^c	>90 ^a	Marti et al. (2010)
				SNr	>400		
6-OHDA (mfb)	Rat	ISH	ppN/OFQ mRNA	VTA	n.e.	>90 ^a	Marti et al. (2010)
				Striatum	n.e.		
				STN	n.e.		
				SNC	+150		
				SNr	+105		
				STN	+45		
				striatum	-25		
				M1/M2	n.e.		
NAcc	n.e.						
GP	n.e.						
VPL/M	n.e.						

(continued)

Table 1 (continued)

Model	Species	Technique	Readout	Area	% change	Extent of lesion	Reference
6-OHDA (mfb), dyskinetic	Rat	Autoradiography on slices	N/O/FQ binding	Striatum SNr/SNc STN M1/M2	+50 -50 n.c. n.c.	>95 ^a	Marti et al. (2012)
Paraquat + maneb (systemic)	Rat	RT PCR	ppN/O/FQ mRNA NOP mRNA	Striatum SN Striatum SN	n.c. +50 n.c. -40	35–40 ^b	Bastias-Candia et al. (2015)
PD patients	–		ppN/O/FQ mRNA	SN			Collins et al. (2015)

ISH in situ hybridization, *mfb* medial forebrain bundle, *n.c.* no change

^aStriatal TH immunohistochemistry

^bTH levels in striatum or SN (western blot)

^cStrain-dependent

^dDopamine neuron count in SNc

DA-depleting parkinsonian toxins substantially replicated what observed with 6-OHDA. An increase of ppN/OFQ in SNc was captured with RT-PCR following i.c.v. injection of MPP+ (di Benedetto et al. 2009) or systemic administration of paraquat/maneb (Bastias-Candia et al. 2015) in rats, and MPTP administration in mice (Gouty et al. 2010), although in this case such effect was strain-dependent. Nonetheless, in this study, BM Cox and collaborators (Gouty et al. 2010) reported a strong elevation of ppN/OFQ in SNr, in line with that found with 6-OHDA. A careful immunohistochemical analysis showed that this elevation occurred in neurons and, specifically, in a subset of GABAergic neurons spanning throughout the SNr (Gouty et al. 2010). Altogether, morphological studies consistently showed that DA loss is accompanied by area-dependent changes of the N/OFQ-NOP receptor system in the basal ganglia: upregulation of ppN/OFQ expression associated with reduction of NOP receptor binding in SNc/SNr and downregulation of ppN/OFQ expression associated with increase of NOP receptor binding in striatum. This picture seems to be confused by the postmortem analysis of the SN of PD patients that instead revealed a downregulation of ppN/OFQ gene expression (Collins et al. 2015). This finding does not easily reconcile with preclinical data and might indicate the occurrence of compensatory mechanisms to prevent excessive NOP receptor activation.

2 N/OFQ Release in PD

In vivo microdialysis showed that the increase in nigral ppN/OFQ mRNA observed after DA depletion was actually accompanied by an elevation of N/OFQ release in the extracellular space (Marti et al. 2005). Radioimmunoassay of dialysates from the microdialysis probes simultaneously implanted in the lesioned and unlesioned SNr of 6-OHDA hemilesioned rats revealed that N/OFQ-like immunoreactivity at baseline was threefold elevated in the lesioned compared to the unlesioned side. Interestingly, when the rat was placed on a rotating cylinder (rotarod test), N/OFQ levels rose in the SNr of both sides, indicating a functional role of N/OFQ during exercise. In these conditions, however, the side difference became larger (>3.5-fold) with highest levels attained in the lesioned SNr. An increase of extracellular N/OFQ levels in SNr was observed not only after destruction of DA neurons but also during functional inhibition of DA transmission caused by haloperidol treatment (Marti et al. 2010). Although the increase was milder (50%), it suggested the existence of an ongoing tonic inhibitory control mediated by striatal DA, likely via the indirect striato-nigral pathway, on nigral N/OFQ release. In striking agreement with studies in models of PD, an increase of N/OFQ levels was observed in the CSF of parkinsonian patients (Marti et al. 2010). In fact, we measured N/OFQ levels in the CSF of PD patients subjected to surgical implantation of electrodes for deep brain stimulation, in comparison with non-PD neurological controls. Both cohorts were balanced for number (17 controls subjects against 20 PD patients), age, and gender. PD patients were affected by advanced and severe PD (Hoehn&Yahr stage 2.9, UPDRS II score 46.1) with a long history of disease (11.8 years). In line with that found in the DA-depleted rat SNr, we reported a 3.5-fold elevation of N/OFQ

levels in the CSF from PD vs non-PD patients. Since human CSF is in equilibrium with parenchymal fluids, this study suggests that N/OFQ might play a role in PD.

3 NOP Receptor Ligands in PD Models: Symptomatic Efficacy

In line with the hypothesis that the increase of N/OFQ expression and release contributes to PD, NOP receptor antagonists proved effective in reducing motor deficits and neurodegeneration in PD models. Preliminary studies in naïve rats somewhat predicted the effectiveness of NOP antagonists in PD models. In fact, while N/OFQ was capable of elevating glutamate (GLU) levels in SNr, the NOP receptor peptide antagonist [Nphe¹]N/OFQ(1–13)NH₂ ([Nphe¹]) inhibited it (Marti et al. 2002). Moreover, while exogenous N/OFQ injected in SNr reduced DA levels in striatum and motor activity, the NOP receptor peptide antagonist UFP-101 injected in SNr and the NOP receptor small molecule J-113397 administered systemically caused opposite effects (Marti et al. 2004b). The symptomatic efficacy of NOP receptor antagonists was consistently shown in different models of PD, as detailed below.

