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**Antiparkinsonian and antidyskinetic profiles of two novel potent and selective nociceptin/orphanin FQ receptor agonists**

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**Running title:** NOP receptor agonists in Parkinson's disease

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**Abstract**

**Background and purpose:** We previously showed that Nociceptin/orphanin FQ opioid peptide (NOP) receptor agonists attenuate the expression of levodopa-induced dyskinesia in animal models of Parkinson's disease. We now investigate the efficacy of two novel, potent and chemically distinct NOP receptor agonists, AT-390 and AT-403, to improve parkinsonian disabilities and attenuate dyskinesia development and expression.

**Experimental approach:** Binding affinity and functional efficacy of AT-390 and AT-403 at the opioid receptors were determined in radioligand displacement assays and in GTP $\gamma$ S binding assay respectively, conducted in Chinese hamster ovary cells. Their antiparkinsonian activity was evaluated in 6-hydroxydopamine hemilesioned rats whereas the antidyskinetic properties were assessed in 6-hydroxydopamine hemilesioned rats chronically treated with levodopa. The ability of AT-403 to inhibit the D1 receptor-induced phosphorylation of striatal extracellular signal regulated kinase (ERK) was investigated.

**Key results:** AT-390 and AT-403 selectively improved akinesia at low doses and disrupted global motor activity at higher ones. AT-403 palliated dyskinesia expression without causing sedation in a narrow therapeutic window, whereas AT-390 delayed AIMs appearance and increased their duration at doses causing sedation. AT-403 did not prevent the priming to levodopa, although it significantly inhibited dyskinesia on the first day of administration. AT-403 reduced the ERK phosphorylation induced by SKF38393 in vitro and by levodopa in vivo.

**Conclusions and Implications:** NOP receptor stimulation can provide significant, albeit mild antidyskinetic effect, at doses not causing sedation. The therapeutic window, however, varies across compounds. AT-403 appears to be a potent and selective tool to investigate the role of NOP receptors in vivo.

**Non standard abbreviations**

AIMs	abnormal involuntary movements
ALO	axial, limb and orolingual
CHO	Chinese hamster ovary
DA	dopamine
$\delta$	delta opioid peptide
ERK	extracellular signal regulated kinase 1 and 2
$\kappa$	kappa opioid peptide
L-DOPA	levodopa
LID	levodopa-induced dyskinesia
$\mu$	mu opioid peptide
MPTP	1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine
MSNs	medium-sized spiny neurons
N/OFQ	nociceptin/orphanin FQ
NOP	nociceptin/orphanin FQ opioid peptide
6-OHDA	6-hydroxydopamine
PD	Parkinson's disease
SNr	substantia nigra reticulata

## Introduction

[Nociceptin/Orphanin FQ](#) (N/OFQ) and the N/OFQ opioid peptide receptor ([NOP receptor](#)) (previously known as Opioid Receptor Like 1 receptor) constitute a neuropeptide system carrying numerous structural and functional analogies with the classical opioid systems (Toll *et al.*, 2016). Nonetheless, the pharmacology of the N/OFQ-NOP receptor is distinct from those of classical opioid receptor systems, since N/OFQ does not bind classical opioid receptors, and most classical opioid receptor ligands (e.g. naloxone) do not bind the NOP receptor (Calo *et al.*, 2000; Zaveri, 2016). Because of its widespread distribution in the central nervous system, the N/OFQ-NOP receptor system is key in the regulation of multiple central functions, such as sensory nociceptive processing, mood, food intake, pain, reward and locomotion. Moreover, the N/OFQ-NOP receptor system is implicated in various neuropsychiatric disorders such as neuropathic pain (Lin *et al.*, 2013), anxiety and depression (Gavioli *et al.*, 2013), drug abuse (Cippitelli *et al.*, 2016; Lutfy *et al.*, 2016), and Parkinson's disease (PD) (Marti *et al.*, 2005; Viaro *et al.*, 2008).

As far as PD is concerned, preclinical models disclosed a potential role for NOP receptor agonists as antidyskinetic agents (Marti *et al.*, 2012). Acute central administration of N/OFQ or systemic administration of the small molecule NOP receptor agonist Ro 65-6570 attenuated the severity of dyskinesia in 6-OHDA hemilesioned rats and 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP)-treated macaques chronically treated with [levodopa](#) (L-DOPA). Dyskinesia represents a major complication of L-DOPA pharmacotherapy of PD (Bastide *et al.*, 2015); however, there are currently no drugs that can prevent dyskinesia development when chronically combined with L-DOPA in de novo PD patients (Bastide *et al.*, 2015; Huot *et al.*, 2013). Therefore, the identification of antidyskinetic drugs is an unmet clinical need. Since the antidyskinetic effect of NOP agonists was observed in rats after acute administration at doses that did not cause

hypolocomotion or sedation (Marti *et al.*, 2012), effects typical of NOP receptor agonists (Zaveri, 2016), we hypothesized that NOP agonists may exert specific antidyskinetic effects and be clinically useful in PD patients that have already become dyskinetic due to chronic L-DOPA therapy. In our first proof-of-concept study, we used the NOP agonist Ro 65-6570, which is only modestly selective for the NOP receptor, as the systemically-active agonist to replicate the effects of centrally administered N/OFQ. However, we did not specifically investigate whether N/OFQ or Ro 65-6570 worsened parkinsonian disabilities, which is worth investigating in view of the symptomatic effects of NOP receptor antagonists in 6-OHDA hemilesioned rats (Marti *et al.*, 2005; Marti *et al.*, 2004a; Marti *et al.*, 2008; Volta *et al.*, 2011), and other models of PD (Viaro *et al.*, 2008; Visanji *et al.*, 2008; Volta *et al.*, 2010).

Further, we recently demonstrated that NOP receptor stimulation opposes D1 receptor signaling in striatum (Marti *et al.*, 2012; Olanas *et al.*, 2008). It is well known that upregulation of D1 signalling cascade in striatal medium-sized spiny GABAergic neurons (MSNs) projecting to SNr (the so-called direct pathway), underlies the appearance of dyskinesia (Bastide *et al.*, 2015). In our previous study, we did not assess whether NOP receptor agonists, in addition to preventing expression of L-DOPA-induced dyskinesia (LID), a symptomatic effect, also attenuated the process of brain sensitization (priming) that underlies the development of dyskinesia when given chronically in combination with L-DOPA.

We recently discovered two high affinity, highly selective NOP receptor agonists AT-390 and AT-403, from two distinct chemical classes, which had greater than 100-fold selectivity over the  $\mu$ ,  $\delta$  and  $\kappa$  opioid receptors. We characterized these compounds for their functional activity and found them to be full agonists at the NOP receptor. AT-403 was recently characterized as a potent nonpeptide surrogate of the natural endogenous peptide

ligand N/OFQ, because it was found to have unbiased functional efficacy at the NOP G-protein as well as the arrestin signalling pathway, like the natural ligand N/OFQ (Ferrari *et al.*, 2017).

With these highly selective NOP agonists in hand, we undertook the present study to more fully investigate the antiparkinsonian and antidyskinetic profiles of these two novel and selective NOP receptor agonists, AT-403 and AT-390. In the first series of experiments, AT-403 and AT-390 were acutely administered to 6-OHDA hemilesioned rats to assess whether they improve or worsen parkinsonian motor deficits. Next, AT-403 and AT-390 were acutely administered to 6-OHDA hemilesioned, L-DOPA-primed dyskinetic rats to confirm that NOP receptor stimulation attenuates LID expression. Thirdly, to determine whether NOP receptor stimulation attenuates priming to L-DOPA, and therefore the development of dyskinesia, AT-403 was chronically administered to 6-OHDA hemilesioned rats in combination with L-DOPA for 20 days. Finally, to investigate whether AT-403 could specifically affect signalling pathways underlying dyskinesia, phosphorylation levels of striatal extracellular signal regulated kinase 1 and 2 (ERK), a D1-dependent biochemical correlate of LID (Santini *et al.*, 2007; Valjent *et al.*, 2005) were monitored both in vitro and ex-vivo.

## Methods

### Materials

AT-403 (2-(1-(1-((1s,4s)-4-isopropylcyclohexyl)piperidin-4-yl)-2-oxoindolin-3-yl)-N-methylacetamide) (Ferrari *et al.*, 2017) and AT-390 ((1-(1-(cis-4-isopropylcyclohexyl)piperidin-4-yl)-1H-indole-2,3-diyl)dimethanol) were synthesized at Astraea Therapeutics (Mountain View, CA, USA) and were dissolved in 1% CH<sub>3</sub>COOH 1M and 3% DMSO in water. Radioligands [<sup>3</sup>H]N/OFQ, [<sup>3</sup>H]DAMGO, and [<sup>3</sup>H]DPDPE

were obtained from the National Institute of Drug Abuse Drug Supply Program. [<sup>3</sup>H]U69593 and [<sup>35</sup>S]GTP $\gamma$ S was purchased from Perkin Elmer. L-DOPA, benserazide, 6-OHDA hydrobromide and d-amphetamine sulphate were purchased from Tocris Bioscience (Bristol, UK). L-DOPA, benserazide and d-amphetamine sulphate were dissolved in saline, 6-OHDA was dissolved in 0.02% ascorbic acid in saline

### ***Biochemical assays***

The binding affinities of AT-403 and AT-390 for the opioid receptors were determined in radioligand competition experiments using human opioid receptor-transfected Chinese hamster ovary (CHO) cells, as previously reported (Adapa *et al.*, 1997; Zaveri *et al.*, 2004). [<sup>3</sup>H]N/OFQ (130 Ci/mmol, 0.04 nM) was used as radioligand for the NOP receptor, [<sup>3</sup>H]U69,593 (120 Ci/mmol, 0.2 nM) for the  $\kappa$  receptor, [<sup>3</sup>H]DAMGO (120 Ci/mmol, 0.2 nM) for the  $\mu$  receptor, and [<sup>3</sup>H]CI-DPDPE (120 Ci/mmol, 0.2 nM) for the  $\delta$  receptor. IC<sub>50</sub> values were determined from at least six concentrations of test compound, and calculated using Graphpad/Prism (ISI, San Diego, CA). K<sub>i</sub> values (nM) were derived from the Cheng-Prusoff equation  $K_i = IC_{50} / (1 + [L] / K_d)$  where [L] is the concentration of the radioligand.

