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RESEARCH PAPER

Acute and chronic antiparkinsonian effects of the novel nociceptin/orphanin FQ receptor antagonist NiK-21273 in comparison with SB-612111

M Marti[1,2], F Mela[1,2], M Budri[1,2], M Volta[1,2], D Malfacini[1,2], S Molinari[1,2], NT Zaveri[3], S Ronzoni[4], P Petrillo[4], G Calò[1,2] and M Morari[1,2]

[1]*Department of Experimental and Clinical Medicine, Section of Pharmacology, University of Ferrara, Ferrara, Italy,* [2]*National Institute of Neuroscience, University of Ferrara, Ferrara, Italy,* [3]*Astraea Therapeutics, Mountain View, CA, USA, and* [4]*NiKem Research srl, Baranzate, Italy*

Correspondence
Michele Morari, Department of Experimental and Clinical Medicine, Section of Pharmacology, University of Ferrara, via Fossato di Mortara 17-19, 44100 Ferrara, Italy.
E-mail: m.morari@unife.it

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BACKGROUND AND PURPOSE

Nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor antagonists have been proposed as a novel therapeutic approach to Parkinson's disease. Main limitations of previous studies were the use of structurally similar compounds and the evaluation of their acute effects only. We report here on the acute and long-term antiparkinsonian effects of the novel compound 2-[3-[4-(2-chloro-6-fluoro-phenyl)-piperidin-1-ylmethyl]-2-(morpholine-4-carbonyl)-indol-1-yl]-acetamide (NiK-21273) in comparison with the potent and selective NOP receptor antagonist SB-612111.

EXPERIMENTAL APPROACH

Basic pharmacological properties of NiK-21273 were studied in cell lines and isolated tissues (mouse and rat vas deferens). Antiparkinsonian effects were studied in reserpinized mice and 6-hydroxydopamine hemilesioned rats under both acute and chronic administration protocols.

KEY RESULTS

In vitro, NiK-21273 behaved as a potent (pA_2 7.7) and selective NOP receptor antagonist. *In vivo*, it reduced hypokinesia in reserpinized mice at 0.1 and 1 but not 10 mg·kg^{-1}, whereas SB-612111 (0.01–1 mg·kg^{-1}) provided a dose-dependent antiparkinsonian effect. NiK-21273 ameliorated motor performance in 6-hydroxydopamine hemilesioned rats at 0.5 and 5 but not 15 mg·kg^{-1}. SB-612111 replicated these effects in the 0.01–1 mg·kg^{-1} range without loss of efficacy. Both antagonists synergized with L-DOPA at subthreshold doses. Chronic administration of NiK-21273 provided delayed improvement in baseline activity at 0.5 and 1.5 mg·kg^{-1}, although tolerance to the higher dose was observed. Conversely, SB-612111 (1 mg·kg^{-1}) maintained its effects over time without modifying baseline activity.

CONCLUSIONS AND IMPLICATIONS

NOP receptor antagonists provide motor benefit in parkinsonism models although the 'therapeutic' window and long-term effects may vary between compounds.

Abbreviations

6-OHDA, 6-hydroxydopamine; DA, dopamine; N/OFQ, nociceptin/orphanin FQ; NOP, N/OFQ peptide; PD, Parkinson's disease; SNr, substantia nigra reticulata

Introduction

Nociceptin/orphanin FQ (N/OFQ) (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995) and its receptor (NOP) constitute a neuropeptide system showing structural and functional analogies with classical opioid systems but with unique pharmacological properties (Calo *et al.*, 2000; Mogil and Pasternak, 2001). The N/OFQ-NOP receptor system is widely distributed throughout the central and peripheral nervous system, and participates in the regulation of a number of biological processes such as nociception, food intake, learning, anxiety, reward and locomotion (Lambert, 2008). Endogenous N/OFQ also appears to play a role in Parkinson's disease (PD) (Marti *et al.*, 2005). PreproN/OFQ expression is elevated in the substantia nigra (SN) (Norton *et al.*, 2002; Marti *et al.*, 2005; 2010) and subthalamic nucleus (Marti *et al.*, 2010) of 6-hydroxydopamine (6-OHDA) hemilesioned rats, and in the SN of MPP^+- or MPTP-treated mice (Di Benedetto *et al.*, 2009; Gouty *et al.*, 2010). Consistently, enhanced extracellular N/OFQ levels were found in the substantia nigra reticulata (SNr) of parkinsonian rats (Marti *et al.*, 2005; 2010), as well as in the CSF of parkinsonian patients (Marti *et al.*, 2010). Such elevation of the N/OFQ tone in SNr may contribute to parkinsonian hypokinesia, since NOP receptor antagonists given systemically or intranigrally alleviate akinesia/bradykinesia in parkinsonian rats (Marti *et al.*, 2005; 2008; 2007; Volta *et al.*, 2010b), mice (Viaro *et al.*, 2008; Volta *et al.*, 2010a) and non-human primates (Viaro *et al.*, 2008; Visanji *et al.*, 2008). These pharmacological data are consistent with genetic evidence showing greater resistance of NOP receptor knockout ($NOP^{-/-}$) mice to haloperidol-induced (Marti *et al.*, 2005; Mabrouk *et al.*, 2010) or reserpine-induced (Volta *et al.*, 2010a) akinesia compared to wild-type ($NOP^{+/+}$) mice, strongly suggesting that NOP receptor antagonists might represent a novel therapeutic approach to PD.