3.1 Models of Functional Parkinsonism: Genetic and Pharmacological Blockade of NOP Receptor

The first evidence that an NOP receptor antagonist could reverse parkinsonian symptoms was obtained in a rat model of neuroleptic-induced parkinsonism (Marti et al. 2004a). In that study, UFP-101 was injected (30 nmol) through a cannula implanted in the SNr of a rat made cataleptic by systemic haloperidol (0.8 mg/kg). Akinesia was assessed with the bar test and GLU levels in SNr were simultaneously monitored via a microdialysis probe. Haloperidol blocks striatal D2 receptors, which causes disinhibition of the striato-pallidal and subthalamo-nigral pathways, leading to stimulation of the nigral output and motor inhibition. Indeed, haloperidol elevated both akinesia and nigral GLU levels whereas UFP-101 transiently reduced akinesia and normalized GLU levels. Moreover, UFP-101 triggered contralateral rotations, more intensely in haloperidol-treated rats than in naïve rats. The role of endogenous N/OFQ in neuroleptic-induced parkinsonism was also investigated in mice (Mabrouk et al. 2010). In this model, the antiakinetic effectiveness of UFP-101 (10 nmol, i.c.v.) was confirmed. In addition, we showed that similar effects were produced also by systemic administration of J-113397 (0.1–10 mg/kg). The role of endogenous N/OFQ in modulating neuroleptic-induced parkinsonism was further supported by the finding that NOP receptor knockout (NOP^{-/-}) mice were less prone to develop motor impairment after administration of low doses (0.3–0.8 mg/kg) of haloperidol (Marti et al. 2005).

The effectiveness of NOP receptor antagonists was also proven in reserpinized animals. Reserpine depletes vesicular stores of monoamines by blocking the vesicular monoamine transporter type 2 (VMAT2). Reserpine administration (0.1–3 mg/

kg) in mice caused a dose-dependent and long-lasting impairment of motor activity lasting for >4 days (Volta et al. 2010a). Repeated daily administration of J-113397 (1 mg/kg) caused acute improvement of motor activity (which, however, underwent tolerance within 2 days) and accelerated recovery of motor function that reached (drag test), approximated (bar test), or even exceeded (rotarod test) baseline values within 4 days. Again, studies in NOP^{-/-} mice substantiated these findings. In fact, in line with that found with haloperidol, NOP^{-/-} mice were less prone to develop motor impairment after administration of reserpine 1 mg/kg. Finally, acute administration of SB-612111 (0.1–10 mg/kg) also dose-dependently improved motor activity in reserpinized mice, an effect partly replicated by the NOP antagonist NiK-21273 (Marti et al. 2013).

3.2 Models of Neurotoxic Parkinsonism: 6-OHDA Hemilesioned Rats

The proof-of-concept that NOP receptor antagonists possess symptomatic antiparkinsonian properties was provided by Morari lab in 2005 (Marti et al. 2005), testing UFP-101 and J-113397 in comparison with a fixed-doses of L-DOPA (1 mg/kg) as a positive control, in 6-OHDA hemilesioned rats, a well-validated model of neurodegenerative PD (Schwartz and Huston 1996a, b). UFP-101 (0.1–30 nmol, intranigral) and J-113397 (0.1–3 mg/kg, i.p.) reduced akinesia in the bar test and akinesia/bradykinesia in the drag test, and improved overall motor performance in the rotarod test, replicating the pattern of responses to L-DOPA. Other small molecules NOP antagonists given i.p. were later proven effective in this model: Trap-101 (a non-chiral analog of J-113397) (Marti et al. 2008), GF-4 (a derivative of Trap-101) (Volta et al. 2010b), NiK-21273 (Marti et al. 2013), and the more potent and selective NOP antagonists Compound 24 (Volta et al. 2011) and SB-612111 (Marti et al. 2013).

All NOP antagonists improved motor function in the three different tests (bar, drag, and rotarod) with no major difference in efficacy. Nonetheless, differences in potency were observed, Compound 24 being the most potent antagonist (effective in all three tests already at 0.1 mg/kg) and Trap-101 the least potent one (effective at 1 mg/kg in the drag test and at 10 mg/kg in the drag and rotarod test).