The functional efficacy of the compounds was determined by their ability to stimulate [<sup>35</sup>S]GTP $\gamma$ S binding to cell membranes, and compared to the standard agonists N/OFQ (NOP), DAMGO ( $\mu$ ), U69,593 ( $\kappa$ ) and DPDPE ( $\delta$ ), as previously described (Adapa *et al.*, 1997; Spagnolo *et al.*, 2008; Zaveri *et al.*, 2004).

### ***Animal Subjects***

Experiments were performed in accordance with the ARRIVE and BJP guidelines. Experimenters were blinded to treatments. Male Sprague-Dawley rats (150 g, 6 week old; Envigo, S. Pietro al Natisone, Italy) were housed in a standard facility with free access to

food (4RF21 standard diet; Mucedola, Settimo Milanese, Milan, Italy) and water, and kept under regular lighting conditions (12 hr dark/light cycle). Animals were housed in groups of 5 for a 55x33x20 cm polycarbonate cage (Tecniplast, Buguggiate, Varese, Italy) with a Scobis Uno bedding (Mucedola, Settimo Milanese, Milan, Italy) and environmental enrichments. The experimental protocols were approved by the Italian Ministry of Health (license n. 170/2013-B). Adequate measures were taken to minimize animal pain and discomfort. At the end of the experiments, rats were sacrificed with an overdose of isoflurane. **Six** male 2 month-old C57Bl6 mice were used for ERK studies *in vitro*. Mice were housed in a standard facility at Cardiff University, under regular conditions of light (12hr light/dark cycle), with food and water *ad libitum*.

#### ***ERK measurement in vitro***

Adult mice were decapitated after cervical dislocation and brain slices were freshly prepared according to the protocol described in (Marti *et al.*, 2012). The brains were rapidly removed and put on a cool glass plate filled with ice-cold sucrose-based dissecting solution (87 mM NaCl, 2.5 mM KCl, 7 mM MgCl<sub>2</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 75 mM sucrose, 25 mM NaHCO<sub>3</sub>, 10 mM D-glucose, 0.5 mM CaCl<sub>2</sub>, 2 mM kynurenic acid), carbogenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>), and subsequently mounted on the vibratome stage (Vibratome, VT1000S-Leica Microsystems). Two-hundred (200) µm-thick slices were cut and transferred into a brain slice chamber (Brain slice chamber-BSC1 - Scientific System design Inc., Mississauga, ON, Canada) and allowed to recover for 1 hr at 32°C, with a constant perfusion of carbogenated artificial cerebrospinal fluid (ACSF: 124 mM NaCl, 5 mM KCl, 1.3 mM MgSO<sub>4</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 10 mM D-glucose, 2.4 mM CaCl<sub>2</sub>). The D1 receptor agonist [SKF38393](#) (100 µM) was applied for 10 min in the presence of AT-403 (30 nM) or vehicle. After fixation in 4% PFA for 15

min at room temperature, slices were rinsed three times in PBS and cryoprotected in 30% sucrose solution overnight at 4°C. On the following day, slices were further cut into 18 µm-thick slices using a cryostat (Leica CM1850) and mounted onto SuperFrost Plus slides (Thermo Scientific). Immunohistochemistry was performed following the protocol described in (Papale *et al.*, 2016): 1 hr after blocking in 5% normal goat serum and 0.1% Triton X-100 solution, slices were incubated overnight at 4°C with anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) (1:1000, Cell Signaling Technology cat. #4370L). Sections were then incubated with biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories, cat. #BA-1000) for 2 hr at room temperature. Detection of the bound antibodies was carried out using a standard peroxidase-based method (ABC-kit, Vectastain, Vector Labs), followed by a DAB and H<sub>2</sub>O<sub>2</sub> solution. Images were acquired from the striatum at 40X magnification using a brightfield microscope (Leica Macro/Micro Imaging System) and the number of pERK positive cells **in the striatum** was counted in each slice.

### ***Unilateral 6-OHDA lesion***

The unilaterally 6-OHDA lesioned rat, the most popular and best validated model of PD (Duty *et al.*, 2011; Schwarting *et al.*, 1996), was used to assess the ability of AT-403 and AT-390 to improve parkinsonian-like motor deficits. Unilateral lesion of dopaminergic neurons was induced under isoflurane anaesthesia as previously described (Marti *et al.*, 2005). Eight micrograms of 6-OHDA freebase (dissolved in 0.9% saline solution containing 0.02% ascorbic acid) were stereotaxically injected in the medial forebrain bundle according to following coordinates from bregma: antero-posterior= -4.4 mm, medio-lateral= -1.2, dorso-ventral=-7.8 mm below dura (Paxinos *et al.*, 1986). Animals were pre-treated with antibiotics (Synulox™, 50 µL/Kg, i.p.). The wound was sutured and

infiltrated with 2% lidocaine solution (Esteve™). In order to select rats that were successfully hemilesioned, two weeks after 6-OHDA injection, rats were injected with a test dose of D-amphetamine (5 mg kg<sup>-1</sup>, i.p., dissolved in saline), and those performing >7 turns per minute in the direction ipsilateral to the lesion were enrolled in the study. In fact, this behaviour has been associated with >95% loss of striatal dopaminergic terminals (Marti *et al.*, 2007) and extracellular dopaminergic levels (Marti *et al.*, 2002).

### **Behavioral Tests**

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

*Drag Test.* The drag test (Marti *et al.*, 2005), 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 backward dragging. Each rat was gently lifted from the abdomen leaving the forepaws on the table and dragged backwards at a constant speed of 20 cm sec<sup>-1</sup> for a fixed distance of 1 m. Two different observers counted the number of touches made by each forepaw.

*Rotarod Test.* This test measures the ability of the animal to run on a rotating cylinder and provides different information on a variety of motor parameters such as coordination, balance, muscle tone, gait and motivation to run (Rozas *et al.*, 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 5 rpm, speed was stepwise increased by 5 rpm every 180 sec, and total time spent on the rod was calculated.

*L-DOPA treatment and abnormal involuntary movements rating*

Rats that successfully performed the amphetamine test were treated for 20 days with L-DOPA (6 mg kg<sup>-1</sup> + benserazide 12 mg kg<sup>-1</sup>, s.c., once daily) to induce abnormal involuntary movements (AIMs), a correlate of LID, as previously described (Bido *et al.*, 2011; Marti *et al.*, 2012; Mela *et al.*, 2012; Mela *et al.*, 2010; Paolone *et al.*, 2015). This represents the best validated model of dyskinesia in rodents, (Bastide *et al.*, 2015; Cenci *et al.*, 1998; Cenci *et al.*, 2007). Rats were observed for 1 min, every 20 min, 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 (Cenci *et al.*, 1998; Cenci *et al.*, 2007): (1) Axial AIM, i.e., twisted posture or turning of the neck and upper body toward the side contralateral to the lesion; (2) forelimb AIM, i.e., jerky and dystonic movements and/or purposeless grabbing of the forelimb contralateral to the lesion; (3) orolingual AIM, 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 scale from 0 to 4 based on a previously validated scale (Cenci *et al.*, 2007). Axial, Limb and Orolingual (ALO) AIMs total value were obtained as the sum of the product between amplitude and frequency of each observation (Cenci *et al.*, 2007). Therefore the theoretical maximum ALO AIMs score is 432; to be considered fully dyskinetic an animal has to score  $\geq 100$ .

### ***Western blot analysis***

Dyskinetic rats were treated with saline or AT-403 (0.1 mg kg<sup>-1</sup>), and 15 min later with L-DOPA (6 mg kg<sup>-1</sup> + benserazide 12 mg kg<sup>-1</sup>, s.c.). Thirty min after L-DOPA, rats were anesthetised with isoflurane, sacrificed by decapitation and striata rapidly dissected, frozen

on dry ice and stored at  $-80^{\circ}\text{C}$  until analysis. Tissues were homogenized in lysis buffer (RIPA buffer, protease inhibitor cocktail and phosphatase inhibitor cocktail) and centrifuged at 13,000 rpm at  $4^{\circ}\text{C}$  for 15 min. Supernatants were collected and protein levels were quantified using the bicinchoninic acid protein assay kit (Thermo Scientific). Thirty micrograms of protein per sample were separated on a 4-12% gradient polyacrilamide precast gels (Bolt® 4-12% Bis-Tris Plus Gels, Life Technologies) in a Bolt® Mini Gel Tank apparatus (Life Technologies). Proteins were then transferred onto polyvinylidene difluoride membrane, blocked for 60 min with 5% non-fat dry milk in 0.1% Tween20 Tris-buffered saline, and incubated overnight at  $4^{\circ}\text{C}$  with anti-Thr202/Tyr204-phosphorylated ERK1/2 (pERK) rabbit monoclonal antibody (Merck Millipore, cat. #05-797R, 1:1000) or with anti-ERK1/2 (totERK) rabbit polyclonal antibody (Merck Millipore cat. #06-182, 1:5000). Membranes were washed, then incubated 1 hr at room temperature with horseradish peroxidase-linked secondary antibodies (Merck Millipore, cat. #12-348, 1:5000). Immunoreactivity was visualized by enhanced chemiluminescence (ECL) detection kit (Perkin Elmer), images were acquired using the ChemiDoc MP System quantified using the ImageLab Software (Bio-Rad). Membranes were then stripped and re-probed with rabbit monoclonal anti-tubulin antibody (Merck Millipore, cat. #04-1117, 1:50000). Data were analyzed by densitometry, and the optical density of specific pERK or totERK bands was normalized to the corresponding tubulin levels.

### ***Experimental protocols and design***

Experiments were performed according to BJP guidelines. Overall, 149 rats and 6 mice were used in this study. Timelines of the different studies are illustrated in Fig. 1.

*Motor behavior in 6-OHDA hemilesioned, L-DOPA naïve rats*

Fifty-two (52) rats were hemilesioned with 6-OHDA, 40 of which passed the amphetamine testing after 2 weeks, meeting the selection criteria (Fig. 1). Ten days later, these rats were subjected to the bar, drag and rotarod tests repeated in a fixed sequence as a training. When motor performance was reproducible (usually after 7-10 days), rats were randomized and treated with AT-403 (0.001, 0.01, 0.03, 0.1 and 0.3 mg kg<sup>-1</sup>), AT-390 (0.03, 0.1, 1 and 3 mg kg<sup>-1</sup>), the NOP receptor antagonist [SB-612111](#), AT-403 + SB-612111 or vehicle. Rats were tested 3-4 times, and a 3-day washout was allowed between treatments. Motor activity was assessed 30 and 90 min after AT-403 administration, or 60, 120 and 180 min after AT-390 administration, and expressed as absolute values.

#### *LID expression*

Seventy-three (73) rats were hemilesioned with 6-OHDA, 60 of which passed the selection criteria (Fig. 1). Fifty-two (52) 6-OHDA hemilesioned rats were chronically treated with L-DOPA, whereas 8 were left untreated as a control for the ERK analysis. At the end of L-DOPA treatment, 24 fully dyskinetic rats (ALO AIMS score  $\geq 100$ ) were randomized to L-DOPA (6 mg kg<sup>-1</sup> plus benserazide 12 mg kg<sup>-1</sup>, s.c.) in combination with AT-403 (0.03 and 0.1 mg kg<sup>-1</sup>), AT-390 (0.3, 1 and 3 mg kg<sup>-1</sup>) or vehicle. Each animal was tested 3-4 times, with a 3-day washout allowed between treatments. On separate days, motor performance was measured using the rotarod test, both before (OFF L-DOPA) and 60 min after L-DOPA administration (ON L-DOPA), to evaluate whether the potential antidyskinetic effect was associated with an improvement of global motor activity. This time point was chosen based on the ALO AIMS time-course, which showed a peak 60-80 min after L-DOPA administration (Bido *et al.*, 2011; Marti *et al.*, 2012; Mela *et al.*, 2012; Mela *et al.*, 2010; Paolone *et al.*, 2015). Sixteen (16) additional, fully dyskinetic rats were used for ERK analysis and parsed into two groups, one treated with saline plus L-DOPA and another with 0.1 mg kg<sup>-1</sup> AT-403 plus L-DOPA.

### *LID induction*

Twenty-four (24) naïve rats were hemilesioned with 6-OHDA, 18 of which passed the selection criteria and were treated with L-DOPA in combination with vehicle (6 mg kg<sup>-1</sup> plus benserazide 12 mg kg<sup>-1</sup>, s.c.; n=9) or AT-403 (0.03 mg kg<sup>-1</sup>, s.c., given 15 min before L-DOPA; n=9) for 20 days. ALO AIMs score was performed at days 1, 5, 10, 15 and 20. At the end of the 20-day treatment, all animals were allowed a 24-hr washout, and then given a L-DOPA challenge (dosage as above) in order to verify whether the antidyskinetic effect was due a reduction of priming rather than a symptomatic effect. During these 3 weeks, motor activity was assessed in 4 different occasions (days 3, 8, 14, 19) with the rotarod test performed both OFF and ON L-DOPA (60 min after injection).

### *Data presentation and statistical analysis*

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Motor performance was expressed as absolute values (time on bar or rod, number of steps), and statistically analysed using PRISM 6.0 (GraphPad Software Inc., San Diego, CA) with two-way repeated measure (RM) ANOVA followed by the Bonferroni test (Fig. 2 and Fig. 4) or one-way ANOVA followed by the Newman-Keuls test (Fig. 3). ALO AIMs score was expressed as absolute values and statistically analysed with two-way RM ANOVA followed by Bonferroni test (time-course; Fig. 5A, 6A, 7A-E) or one-way ANOVA followed by the Newman-Keuls test (cumulative AIMs score; Fig. 5B and 6B). Rotarod performance in dyskinetic animals was expressed as time on rod (in sec) and analysed with two-way ANOVA followed by Bonferroni test (Fig. 5C and 6D). In vitro ERK data (Fig. 8A) were analysed using two-way ANOVA followed by the Bonferroni test, whereas ex-vivo ERK data were analysed

by one-way ANOVA followed by the Newman-Keuls test. Statistical significance was set at  $p < 0.05$ .

### Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

### Results

#### *Receptor and GTP $\gamma$ S binding*

AT-403 and AT-390 showed high nanomolar affinity for the NOP receptor expressed in CHO cells ( $K_i$  of 1.1 nM and 0.9 nM, respectively), and significantly lower affinity for the other opioid receptors (Table 1). The selectivity ratios for NOP over classical opioid receptors were: AT-390, 59-fold ( $\mu$ ), 125-fold ( $\delta$ ) and 95-fold ( $\kappa$ ); AT-403, 89-fold ( $\mu$ ), 3700-fold ( $\delta$ ) and 1420-fold ( $\kappa$ ). Both compounds were characterized for their functional activity in the [ $^{35}$ S]GTP $\gamma$ S binding assay using a range of concentrations up to 10  $\mu$ M. In this assay, AT-403 and AT-390 mimicked the stimulatory effects of N/OFQ at the NOP receptor, showing similar efficacy (110% and 104%, respectively) and slightly lower potency ( $EC_{50}$ : N/OFQ 3.6 nM, AT-403 6.3 nM AT-390 15.2 nM; Table 1), suggesting they are full agonists at the NOP receptor. Higher concentrations of AT-403 and AT-390 appeared to stimulate the  $\mu$  opioid receptor, although with significantly lower potency (overall <60%). Potency selectivity ratios of AT-403 and AT-390 for NOP over the  $\mu$  receptor were 33-fold and 10-fold, respectively, and maximal stimulation was 33% and 54% of positive controls, respectively (Table 1).

*Effect on parkinsonian disabilities*

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 (s.c.) in the 0.001-0.3 mg kg<sup>-1</sup> dose-range, and motor activity evaluated by the bar, drag and rotarod tests 30 and 90 min after injection. After preliminary screening, we observed that rats (n=3) administered with AT-403 0.3 mg kg<sup>-1</sup> were unable to perform for 1 hour after drug administration, due to profound, though reversible, sedation/hypolocomotion. Therefore, the study was continued with the lower doses.

In the bar test (Fig. 2A), ANOVA showed a significant effect of treatment ( $F_{4,2}=5.99$ ;  $p=0.0004$ ), time ( $F_{2,130}=57.33$ ;  $p<0.0001$ ) and time x treatment interaction ( $F_{8,130}=6.07$ ;  $p<0.0001$ ) at the contralateral paw. Post hoc analysis revealed that AT-403 caused a transient reduction (30 min time point) of the time on bar at the contralateral paw at 0.01 mg kg<sup>-1</sup>, showing more profound and long lasting effects at the higher doses (Fig. 2A). AT-403 did not affect stepping activity at the contralateral paw (Fig. 2B). In the rotarod test (Fig. 2C), ANOVA did not show an overall effect of treatment ( $F_{4,2}=1.45$ ;  $p=0.22$ ), but a significant effect of time ( $F_{2,130}=6.34$ ;  $p=0.0023$ ) and time × treatment interaction ( $F_{8,130}=3.08$ ;  $p=0.0008$ ). Post hoc analysis revealed a significant decrease of rotarod performance with the highest dose of AT-403 (0.1 mg kg<sup>-1</sup>) at 30 min after administration.

To investigate the NOP selectivity of AT-403, the effects of systemic administration of AT-403 (0.01 mg kg<sup>-1</sup>, s.c.) in combination with the NOP receptor antagonist SB-612111 were evaluated in the bar test (Fig. 3). SB-612111 was administered at a dose (0.01 mg kg<sup>-1</sup>, s.c.) ineffective per se on motor behaviour (Marti *et al.*, 2013). ANOVA showed a significant effect of treatment ( $F_{3,31}=3.77$ ;  $p=0.020$ ) (Fig. 3). Post hoc analysis revealed

that AT-403 reduced the time on bar and SB-612111, ineffective by itself, prevented such effect.