What also emerged from these studies was the bell-shaped profile of the dose–response curve of NOP receptor antagonists. In fact, NOP receptor antagonists lost their positive effect or even caused overt motor inhibition when given at high doses. This applied to J-113397, GF-4, and Compound 24 administered at 30 mg/kg. We could not observe such a phenomenon for Trap-101 (tested up to 30 mg/kg) and SB-612111 (tested up to 1 mg/kg), although we cannot rule out that this might occur at higher doses. Indeed, Trap-101 induced motor inhibition when administered at 30 mg/kg to naïve rats or mice (Marti et al. 2008). The mechanisms underlying motor facilitation and inhibition induced by NOP receptor antagonists were investigated (Viaro et al. 2013) using selective DA receptor antagonists, and mice constitutively lacking the D2 receptor (D2R^{-/-} mice) (Baik et al. 1995) or its long

(L) isoform (D2L^{-/-} mice) (Usiello et al. 2000), which is preferentially expressed at the postsynaptic level (Picetti et al. 1997; Usiello et al. 2000). Motor inhibition induced by UFP-101 30 mg/kg or J-113397 10 mg/kg was abolished (and in some cases reversed into motor facilitation) by low doses of the atypical D2 antagonist amisulpride but not by the typical D2 antagonist raclopride. In addition, it was abolished in D2R^{-/-} mice but remained unchanged in D2L^{-/-} mice. Considering the affinity of low doses of amisulpride for presynaptic D2 receptors (Scatton et al. 1997; Schoemaker et al. 1997), these data suggested that motor inhibition induced by high doses of NOP receptor antagonists is mediated by activation of presynaptic D2 receptors. To support this view, also motor inhibition induced by high doses of dopaminergic agonists (L-DOPA 100 mg/kg or pramipexole 0.1–1 mg/kg) was reversed by amisulpride and/or cancelled in D2R^{-/-} mice. The most parsimonious explanation is that blockade of presynaptic NOP receptors located on dopaminergic terminals elevates DA release (Marti et al. 2004b), and extracellular DA binds to presynaptic D2 receptors activating the inhibitory auto feed-back.

In one study, we addressed the question whether the antiparkinsonian effect of SB-612111 undergoes tolerance (Marti et al. 2013). SB-612111 was chronically administered for 16 days at a low, ineffective (0.01 mg/kg) or high, maximally effective (1 mg/kg) dose. Motor function was assessed before and after acute administration. Essentially, there was no chronic effect of SB-612111 over motor function over time (i.e., no changes of baseline motor activity) and the acute effect of the drug remained unchanged during the study, indicating there was no development of tolerance. We also repeated the experiment using ineffective (0.5 mg/kg) and effective (1.5 mg/kg) doses of NiK-21273. In this case, we noticed a late improvement of motor function at baseline in the bar and drag tests (12–16 days after the onset of administration), but a rapid tolerance (within 4 days) to the acute effects.

One particular aspect of the symptomatic antiparkinsonian effect of NOP antagonists that was investigated in 6-OHDA rats was the ability to interact with L-DOPA. The first study addressing this issue (Marti et al. 2007) showed that fully effective doses of J-113397 (1 mg/kg) caused additive improvement of motor activity (in the drag and rotarod tests) when challenged with a submaximal dose of L-DOPA (1 mg/kg). Interestingly, we found an additive stimulation of the nigro-thalamic pathway as a neurochemical correlate of this behavior (discussed below). The ability of NOP receptor antagonists to positively interact with L-DOPA was later confirmed using maximally effective doses of Trap-101 (10 mg/kg) in combination with subthreshold (ineffective) doses of L-DOPA (0.1 mg/kg) (Marti et al. 2008). In this case, the combination produced greater antiakinetic effect (bar test) and improvement of overall gait ability (rotarod test) than that produced by each compound alone. Interestingly, this effect was reproduced when L-DOPA was administered systemically and Trap-101 was perfused in SNr via a microdialysis probe. In fact, simultaneous monitoring of GLU and GABA levels in SNr and ipsilateral ventromedial thalamus (VMTh) in these animals establishes that the neurobiological substrate of the positive interaction was the nigro-thalamic pathway (discussed below). Finally, a marked synergistic interaction between subthreshold doses of SB-612111 (0.01 mg/kg) and L-DOPA (0.1 mg/kg) was demonstrated (Marti et al. 2013), an effect replicated by subthreshold doses of NiK-21273 (0.5 mg/kg).

Whether an NOP antagonist, in addition to potentiating the therapeutic effect of L-DOPA, would also exacerbate L-DOPA-induced dyskinesia (LID) was specifically assessed (Marti et al. 2012) in the classical 6-OHDA rat model of L-DOPA-induced dyskinesia (Cenci et al. 1998). In fact a previous study in MPTP-treated nonhuman primates (marmosets) reported that a high dose of J-113397 (30 mg/kg s. c.) potentiated the effect of L-DOPA, at the cost of exacerbating dyskinesia (Visanji et al. 2008). Consistently, when 10 nmol UFP-101 (i.c.v.) or 3 mg/kg J-113397 (i.p.) were co-administered with L-DOPA to L-DOPA-primed dyskinetic rats, exacerbation of abnormal involuntary movements (AIMs), i.e., the rodent correlate of dyskinesia, was observed. In this model, however, the effect was mild and limited to the limb subgroup of AIMs (50% with UFP-101, 20% with J-113397), the axial and orolingual (facial) muscles being spared. Interestingly, in that study we found that intranigral but not intrastriatal UFP-101 injection replicated the effect of i.c.v. injections, suggesting that NOP antagonists act where NOP tone is elevated (i.e., in SNr) but not where N/OFQ is reduced (i.e., striatum). Consistently, in that study we provided the proof-of-concept that NOP receptor agonists exert antidyskinetic actions.