To confirm that NOP receptor stimulation dually modulated parkinsonian disabilities, behavioural observations were extended up to 180 min after AT-390 injection (Fig. 4). In a dose-finding experiment, a profound and long lasting sedation/hypolocomotion was observed with  $3 \text{ mg kg}^{-1}$  AT-390. Animals were unable to perform for at least 2 hours after drug administration; therefore, the study was continued with lower doses. Very much like AT-403, AT-390 improved the immobility time at the contralateral paw (Fig. 4A; treatment  $F_{3,3}=5.39$   $p=0.0047$ ; time  $F_{3,84}=11.09$ ,  $p<0.0001$ , time  $\times$  treatment interaction  $F_{9,84}=4.59$   $p<0.0001$ ). A transient reduction of immobility time was observed at 120 min after injection of  $0.1 \text{ mg kg}^{-1}$  AT-390, whereas a sustained (up to 180 min) inhibition was observed with both  $0.3$  and  $1 \text{ mg kg}^{-1}$  AT-390, with the effect of the former showing a tendency to fade over time. In the drag test, no significant effect was observed at the contralateral paw (Fig. 4B). Finally, in the rotarod test (Fig. 4C), transient inhibition was detected at 60 min with all doses (treatment  $F_{3,3}=4.07$   $p=0.016$ ; time  $F_{3,84}=19.65$   $p<0.0001$ ; time  $\times$  treatment interaction  $F_{9,84}=5.54$   $p<0.0001$ ). The effect was particularly dramatic with  $1 \text{ mg kg}^{-1}$  AT-390. A milder, though significant, inhibition was still observed at 2 hours with  $0.3$  and  $1 \text{ mg kg}^{-1}$  AT-390, whereas the effect disappeared 3 hrs after treatment.

#### *Effects on AIMs expression*

To investigate whether NOP receptor stimulation prevents the expression of AIMs, AT-403 and AT-390 were administered 15 min before an L-DOPA challenge in dyskinetic rats (Fig. 5). AT-403 caused a delay in LID appearance without significantly affecting the overall duration of the response to L-DOPA (treatment  $F_{2,8}=7.92$ ;  $p=0.0012$ ; time  $F_{8,352}=47.47$ ,  $p<0.0001$ ; time  $\times$  treatment interaction  $F_{16,352}=15.17$ ,  $p<0.0001$ ; Fig. 5A). ANOVA on itemized cumulative AIMs (Fig. 5B) showed that AT-403 at  $0.1 \text{ mg kg}^{-1}$

markedly inhibited all AIM subtypes whereas AT-403 at 0.03 mg kg<sup>-1</sup> cause a mild and selective inhibition of limb AIMS (Fig. 5B). The beneficial effect of 0.1 mg kg<sup>-1</sup> AT-403 was accompanied by overt sedation/hypolocomotion within the first hour after compound administration. This was also evident when rats were challenged on the rotarod. Indeed, control animals displayed dramatic reduction (75%) of the rotarod performance at peak dyskinesia (Fig 5C), whereas animals treated with 0.03 mg kg<sup>-1</sup> AT-403 were significantly less impaired (55% reduction). Animals treated with 0.1 mg kg<sup>-1</sup> AT-403 instead, were inhibited as much as controls, indicating that the absence of dyskinetic movements was not associated with better rotarod performance.

AT-390 significantly modified AIMS time-course, although in a quite unexpected way (Fig. 6A). While 0.3 mg kg<sup>-1</sup> AT-390 did not significantly affect the response to L-DOPA, 1 mg kg<sup>-1</sup> AT-390 caused a delay in AIMS appearance and a prolongation of AIMS duration up to 240 min after L-DOPA injection, such that the overall response to L-DOPA was quantitatively unchanged (treatment  $F_{2,13}=0.14$ ;  $p=0.87$ , time  $F_{13,260}=51.43$ ;  $p<0.0001$ , time  $\times$  treatment:  $F_{26,260}=9.34$ ,  $p<0.0001$ ) (Fig 6A). To confirm this profile, we injected a higher dose of AT-390 (3 mg kg<sup>-1</sup>) in a small number of rats ( $n=4$ , Fig 6C). The effect was particularly dramatic as a further rightward shift in the time-course was observed, with longer delay and duration of the AIMS response. As observed for AT-403, a clear sedative/hypolocomotive effect was associated with the delayed response to 1 mg kg<sup>-1</sup> AT-390. Indeed, even though AIMS were significantly reduced at 60 min after L-DOPA injection, rotarod performance was dramatically impaired (90%), similar to controls (Fig. 6D).

#### *Effect on AIMS development (priming protocol)*

The experiments in dyskinetic animals revealed that only AT-403 (0.03 mg kg<sup>-1</sup>) provided an acute, albeit mild, antidyskinetic effect, without causing primary

sedation/hypolocomotion. We therefore tested whether this dose could attenuate AIMs development when chronically combined to L-DOPA.

Animals treated with L-DOPA alone developed severe AIMs within the first week of treatment (Fig. 7A). Then, dyskinesia scores remained maximal until the end of the 20-day period. AT-403 did not confer overall protection against AIMs development, although AT-403 treated rats showed a tendency to be less dyskinetic than controls throughout the study. Nonetheless, a significant attenuation of dyskinesia was observed at first administration (day 1; treatment,  $F_{1,8}=2.55$ ,  $p=0.13$ ; time,  $F_{8,128}=8.99$ ,  $p<0.0001$ , time  $\times$  treatment interaction  $F_{8,128}=2.51$ ,  $p=0.0143$ ) (Fig. 7B). This effect, however, disappeared in subsequent experimental sessions (Fig. 7C-E).

At days 3, 8, 14 and 19, rotarod activity was evaluated at 60 min after L-DOPA administration (treatment,  $F_{3,3}=5.89$ ,  $p=0.0025$ ; time,  $F_{3,96}=3.14$ ,  $p=0.0289$ , time  $\times$  treatment interaction  $F_{9,96}=0.62$ ,  $p=0.77$ ) (Fig 7F). ANOVA revealed that rats treated with L-DOPA alone poorly performed on the rotarod from the first through the last session, whereas animals treated with  $0.03 \text{ mg kg}^{-1}$  AT-403 were significantly impaired only at day 19.

#### *Effects on ERK phosphorylation*

D1 receptor activation increases pERK levels in striatal MSNs (Valjent *et al.*, 2005), and elevated pERK levels are associated with LID (Pavon *et al.*, 2006; Santini *et al.*, 2007). We therefore investigated whether AT-403 could reduce D1 agonist-stimulated pERK levels in striatum. In the first set of experiments (Fig. 8A), application of the D1 agonist SKF38393 ( $100 \mu\text{M}$ ) to striatal slices of naïve mice caused a ~4-fold increase in the number of pERK immunoreactive cells. AT-403 alone tended to reduce basal pERK levels and, when co-applied with SKF38393, prevented its effect (AT-403  $F_{1,44}=29.20$   $p<0.0001$ ; SKF38393  $F_{1,44}=43.30$   $p<0.0001$ ; interaction  $F_{1,44}=29.61$   $p<0.0001$ ). We therefore investigated

whether AT-403 could also be effective in vivo (Fig. 8B-C; Supporting Information Figure S1). 6-OHDA dyskinetic rats were pretreated with saline or AT-403 0.1 mg kg<sup>-1</sup> and 15 min later, challenged with L-DOPA. Dyskinetic animals showed a 40% increase of pERK levels in the ipsilateral compared to the contralateral striatum whereas AT-403-treated dyskinetic and 6-OHDA hemilesioned rats showed similar levels in both striata (Fig. 8B; F<sub>5,42</sub>=5.96, p=0.0003). Indeed, pERK levels at the ipsilateral side were normalized in AT-403-treated dyskinetic rats compared to dyskinetic controls and 6-OHDA hemilesioned rats. Interestingly, statistical analysis also revealed a ~30% reduction in pERK levels at the contralateral side in AT-403-treated dyskinetic rats compared to 6-OHDA hemilesioned rats. AT-403 did not affect total ERK levels (Fig. 8C), showing the effect was specific for the phosphorylated form of the protein.

## Discussion

AT-403 and AT-390 are novel small-molecule NOP receptor agonists with affinity, potency, and selectivity for the NOP receptor better than those of our previously used NOP agonist Ro 65-6570 (Marti *et al.*, 2012), and in line with most potent and selective NOP receptor agonists described thus far (Zaveri, 2016). In vivo, both AT-403 and AT-390 significantly improved parkinsonian-like akinesia at low doses and disrupted motor coordination and global behaviour at higher ones. AT-403 was remarkably potent, 10-fold more than AT-390, causing behavioural effects at doses as low as 0.01 mg kg<sup>-1</sup>, and overt sedation at 0.3 mg kg<sup>-1</sup>. Various studies have shown that genetic deletion or pharmacological blockade of the NOP receptor improve motor activity in naïve mice, rats and nonhuman primates (Candeletti *et al.*, 2000; Marti *et al.*, 2004b; Marti *et al.*, 2008; Rizzi *et al.*, 2011; Viaro *et al.*, 2008) suggesting that endogenous N/OFQ tonically inhibits

motor behaviour. Motor inhibition is also typically observed when intermediate-to-high doses of the natural agonist N/OFQ are exogenously administered. However, exogenous N/OFQ is capable of promoting movement when given at low doses (Florin *et al.*, 1996; Kuzmin *et al.*, 2004; Marti *et al.*, 2009; Viaro *et al.*, 2013), an effect that originates from changes of primary motor cortex activity (Marti *et al.*, 2009). The biphasic effect on motor function produced by AT-403 and AT-390 is similar to that of the NOP receptor agonist SCH 655842 in mice, where low doses stimulated and higher doses inhibited total distance travelled (Lu *et al.*, 2011). Nonetheless, not all small-molecule NOP receptor agonists replicate this profile, since Ro 64-6198 caused only inhibition (Kuzmin *et al.*, 2004). The finding that low doses of AT-403 and AT-390 reduced the immobility time in the catalepsy test, and that this effect persisted also at doses inhibiting rotarod performance, would indicate a specific control operated by NOP receptors on neuronal circuits regulating time to initiate movement, i.e. akinesia. Indeed, the effect is clearly mediated by NOP receptors, since it was reversed by SB-612111, at doses per se ineffective on motor function. The reason why AT-403 and AT-390 did not also affect stepping activity or rotarod performance at these low doses may be due to plastic changes of N/OFQ transmission in the parkinsonian brain. In particular, since N/OFQ tone is reduced in some areas (e.g. striatum) and elevated in others (e.g. SNr), we speculate that AT-403 and AT-390 may target specific subpopulations of NOP receptors that are not saturated by endogenous N/OFQ, or that are even up-regulated, perhaps as a compensatory response following the drop of N/OFQ levels (Marti *et al.*, 2012). Alternatively, we must consider the possibility that the reduction of immobility time may reflect changes in non-motor functions. Indeed in rats, NOP receptor agonists exert anxiolysis at low, non-sedative doses (Jenck *et al.*, 2000; Lu *et al.*, 2011; Varty *et al.*, 2008). However, the finding that Ro 65-6570 ameliorated axial symptoms in MPTP-treated nonhuman primates (Marti *et al.*, 2012)

seems to confirm the view that NOP receptor stimulation can exert positive motor effects, even under parkinsonian conditions, in different animal species.

We previously reported that the poorly NOP-selective (10-fold over  $\mu$  receptor) small-molecule NOP agonist Ro 65-6570, reduced LID expression without causing primary hypolocomotion (Marti *et al.*, 2012). The present study confirms that NOP receptor stimulation counteracts the emergence of dyskinesia. Nonetheless, only for AT-403 it was possible to find a (narrow) therapeutic window where inhibition of LID was not associated with sedation/hypolocomotion. In fact, the antidyskinetic effect of 0.03 mg kg<sup>-1</sup> AT-403 was associated with improvement of rotarod performance ON L-DOPA, as previously shown for Ro 65-6570, and as expected from drugs that reduce dyskinetic movements thereby improving motor coordination (Marti *et al.*, 2012). We cannot prove whether the antidyskinetic and the sedative effects are temporally-spaced aspects of a common behavioural response or, if they are independent phenomena instead. In support of the latter however, we showed that N/OFQ injection in the striatum, i.e. a brain area not specifically involved in attention control, inhibited AIMs expression (Marti *et al.*, 2012). Moreover, we found that AT-403 was capable of inhibiting D1-receptor stimulated expression of Extracellular Signal-Regulated Kinase (ERK), a biochemical fingerprint of LID, in vitro and ex-vivo, suggesting that the antidyskinetic effect of NOP agonists specifically relies on the NOP receptor ability to negatively interfere with D1 signaling in direct pathway striatal MSNs neurons (Marti *et al.*, 2012). **The finding that pERK levels at the contralateral side were reduced in AT-403-treated rats compared to 6-OHDA hemilesioned rats is consistent with this view.** This raises the possibility that the antidyskinetic and sedative/hypolocomotive have different neuroanatomical substrates and can be pharmacologically parsed. In fact, not all NOP agonists tested thus far displayed the same thresholds for antidyskinetic and sedative responses. For instance, Ro 65-6570

exerted motor inhibition at doses ( $1 \text{ mg kg}^{-1}$ ) higher than those attenuating dyskinesia ( $0.01 \text{ mg kg}^{-1}$ ) whereas for AT-390 the two responses were superimposable. The wideness of the therapeutic window may thus be compound-dependent and relate to several pharmacodynamic/pharmacokinetic reasons, among which selectivity/activity against different populations of NOP receptors (or NOP receptor-regulated motor pathways), different brain penetrance in brain areas mediating the two effects, or different off-targets. Moreover, recent studies pointed out that Ro 65-6570 is a G protein biased ligand, being less efficient in activating the  $\beta$ -arrestin2 pathway than N/OFQ (Ferrari *et al.*, 2016) or AT-403 (Ferrari *et al.*, 2017). A different efficacy towards the  $G_i$  and  $\beta$ -arrestin2 pathways may thus result in a dissociation between the antidyskinetic and sedative effects.

The ability of AT-390 to prolong AIMs duration was surprising. This effect was dose-dependent, and likely unrelated to the mechanisms underlying the delay in AIMs appearance since it was not shared by AT-403. Interestingly, it was shown that Ro 65-6570 potentiated cocaine-induced locomotion. Both L-DOPA and cocaine are capable of elevating striatal dopamine (DA) levels, and indirect evidence that NOP agonists improve DA transmission has been presented. In fact, the motor stimulating effect of N/OFQ is blocked by a D2 antagonist or by genetic removal of the D2 receptor (Florin *et al.*, 1996; Viaro *et al.*, 2013). Therefore, the prolongation of L-DOPA effect might rely on a prolonged DA output. Interestingly, we previously showed that NOP antagonists can also promote movement by elevating D2 transmission (Viaro *et al.*, 2013), and are capable of potentiating L-DOPA therapeutic effects, at the cost of inducing dyskinesia (Marti *et al.*, 2012; Visanji *et al.*, 2008). Therefore, low degree stimulation of NOP receptors and NOP receptor blockade appear to share similar mechanisms and motor effects (Viaro *et al.*, 2013). However, prolongation of AIMs duration was not previously reported with Ro 65-6570 or N/OFQ itself (Marti *et al.*, 2012). Therefore, alternative explanations should be

taken into account, such that AT-390 stimulates specific subpopulation of NOP receptors, inhibits enzymatic degradation of synaptic DA (newly formed from L-DOPA) or even L-DOPA itself, or has off-target effects. Nonetheless, the ability of AT-390 to prolong L-DOPA action deserves further investigation. In fact, the prolongation of L-DOPA action might help minimize long-term side effects of the drug, such as dyskinesia and motor fluctuations, which are due to oscillations of L-DOPA plasma levels and, consequently, DA brain levels.

Chronic administration of  $0.03 \text{ mg kg}^{-1}$  AT-403 failed to affect overall LID development, suggesting that NOP receptor stimulation with such low dose of AT-403 does not prevent the sensitization (i.e. priming) of striatal MSNs to L-DOPA. Nonetheless, AT-403 significantly reduced AIMs appearance at first administration and, consistently, spared rotarod performance ON L-DOPA. Thus, we might speculate that the inability of AT-403 to prevent the priming to L-DOPA is due to development of NOP receptor desensitization. Indeed, receptor tolerance to the analgesic effect can develop after prolonged exposure to NOP agonists (Khroyan *et al.*, 2007; Micheli *et al.*, 2015). If this were the case, a NOP receptor partial agonist might exert a prolonged efficacy, due to its lower propensity to desensitize the receptor. In addition, such a molecule might provide additional symptomatic benefit due to its ability to attenuate overactive N/OFQ transmission in SNr, which appears to contribute to motor deficit in parkinsonism models (Marti *et al.*, 2005; Marti *et al.*, 2012). However, we should note that other behavioural responses to NOP receptor agonists, such as the anxiolytic effect of Ro 64-6198 (Dautzenberg *et al.*, 2001) or the protective effect on alcohol drinking of MT-7716 (Ciccocioppo *et al.*, 2014) do not undergo tolerance after repeated administration. Alternatively, we can speculate that the modulation operated by such a low dose of AT-403 is insufficient to fully counteract LID development, being effective only when the process is in its initial phase. In this respect,

since the antidyskinetic effect of AT-403 might rely on a negative NOP-D1 receptor functional interaction in direct pathway striatal MSNs, higher AT-403 doses (e.g. 0.1 mg kg<sup>-1</sup>) would prove more effective. However, these doses are burdened by severe sedation, and whether repeated administration widens the therapeutic window, having a different impact on the thresholds of the antidyskinetic and the sedative effects, cannot be predicted.

### *Conclusions*

The novel small molecule NOP receptor agonists, AT-390 and AT-403, were used to investigate the effect of NOP receptor stimulation on parkinsonian-like disabilities and LID in 6-OHDA hemilesioned rats. Both compounds selectively improved akinesia at low doses, and disrupted motor coordination and general behaviour at higher ones, confirming that NOP receptor stimulation biphasically regulates motor function. AT-403 also provided a specific, albeit mild, therapeutic effect in protocols of LID expression and development, which was associated with inhibition of biochemical marker of D1 receptor-induced striatal MSNs activation. These data confirm that, beyond their well characterized anti-reward, anxiolytic and analgesic effects (Zaveri, 2016), NOP receptor agonists may be therapeutically useful to attenuate dyskinesia in L-DOPA-treated parkinsonian patients (Marti *et al.*, 2012). Nonetheless, further studies are needed to pin down the neurobiological substrates of the antidyskinetic and sedative/hypolocomotive effects in order to develop NOP receptor agonists with an optimal therapeutic window.

### **Author contributions**

LA and CAP performed surgery, L-DOPA treatments and AIMs scoring, LA, SN, MF carried out motor tests. BVJ and MEM synthesised AT-403 and AT-390 and WEP performed binding and GTP $\gamma$ S experiments. IM and SF carried ERK studies in slices, DM

carried out Western blot analysis. RB drafted the manuscript, NTZ and MM conceived the study and drafted the manuscript.