3.3 Models of Neurotoxic Parkinsonism: MPTP-Treated Mice

MPTP-treated mice are not routinely used as a model for studying the symptomatic antiparkinsonian effects perhaps due to the inconsistencies of MPTP effects across different labs and protocols, though motor deficits can be captured and quantified in these mice (Sedelis et al. 2000). Using a classical protocol of acute MPTP administration (4 × 20 mg/kg, 90 min apart) causing ~60% loss of striatal dopaminergic terminals, we were able to capture a robust increase in the immobility time (from <1 to 26 s, bar test) and a ~30% reduction in stepping activity (drag test) and time on rod (rotarod test) a week after MPTP administration. J-113397 reversed these changes just as our positive control, L-DOPA. Indeed, J-113397 caused a reduction of immobility time and an increase in stepping activity and rotarod performance in the dose range of 0.01–0.03 mg/kg, that were quantitatively similar, although not as prolonged as those evoked by 10 mg/kg L-DOPA. In addition, we observed a reversal of action at 1 mg/kg, again pointing out the bell-shaped nature of the dose–response curve of this molecule.

3.4 Models of Neurotoxic Parkinsonism: MPTP-Treated Nonhuman Primates

We investigated the effect of J-113397 in MPTP-treated, stably parkinsonian macaques (Viaro et al. 2008), the gold standard in preclinical models of PD. Four macaques were enrolled in this study: their motor performance was assessed and quantified via computerized time reaching tasks (MAP test, i.e., the platform and straight rod tests) or by post hoc videotape analysis (UPDRS scale) by a neurologist

blind to the experiment. Preliminary dosing in these animals indicated a positive effect of 0.01 mg/kg J-113397 in the MAP test. This dose also caused symptomatic benefit in all four animals, improving various motor parameters such as hypokinesia, bradykinesia, tremor, balance, and rigidity. J-113397 was overall 50% less effective than L-DOPA (30 mg/kg i.m.), although it was as effective as L-DOPA on hypokinesia. Very much like that observed in rodent models, higher doses of J-113397 negatively affected behavior; in particular, 1 mg/kg J-113397 caused long episodes of freezing. A study in MPTP-treated marmosets (Visanji et al. 2008) revealed that 30 mg/kg J-113397 (s.c.) was capable of potentiating the effect of a low subtherapeutic dose (12.5 mg/kg) of L-DOPA, at the cost, however, of inducing dyskinesia. These authors raised the concern that the L-DOPA sparing effect of NOP antagonists would be counterbalanced by the appearance of dyskinetic movements.

3.5 Models of α -Synucleinopathy

The discovery that mutations in the SNCA gene coding for α -synuclein were associated with autosomal dominant PD (Polymeropoulos et al. 1997), and that Lewy Bodies, a neuropathological feature of PD, are mainly composed by α -synuclein (Spillantini et al. 1997), paved the way for the research on the genetics of PD. It is well accepted that the fibrillization process of α -synuclein that leads to LB formation also determines the formation of toxic species that cause neuronal death. Thus, overexpression of native or mutated α -synuclein has been used to replicate synucleinopathy observed in PD brains. To extend the previous findings in functional and neurodegeneration models of PD, we tested SB-612111 in a synucleinopathy model. In this model, a recombinant adeno-associated virus of serotype 2/9, carrying human p.A53T α -synuclein transgene (AAV2/9-h α -syn), a mutated, toxic form of α -synuclein (α -syn), under the promotor of synapsin 1, was injected into the SNc of naïve rats. Although the primary endpoint of the study was the assessment of the neuroprotective properties of SB-612111, motor behavior was monitored along with chronic administration of the compound. Transgene expression was associated with nigro-striatal degeneration and progressive motor deficits, namely reduction of stepping activity (Arcuri et al. 2016). SB-612111, administered daily for 8 weeks, starting a week after virus injection, was able to attenuate nigro-striatal degeneration (see below) and prevent motor impairment.

4 Mechanisms of Symptomatic Action of NOP Receptor Antagonists

The mechanism underlying the motor promoting action of NOP receptor antagonists was well defined. Several lines of evidence converge in suggesting that NOP receptor antagonists act to reset the balance between excitatory and inhibitory inputs

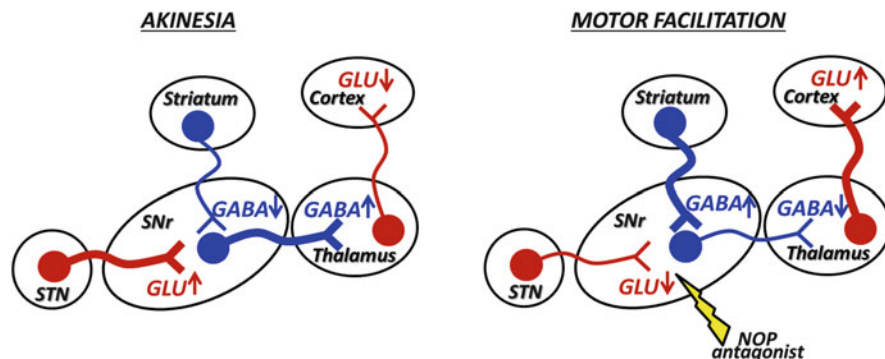


Fig. 1 Neurochemical and functional consequences of NOP receptor blockade in the substantia nigra reticulata (SNr) of parkinsonian rats. Parkinsonian akinesia is characterized by disinhibition of the activity of the glutamatergic neurons located in subthalamic nucleus (STN), and reduction of the activity of the striatal GABAergic neurons projecting to SNr (left panel). Microdialysis studies in 6-OHDA hemilesioned rats revealed that NOP receptor antagonists oppose these changes, reducing glutamate (GLU) and increasing GABA levels in SNr (right panel). This causes overinhibition of the nigro-thalamic pathway and thalamic disinhibition, ultimately resulting in motor facilitation

impinging on nigro-thalamic GABAergic neurons, thus causing disinhibition of thalamo-cortical pathways and movement facilitation (Fig. 1). According to the classical model of basal ganglia functioning (Albin et al. 1989; Alexander et al. 1990), the parkinsonian condition is associated with an increased activity of the glutamatergic projections from the subthalamic nucleus to the output nuclei of the basal ganglia, namely SNr and the pars interna of the globus pallidus (GPi). Simultaneously, the inhibitory projection from striatal GABAergic medium-sized spiny neurons (MSNs) to the SNr/GPi (the so-called “direct pathway”) becomes hypoactive. This causes a net increase of the excitatory inputs over the tonically active nigrofugal GABAergic neurons, leading to further inhibition of thalamo-cortical projections.

4.1 NOP Receptor Antagonists and GLU Release

Microdialysis studies in rodents consistently pointed out that NOP receptor antagonists are capable of reducing GLU release in SNr. Haloperidol-induced catalepsy/akinesia is associated with elevation of nigral GLU release (Mabrouk et al. 2010; Marti et al. 2004a). This is due to the disinhibition of subthalamo-nigral pathway as a consequence of blockade of inhibitory D2 receptors expressed on striato-pallidal MSNs. UFP-101 (10 nmol, i.c.v.) and J-113397 (1 mg/kg, i.p.) reversed haloperidol-induced nigral GLU release in the rat (Marti et al. 2004a, 2005). J-113397 was also effective in the mouse (Mabrouk et al. 2010). In both species, normalization of haloperidol-elevated GLU release was accompanied by reversal of akinesia. Consistently, genetic deletion of the NOP receptor attenuated

haloperidol-induced catalepsy and its neurochemical correlate. In fact, $NOP^{-/-}$ mice did not show the typical rise of GLU levels observed in $NOP^{+/+}$ and wild-type mice following haloperidol (0.3 mg/kg), actually showing a reduction, and were also insensitive to the cataleptic action of this dose of haloperidol. Since there was no difference in basal GLU levels between $NOP^{+/+}$ and $NOP^{-/-}$ mice, we conclude that there is a close relationship between catalepsy and endogenous N/OFQ and glutamate in SNr. To confirm this view, a microdialysis study showed that $NOP^{-/-}$ mice treated with reserpine 1 mg/kg developed a milder increase in nigral GLU levels than $NOP^{+/+}$ controls, which was accompanied by a 50% reduction of catalepsy severity (Volta et al. 2010a).

Microdialysis studies in 6-OHDA rats substantially confirmed the ability of NOP receptor antagonists to reduce nigral GLU. In fact, in a microdialysis study where probes were simultaneously implanted in the lesioned and unlesioned SNr, UFP-101 (1–10 μ M through the probe) or J-113397 (0.1–3 mg/kg, i.p.) reduced GLU levels (20–30%) in both hemispheres, although more potently in the lesioned one. We should recall that GLU levels detected at baseline via microdialysis only minimally (~20%) derive from neuronal sources (Morari et al. 1996), so it is possible that a “floor effect” prevented to detect further reduction. Similar reductions of basal GLU release were observed administering Trap-101 (10 mg/kg) (Marti et al. 2008) or GF-4 (1 mg/kg) (Volta et al. 2010b) systemically, or Trap-101 (10 μ M) (Marti et al. 2008) and Compound 24 (0.03 μ M) (Volta et al. 2011) through a microdialysis probe implanted in SNr. Also, during these studies microdialysis was coupled to behavioral monitoring (the immobility time in the bar test), confirming that these procedures led to significant attenuation of akinesia. Consistently, combined administration of Trap-101 and L-DOPA caused slightly more profound (~30%) and/or faster inhibition of nigral GLU release (depending on the way Trap-101 was administered) which was associated with additive attenuation of akinesia.

Overall, these data suggest that DA loss amplifies a tonic excitatory action of endogenous N/OFQ over nigral GLU terminals (Marti et al. 2002). Since an increase of excitatory input over nigro-thalamic GABA neurons causes thalamic inhibition and impairment of motor initiation, it is plausible that NOP receptor antagonists reduce akinesia by blocking this action.