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### Conflicts of interest

All authors declare no conflicts of interest.

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## Figure legends

**Figure 1. Timelines of studies in 6-OHDA rats.** Timelines of studies on motor disabilities (upper panel), dyskinesia expression (middle panel) and dyskinesia development (lower panel). RBD, bar, drag e rotarod sessions. A, AIMs scoring. R, rotarod sessions.

**Figure 2. Effect of AT-403 on parkinsonian disabilities.** Motor activity of 6-OHDA hemilesioned rats was evaluated in the bar (A), drag (B) and rotarod (C) tests, before, 30 min and 90 min after vehicle or AT-403 administration (0.001, 0.001, 0.03 and 0.1 mg kg<sup>-1</sup>, s.c.). Data are expressed as immobility time (in sec, A), number of steps (B) and time on rod (in sec, C), and are means ± SEM of n=14 determinations per group. Statistical

analysis was performed by two-way RM ANOVA followed by the Bonferroni test for multiple comparisons \* $p < 0.05$ , different from vehicle.

**Figure 3. NOP receptor specificity of the AT-403 effect.** The NOP receptor antagonist SB-612111 (0.01 mg kg<sup>-1</sup>, s.c.) was challenged against a low dose of AT-403 (0.01 mg kg<sup>-1</sup>, s.c.), and the effect evaluated in the bar test. Data are expressed as immobility time (in sec) and are means  $\pm$  SEM of  $n=9$  (vehicle, AT-403, SB-612111) or  $n=8$  (AT-403+SB-612111; one data discarded due to experimental loss) determinations per group. Statistical analysis was performed by one-way ANOVA followed by the Newman-Keuls test for multiple comparisons. \* $p < 0.05$ , different from vehicle, # $p < 0.05$ , different from AT-403.

**Figure 4. Effect of AT-390 on parkinsonian disabilities.** Motor activity of 6-OHDA hemilesioned rats was evaluated in the bar (A), drag (B) and rotarod (C) tests, before, 60 min, 120 min and 180 min after vehicle or AT-390 administration (0.1, 0.3 and 1 mg kg<sup>-1</sup>, s.c.). Data are expressed as immobility time (in sec, A), number of steps (B) and time on rod (in sec, C), and are means  $\pm$  SEM of  $n=9$  (vehicle, AT-390 0.3 mg kg<sup>-1</sup>, AT-390 1 mg kg<sup>-1</sup>) determinations per group. The effect of AT-390 0.01 mg kg<sup>-1</sup> was also assessed, although in a lower number of rats ( $n=5$ ). Statistical analysis was performed by two-way RM ANOVA followed by the Bonferroni test for multiple comparisons \* $p < 0.05$ , \* $p < 0.05$ , significantly different from vehicle.

**Figure 5. AT-403 attenuated the expression of L-DOPA-induced dyskinesia.** Axial, limb and orolingual (collectively ALO) abnormal involuntary movements (AIMs) were scored in 6-OHDA hemilesioned dyskinetic rats following challenge with L-DOPA (6 mg kg<sup>-1</sup> plus benserazide 12 mg kg<sup>-1</sup>, s.c.,  $n=16$ ) combined with vehicle (s.c.,  $n=16$ ), AT-403 0.03

mg kg<sup>-1</sup> (s.c., n=15; one data point was discarded as the animal did not respond to L-DOPA) or AT-403 0.1 mg kg<sup>-1</sup> (s.c., n=16). Data (means ± SEM) are expressed as ALO AIMs score for each-time point in absolute values (A) or as separate axial, limb and orolingual AIMs scores over the 3 hr observation period (i.e. cumulative scores calculated as the sum of scores at each time-point; B). On a separate day, treatments were replicated in the same animals, and rotarod performance (time on rod in sec, n=10 determinations per group) was evaluated before and 60 min after drug administration (C). \*p<0.05, significantly different from vehicle. #p<0.05 different from vehicle ON L-DOPA. Statistical analysis was performed by two-way RM (A) or two-way (C) ANOVA followed by the Bonferroni test for multiple comparisons or one-way ANOVA followed by the Newman-Keuls test for multiple comparisons (B).

**Figure 6. AT-390 delayed and prolonged the expression of L-DOPA-induced dyskinesia.**

Axial, limb and orolingual (collectively ALO) abnormal involuntary movements (AIMs) were scored in 6-OHDA hemilesioned dyskinetic rats following challenge with L-DOPA (6 mg kg<sup>-1</sup> plus benserazide 12 mg kg<sup>-1</sup>, s.c.) combined with vehicle or AT-390 (0.3 or 1 mg kg<sup>-1</sup>, s.c., n=8 determinations per group). The effect of AT-390 3 mg kg<sup>-1</sup> was evaluated separately in a small number of rats (n=4). Data (means ± SEM) are expressed as ALO AIMs score for each-time point in absolute values (A,C) or as separate axial, limb and orolingual AIMs scores over the 3 hr observation period (i.e. cumulative scores calculated as the sum of scores at each time-point; B). On a separate day, treatments were replicated in the same animals, and rotarod performance was evaluated (as time on rod in sec; n=6 determinations per group) before and 60 min after drug administration (D).

\* $p < 0.05$ , significantly different from L-DOPA+vehicle. Statistical analysis was performed by two-way RM (A) or two-way (D) ANOVA followed by the Bonferroni test for multiple comparisons or one-way ANOVA followed by the Newman-Keuls test for multiple comparisons (B).

**Figure 7. Chronic treatment with AT-403 significantly inhibited L-DOPA-induced dyskinesia at day 1, however without interfering with its overall development.** Axial, limb and orolingual (collectively ALO) abnormal involuntary movements (AIMs) were scored in 6-OHDA hemilesioned rats chronically-treated for 20 days with L-DOPA ( $6 \text{ mg kg}^{-1}$  plus benserazide  $12 \text{ mg kg}^{-1}$ , s.c.) combined with vehicle or AT-403  $0.03 \text{ mg/Kg}$  (s.c.,  $n=9$  rats per group). The time-course of AIMs development over the 20-day period (A), and the AIMs response separately at days 1 (B), 9 (C), 20 (D) and 21 (challenge, E) are shown. In days different from AIMs scoring, rotarod performance was evaluated (as time on rod in sec) before and 60 min after drug administration (F). \* $p < 0.05$ , significantly different from vehicle. Statistical analysis was performed by two-way RM ANOVA followed by Bonferroni test for multiple comparisons.

**Figure 8. AT-403 inhibited D1 receptor-stimulated ERK signalling in striatum.** Number of ERK-positive cells in striatal slices of naïve mice following simultaneous application of SKF38393 and AT-403 ( $30 \text{ nM}$ ) (A) and representative microphotographs of treated slices. Data are mean $\pm$ SEM of  $n=12$  slices per group, obtained from 6 mice and pooled together. \* $p < 0.05$ , significantly different from not stimulated vehicle, # $p < 0.05$  significantly different from SKF38393 alone (two-way ANOVA followed by the Bonferroni post-hoc test). Western blot representative images (upper panel) and quantification (lower panel) of pERK (B) and total ERK (C) in the striatum of 6-OHDA hemilesioned, L-DOPA-naïve or

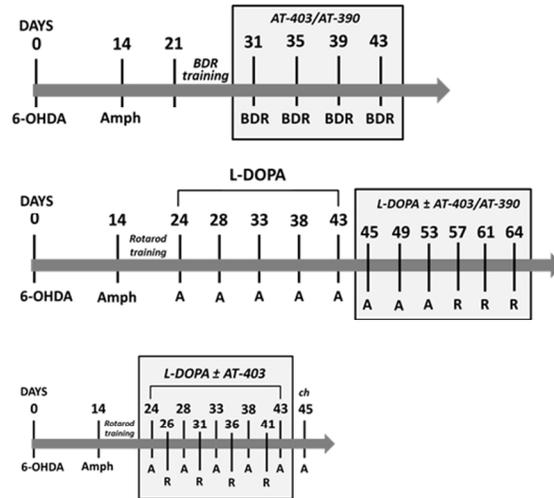
L-DOPA primed, dyskinetic rats. Dyskinetic rats were treated with 0.1 mg kg<sup>-1</sup> AT-403 or vehicle, and 15 min later challenged with L-DOPA. Data are means ± SEM of n=8 rats per group. \*p<0.05, significantly different from the ipsilateral side of L-DOPA treated rats, #p<0.05 significantly different from the contralateral side of control rats (one-way ANOVA followed by the Newman-Keuls post-hoc test).

For Peer Review

**Table 1.** Receptor binding assays (upper panel) and [<sup>35</sup>S]GTPγS functional activity (lower panel) of NOP agonists in membranes of CHO cells stably expressing the human recombinant NOP and classical μ, δ and κ receptors. Experiments were performed as previously described (references in text). Values represent average ± SEM for three experiments conducted in triplicate. Standard ligands in binding and [<sup>35</sup>S]GTPγS experiments were N/OFQ (NOP), DAMGO (μ), DPDPE (δ) and U69,593 (κ). Affinity values (K<sub>i</sub>, nM) were 0.12±0.01 for N/OFQ, 2.96±0.54 for DAMGO, 1.11±0.07 for DPDPE and 1.05±0.02 for U69,593. Efficacy (EC<sub>50</sub> nM) of standard ligands in the [<sup>35</sup>S]GTPγS was 3.6±0.7 for N/OFQ, 32.6±4.06 for DAMGO, 8.98±2.31 for DPDPE and 60.14±7.45 for U69,593. Agonist stimulation by AT-390 and AT-403 are expressed as a percentage of that of the standard agonist taken as 100.