4.2 NOP Receptor Antagonists and GABA Release

The resetting of GLU inputs in SNr is perhaps not the only mechanism through which NOP receptor antagonists ameliorate parkinsonian motor symptoms. In fact, a positive effect of NOP receptor antagonists on GABA levels in SNr was disclosed using microdialysis. Elevation of GABA levels in SNr might mimic the physiological inhibitory control operated by the striato-nigral direct pathway over nigro-thalamic neurons, which is reduced due to the loss of nigro-striatal dopaminergic innervation (Albin et al. 1989; Alexander et al. 1990). The first evidence was obtained in 6-OHDA hemilesioned rats where both L-DOPA and J-113397 (1 mg/kg) elevated nigral GABA levels, and their combination caused an additive effect,

which correlated with an additive antiakinetic effect (Marti et al. 2007). Interestingly, the additive increase of nigral GABA was prevented by perfusion of the voltage-dependent sodium channel blocker tetrodotoxin (TTX) indicating that GABA levels monitored by microdialysis were indeed the result of neuronal activity. This GABA facilitating effect was later replicated by systemic administration of Trap-101 (10 mg/kg) (Marti et al. 2008) and GF-4 (1 mg/kg) (Volta et al. 2010b) as well as by reverse dialysis of Trap-101 (10 μ M) (Marti et al. 2008) and Compound 24 (3 μ M) (Volta et al. 2011) in SNr. This latter approach, in particular, proved that NOP receptors located in SNr tonically inhibit GABA release in this area.

4.3 NOP Receptor Antagonists and Nigro-Thalamic GABAergic Transmission

Whether the changes of GLU and GABA levels induced by NOP antagonists in SNr effectively impacted over the activity of nigro-thalamic GABAergic neurons and, through them, on motor function, was specifically addressed in dual probe microdialysis studies coupled to behavioral testing (Marti et al. 2007, 2008; Volta et al. 2010b, 2011) where one probe was implanted in the lesioned SNr and another in the ipsilateral ventro-medial thalamus (VMTh), which is a target of nigral projections. We first showed that GABA release in VMTh was reduced (~30%) by intranigral perfusion with TTX, indicating that GABA levels were partly due to nigro-thalamic neuron activity (Marti et al. 2007). Consistently, blockade of nigro-thalamic activity with TTX also reduced the immobility time, since it disinhibited thalamo-cortical projections (Marti et al. 2007). A similar effect was produced by the combination of L-DOPA plus J-113397 that, in addition to elevating GABA and reducing GLU in SNr (see above), also reduced GABA in VMTh (Marti et al. 2007). These effects were occluded by TTX, suggesting that these neurochemical changes reflected ongoing neuronal activity and the involvement of nigro-thalamic neurons. To confirm this view, TTX also prevented the reduction of nigral GLU release induced by the L-DOPA/J-113397 combination (Fig. 2). The involvement of the nigro-thalamic pathway in the motor promoting action of NOP antagonists was further proven by reverse dialysis of the GABA_A receptor antagonist bicuculline in SNr (Marti et al. 2007). In fact, we reasoned that if an elevation of GABA in SNr was responsible for the inhibition of nigro-thalamic neurons, blockade of nigral GABA_A receptors, which are expressed by nigro-thalamic neurons, would prevent this effect. In fact, bicuculline did not block the rise of nigral GABA induced by L-DOPA plus J-113397 but prevented its inhibitory effect over nigro-thalamic activity and thalamic GABA levels, and also abolished its behavioral correlate (i.e., the antiakinetic effect) (Marti et al. 2007). Bicuculline also delayed, although it did not abolish, the inhibitory effect on GLU release, possibly indicating that the potentiation of nigral GABA rather than the inhibition of nigral GLU was instrumental for the antiakinetic effect (Marti et al. 2007).

The role of nigral NOP receptors on nigro-thalamic transmission was further proven by directly perfusing the NOP antagonists Trap-101 (Marti et al. 2008) and

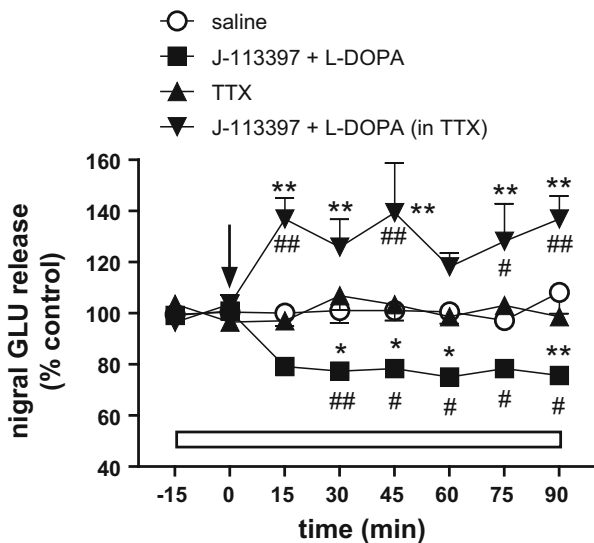


Fig. 2 Tetrodotoxin (TTX) prevented the reduction of GLU release in SNr induced by combined administration of L-DOPA and J-113397. Perfusion with TTX (1 μ M; open bar) in the SNr started 90 min before systemic (i.p.) co-administration (arrow) of J-113397 (1 mg/kg) and L-DOPA (1 mg/kg plus benserazide 15 mg/kg). Data are means \pm SEM of 5–6 experiments per group. Statistical analysis was performed by two-way ANOVA with repeated measures followed by the Bonferroni test. * p < 0.05, ** p < 0.01 different from saline; # p < 0.05, ## p < 0.01 different from TTX

Compound 24 (Volta et al. 2011) in SNr. In both studies, not only did the NOP antagonists reduce nigral GLU and elevate nigral GABA (see above) but they also reduced thalamic GABA along with akinesia, directly proving that blockade of nigral NOP receptors promotes movement by overinhibiting the nigro-thalamic input. Consistently, when combined with L-DOPA, Trap-101 produced a larger reduction of thalamic GABA (Marti et al. 2008).

Reverse dialysis of Compound 24 in SNr also provided valuable information on the mechanisms underlying the *motor inhibiting* effect caused by high doses of NOP receptor antagonists (Volta et al. 2011). In fact, perfusion with a high concentration (3 μ M) of Compound 24 increased akinesia and evoked neurochemical changes opposite to those associated with a 100-fold lower, antiakinetik concentration, i.e., reduction of GABA release in SNr and elevation of GABA release in VMTh (a tendency for an elevation of GLU release in SNr was also evident). Reverse dialysis of the D2 receptor antagonist raclopride in combination with Compound 24 demonstrated that nigral DA was involved in the inhibitory action. In fact, when nigral D2 receptors were blocked, 3 μ M of Compound 24 reversed its action, reducing akinesia, it also elevated nigral GABA and reduced nigral GLU and thalamic GABA, very much like 0.03 μ M of Compound 24 in the absence of raclopride. Conversely, the lower concentration of Compound 24 became behaviorally and neurochemically ineffective in the presence of raclopride (a significant

reduction of GABA release in SNr was detected, though). Therefore, nigral DA regulates the responsiveness of nigro-thalamic GABA neurons to the NOP antagonists. Using a combined pharmacological and genetic approach, we have next demonstrated that motor facilitation induced by NOP antagonists involves D2 postsynaptic receptors and motor inhibition D2 presynaptic receptors (Viaro et al. 2013).

5 NOP Receptor Ligands in PD Models: Neuroprotective Efficacy

Cox and collaborators provided the first evidence that endogenous N/OFQ contributes to parkinsonian degeneration (Marti et al. 2005). In fact, ppN/OFQ^{-/-} mice were reported to be more resistant than ppN/OFQ^{+/+} mice to the neurotoxic action of acute MPTP, showing a greater number of DA neurons and striatal DA terminals spared a week after acute MPTP administration. Since no changes of MPTP metabolism or uptake were observed in ppN/OFQ mice (Marti et al. 2005), the authors attributed the different responsiveness to MPTP to a possible neurotoxic role of endogenous N/OFQ. Interestingly, N/OFQ was not effective against methamphetamine-induced neurotoxicity, which is mainly targeted to striatal terminals, suggesting that N/OFQ could attenuate MPTP-induced toxicity acting at the nigral level (Brown et al. 2006). Since ppN/OFQ codes for other two biologically active neuropeptides beyond N/OFQ, namely, N/OFQ II and nocistatin, we thought mandatory to confirm the toxicity of endogenous N/OFQ in NOP^{-/-} mice (Arcuri et al. 2016). Indeed, NOP^{-/-} mice responded to acute MPTP (4 × 25 mg/kg i.p., every 90 min) exactly as ppN/OFQ^{-/-} mice, showing greater resistance to the toxin than NOP^{+/+} mice (50 vs 75% dopamine neuron loss, respectively). The greater resistance to MPTP was also accompanied by better motor performances in the bar and drag tests (Arcuri et al. 2016). The idea that endogenous N/OFQ could play a neurotoxic role in PD was further corroborated using a clinically driven study design in more progressive models, which allow a window for therapeutic intervention. In these experiments, the NOP receptor antagonist SB-612111 was used, and its administration was delayed with respect to the neurotoxic insult, as it occurs in the clinics where the patient comes to the attention of the neurologist when motor symptoms appear, i.e., far later the disease has started its course. Thus, SB-612111 (10 mg/kg, twice daily for 10 days starting at the 4th day after the onset of MPTP) was capable of preventing the nigro-striatal degeneration induced by subacute MPTP administration (25 mg/kg, i.p., once daily for 7 days) (Arcuri et al. 2016). Moreover, SB-612111 (1 mg/kg, twice daily for 8 weeks, commencing a week after AAV2/9 h-α-syn injection) attenuated the nigro-striatal neurodegeneration induced by α-syn overexpression. The percentage of DA cells spared was significantly greater in SB-612111-treated (50%) than in saline-treated (25%) rats. Considering that about 50% of nigral DA cells die a week after AAV2/9 h-α-syn injection, i.e., at the time when SB-612111 administration is commenced, this is a remarkable result.

The proof that exogenous N/OFQ is harmful for DA neurons was provided by our laboratory in collaboration with O’Keeffe laboratory (Collins et al. 2015). N/OFQ (1 μ M) and the NOP agonist UFP-112 (3 μ M), ineffective alone, were able to potentiate the toxic action of 6-OHDA on SH-SY5Y cell viability, this effect being reversed by NOP antagonists, UFP-101 and SB-612111. Remarkably, N/OFQ alone exerted detrimental effects on neuronal survival and complexity (neurite length and branching) in primary cultures of DA neurons, being as effective as the parkinsonian toxins MPP⁺ and 6-OHDA (Collins et al. 2015). In addition, N/OFQ caused additive effects when combined with either parkinsonian neurotoxin. N/OFQ effects were observed at relatively low concentrations (10–500 nM) and were specifically reversed by SB-612111, indicating that they were mediated by NOP receptors.