	Binding (K <sub>i</sub> nM)			
	NOP	M	δ	κ
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

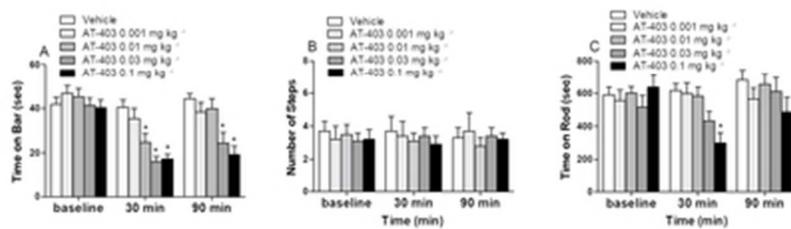
[ <sup>35</sup> S]GTPγS assay								
	NOP		M		δ		κ	
	EC <sub>50</sub>	% Stim	EC <sub>50</sub>	% Stim	EC <sub>50</sub>	% Stim	EC <sub>50</sub>	% Stim
AT-390	15.20 ± 0.4	110.1 ± 11.4	143.80 ± 0.6	54.3 ± 9.4	3847.0 ± 73	61.3 ± 6	534.3 ± 147.2	19.25 ± 1.3
AT-403	6.3 ± 1.42	104.6 ± 1.2	206.4 ± 78.9	33.5 ± 14.6	-----	-----	-----	-----



Timelines of studies in 6-OHDA rats

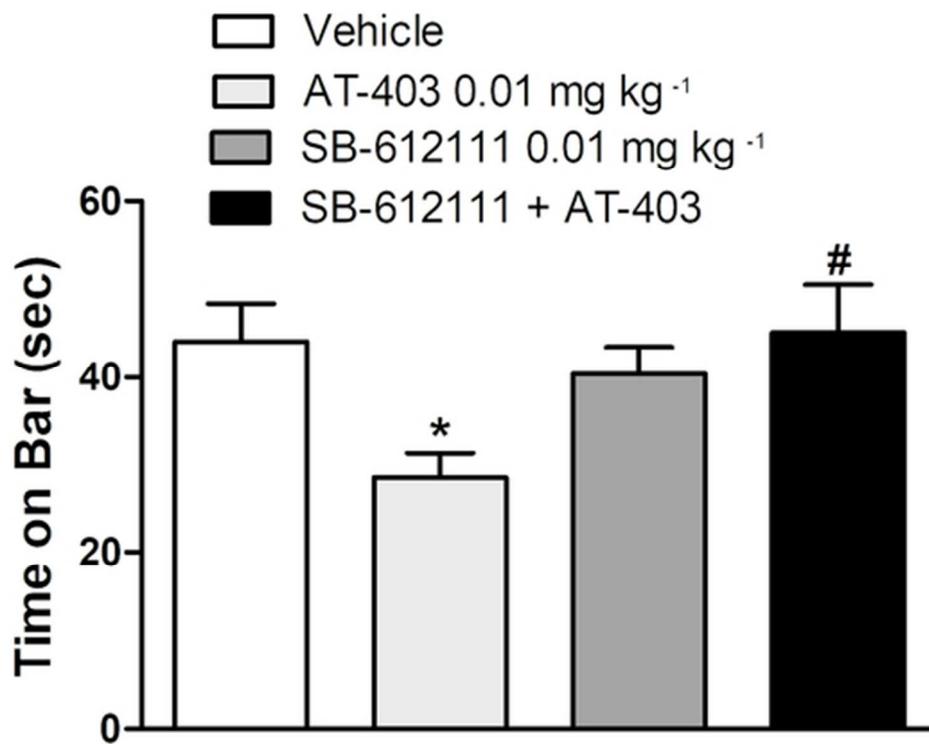
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Review



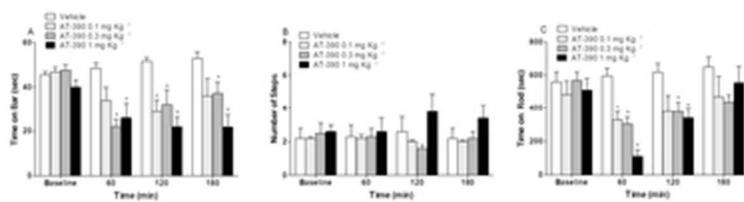
Effect of AT-403 on parkinsonian disabilities

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NOP receptor specificity of the AT-403 effect

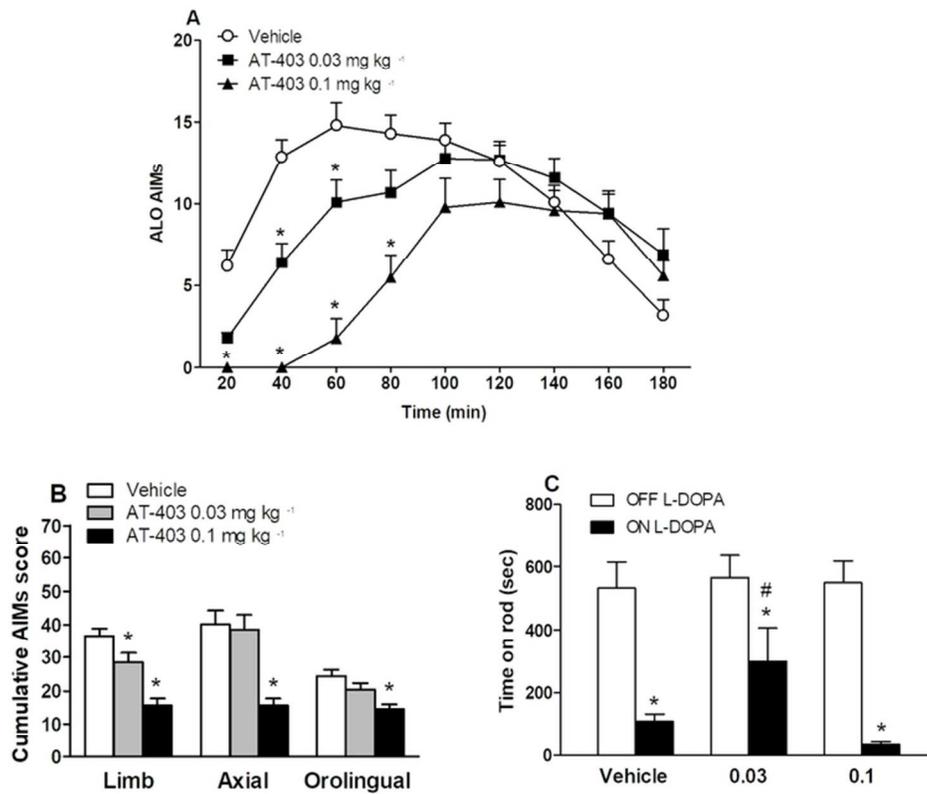
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Effect of AT-390 on parkinsonian disabilities

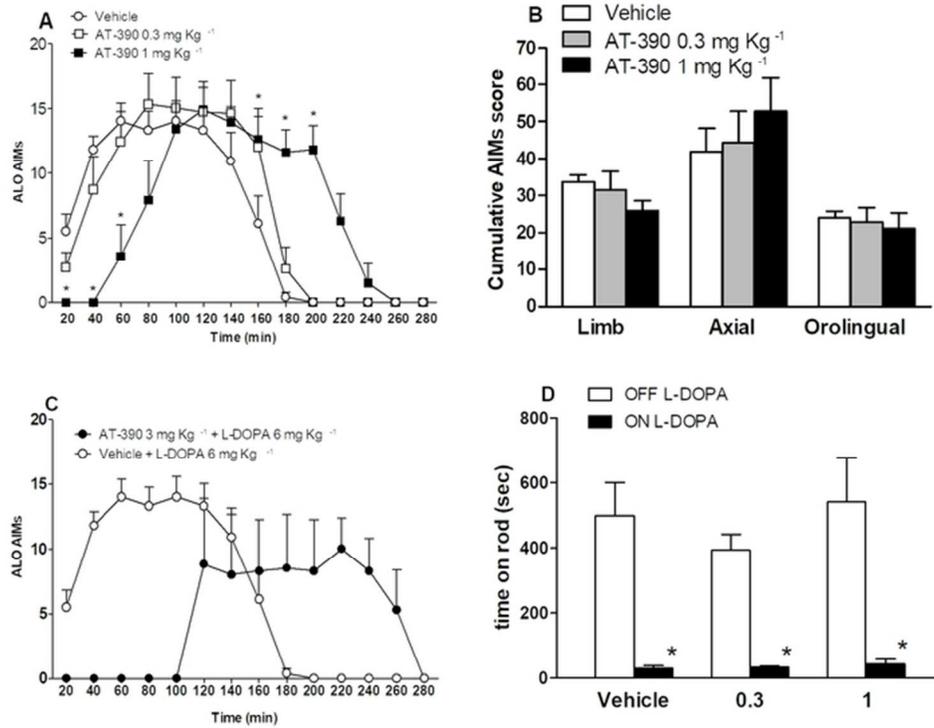
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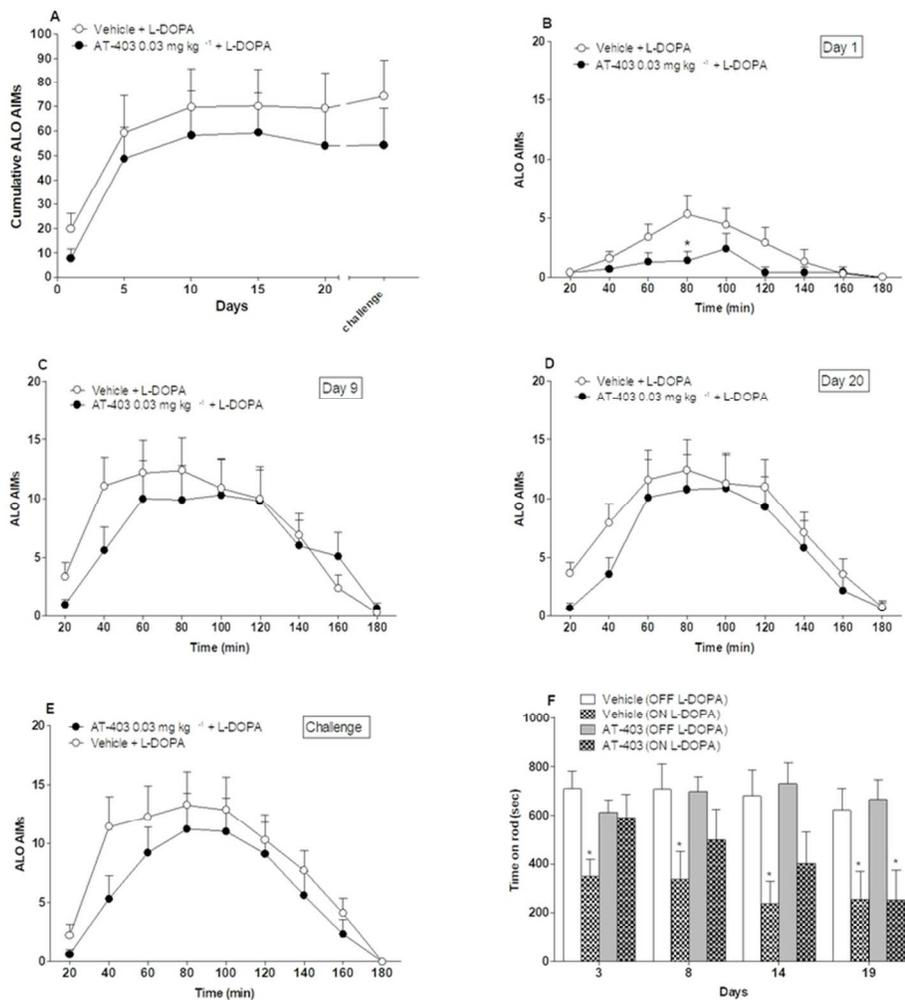
AT-403 attenuated the expression of L-DOPA-induced dyskinesia

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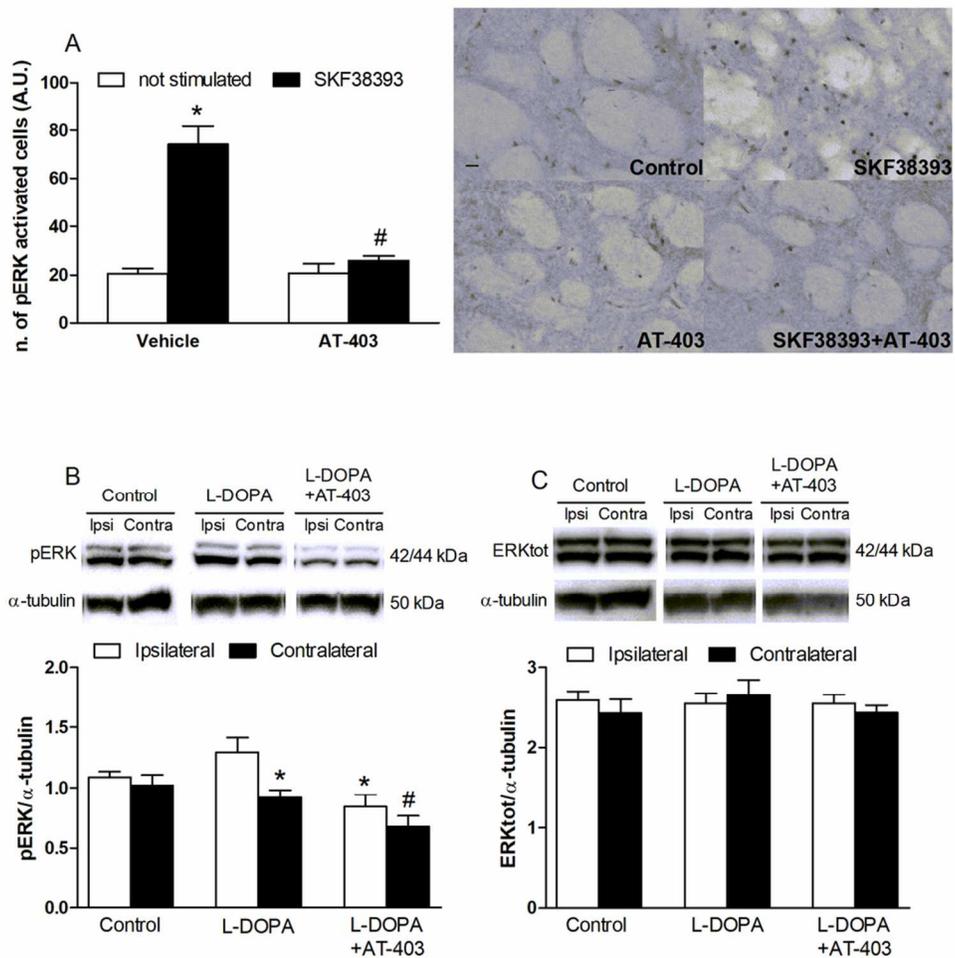
AT-390 delayed and prolonged the expression of L-DOPA-induced dyskinesia

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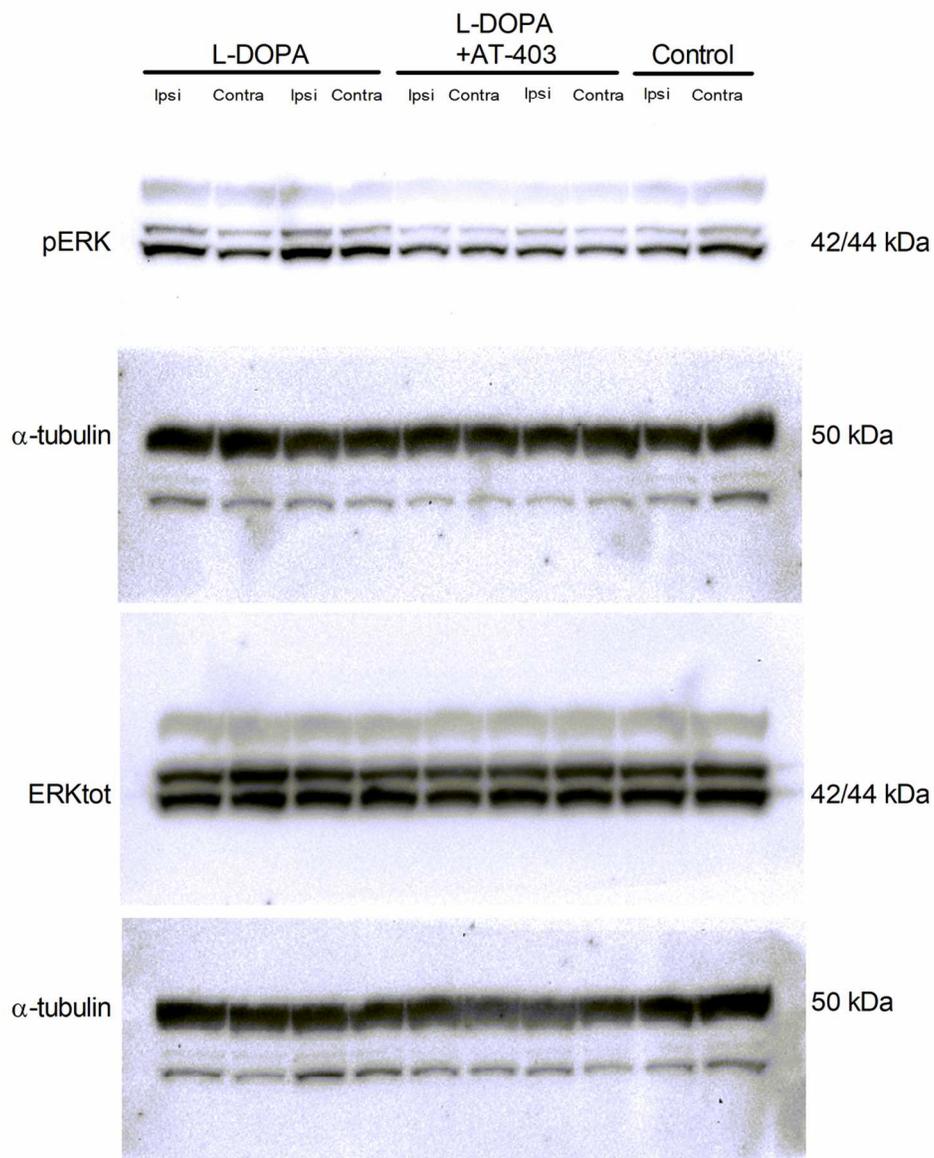
Chronic treatment with AT-403 significantly inhibited L-DOPA-induced dyskinesia at day 1, however without interfering with its overall development

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AT-403 inhibited D1 receptor-stimulated ERK signalling in striatum

89x90mm (300 x 300 DPI)



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