Studies are ongoing to identify the mechanisms underlying the neurotoxic pathways activated by N/OFQ. Since exogenous N/OFQ elevates whereas NOP receptor antagonists reduce glutamate release in the rodent SNr (see Sect. 4.1), we first hypothesized that endogenous N/OFQ can cause DA neuron degeneration through GLU-mediated excitotoxicity (Brown et al. 2006; Marti et al. 2005). Indeed, changes in mitochondrial potential due to inhibition of complex I by MPP⁺ (the active metabolite of MPTP) lead to oxidative stress and GLU-mediated excitotoxicity, which contribute to degeneration of DA neurons (Meredith and Rademacher 2011; Serra et al. 2002). In addition, NOP receptor antagonists can modulate N/OFQ-induced microglial activation (Laudenbach et al. 2001). This is particularly relevant for PD, since neuroinflammation plays an important role in neurodegeneration (Nolan et al. 2013; Poewe et al. 2017). N/OFQ modulates the inflammatory and microglial responses, although both pro- and anti-inflammatory effects of N/OFQ have also been described (Mallimo and Kusnecov 2013). Indeed, NOP receptor seems to bidirectionally modulate the expression and release of cytokines. In particular, it has been proven that N/OFQ inhibits the production of pro-inflammatory cytokines such as IL-6, IL1 β , and TNF α in different tissues and cell types, including glial cells. On the contrary, prolonged activation of the NOP receptor causes a dramatic activation of NF- κ B, a key modulatory transcription factor of the pro-inflammatory response (Toll et al. 2016). How NOP receptor exerts its action on microglia is not clear. Since cytokine activation and NOP receptor signaling share a common transduction pathway, i.e., the mitogen activated protein kinase (MAPK) pathway, we can speculate a cross-talk between NOP and cytokine signals in the modulation of the inflammatory response. Preliminary data in support of this hypothesis come from the study of O’Keeffe and colleagues (Collins et al. 2015), showing that N/OFQ inhibits the survival and growth of DA neurons in cultures through the p38-MAPK cascade. The p38-MAPK signaling is known to be implicated in different neurodegenerative diseases through its regulatory action on apoptosis and inflammation (Cuenda and Rousseau 2007; Zarubin and Han 2005) and increased phospho-p38 levels have been shown in the SNc DA neurons of PD patients (Karunakaran et al. 2008).

6 Conclusions

Major unmet clinical needs in the field of PD are a disease-modifying therapy, a good pharmacological control over non-motor symptoms, among which cognitive impairment, depression, pain, dysautonomias (e.g., orthostatic hypotension and stipsis) and drugs preventing the development of motor complications associated with L-DOPA therapy (e.g., dyskinesia). Preclinical data strongly suggest that the N/OFQ-NOP receptor system is a novel target in PD therapy and, in particular, that NOP receptor antagonists might provide both symptomatic and neuroprotective/neurorescue benefits, also acting as L-DOPA sparing agents. Although the risk of worsening L-DOPA-induced dyskinesia in advanced, complicated PD patients, or to accelerate dyskinesia development in de novo PD patients treated with L-DOPA, should be weighed, this might be overcome by careful titration of the dose of NOP receptor antagonist. Alternatively, we have proposed NOP receptor partial agonists as a possible alternative to NOP receptor antagonists (Marti et al. 2013). In fact, we proved that N/OFQ or NOP receptor agonists Ro 65-6590 or AT-403 improve established dyskinesia in L-DOPA-primed rats or nonhuman primates (Arcuri et al. 2018; Marti et al. 2012), acting on NOP receptor expressed in striatum where N/OFQ tone is low (Marti et al. 2012). Therefore, an NOP receptor partial agonist would improve motor deficits acting on NOP receptors in SNr, where N/OFQ is abnormally elevated, without exacerbating, or perhaps even ameliorating, L-DOPA-induced dyskinesia through stimulation of up-regulated striatal NOP receptors. Interestingly, the therapeutic benefit afforded by an NOP antagonist might extend over non-motor symptoms of PD. In fact, endogenous N/OFQ also contributes to depression (Gavioli and Calo 2013; Post et al. 2016), cognitive impairment (Khan et al. 2018; Redrobe et al. 2000) as well as to impairment of cardiovascular (bradycardia and hypotension) (Malinowska et al. 2002) and gastrointestinal (reduced motility) (Sibaev et al. 2015) functions. Consistently, NOP receptor antagonists were shown to improve depression in a number of preclinical tests and also in humans (Gavioli and Calo 2013; Post et al. 2016). The availability of the first orally active NOP receptor antagonist (LY2940094) (Toledo et al. 2014) has opened the way for the first clinical trial in PD, which has been press launched early 2018. We are eagerly awaiting for the results of this study to confirm that NOP receptor antagonists might really represent a new hope for PD patients (Arcuri et al. 2017).

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