Previous studies, however, only assessed the acute antiparkinsonian effects of NOP receptor antagonists, leaving uncertainty about their efficacy over a prolonged treatment. In addition, the antagonists previously characterized, that is, J-113397, and its closely related analogues Trap-101 and GF-4 are structurally similar. The use of structurally similar molecules represents a limitation because they likely engage the receptor in similar manner and share the overall *in vivo* profile. Indeed, J-113397 and both analogues showed loss of effectiveness or even inhibitory effects at high doses (bell-shaped curves) (Marti *et al.*, 2005; 2008; 2007; Volta *et al.*, 2010b). Although this behaviour is shared by the spiropiperidine Compound 24 (Volta *et al.*, 2011), we noted that this compound had a larger therapeutic window, possibly indicating that even if a bell-shaped curve is a 'class' feature of NOP receptor antagonists, the 'therapeutic window' is likely to vary among structurally different compounds. This may have a significant impact on the clinical utility of these compounds.

The present study was therefore aimed at investigating the antiparkinsonian properties of two piperidine compounds: the novel NOP receptor selective antagonist 2-[3-[4-(2-chloro-6-fluoro-phenyl)-piperidin-1-ylmethyl]-2-(morpholine-4-carbonyl)-indol-1-yl]-acetamide (NiK-21273; Ronzoni, 2010) in comparison with the very potent and selective compound SB-612111 (Zaratin *et al.*, 2004). First, basic pharmacological properties of NiK-21273 were investigated on human recombinant (cell lines) and animal native (mouse and rat vas deferens) NOP receptors *in vitro*. The antiparkinsonian effects of these small molecules were then investigated in reserpinized mice and 6-OHDA hemilesioned rats *in vivo*, using a battery of behavioural tests that provide complementary information of akinesia/bradykinesia and overall gait abilities. Combination studies with L-DOPA were also performed in 6-OHDA hemilesioned rats, testing subthreshold and submaximal/maximal doses of NOP antagonists. Finally, the impact of prolonged administration of NiK-21273 and SB-612111 on motor function in hemiparkinsonian rats was assessed. Compounds were administered once a day for 16 days, and motor activity was evaluated before and after drug administration at days 1, 4, 8, 12 and 16.

Methods

Animals employed in this study (see below) were kept under regular lighting conditions (12 h dark/light cycle) with free access to food and water. Adequate measures were taken to minimize animal pain and discomfort. This study was compliant with the European Council Directive of 24 November 1986 (86/609/EEC) and approved by both the Ethical Committee of the University of Ferrara and the Italian Ministry of Health (license nos. 94-2007-B and 194-2008-B). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

In vitro *studies*

Ca^{2+} mobilization studies. CHO cells stably expressing the human recombinant NOP receptor, the mu opioid peptide (MOP) or the kappa opioid peptide (KOP), together with the C-terminally modified $G\alpha_{qi5}$ protein were used. CHO cells co-expressing the human delta opioid peptide (DOP) receptor and the chimeric G protein $G\alpha_{qG66Di5}$ were also used. Details about the generation of these cell lines and their use in Ca^{2+} mobilization studies have been previously described (Camarda *et al.*, 2009; Fischetti *et al.*, 2009). Briefly, cells were cultured in culture medium consisting of Dulbecco's MEM/HAMS F12 (50/50) supplemented with 10% fetal calf serum, penicillin (100 $IU \cdot mL^{-1}$), streptomycin (100 $mg \cdot mL^{-1}$), geneticin (G418; 200 $\mu g \cdot mL^{-1}$) and hygromycin B (100 $\mu g \cdot mL^{-1}$). Cell cultures were kept at 37°C in 5% CO_2/humidified air. When confluence was reached (3–4 days), cells were seeded at a density of 50 000 cells/well into 96-well black, clear bottom plates. After 24 h incubation, the cells were loaded with medium supplemented with 2.5 mM probenecid, 3 μM of the Ca^{2+} sensitive fluorescent dye Fluo-4 AM, and 0.01% pluronic acid for 30 min at 37°C. Afterwards, the loading solution was aspirated and 100 μL/well of assay buffer (Hank's balanced salt solution supplemented with 20 mM HEPES, 2.5 mM probenecid and 500 μM Brilliant Black) was added. Serial dilutions of ligands were made in Hank's balanced salt solution/HEPES (20 mM) buffer (containing 0.02% BSA fraction V). After placing both plates (cell culture and compound plate) into the FlexStation II (Molecular Device, Union City, CA, USA), fluorescence changes were

measured at 37°C. Online additions were carried out in a volume of 50 µL/well. Antagonists were incubated 24 min before the agonist. Maximum change in fluorescence, expressed as percent of baseline fluorescence, was used to determine agonist response.

Bioassay studies. The mouse and rat vas deferens was taken from male CD-1 mice (30–35 g) and Sprague–Dawley rats (300–350 g), as previously described (Calo *et al.*, 1996; Bigoni *et al.*, 1999). Tissues were suspended in 5 mL organ baths containing heated Krebs solution (composition in mM: NaCl 118.5, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10 and $CaCl_2$ 2.5 for mouse, and $CaCl_2$ 1.8 and $MgSO_4$ 1.2 for rat tissues) oxygenated with 95% O_2 and 5% CO_2. The bath temperature was set at 33°C for mouse and 37°C for rat tissues. A resting tension of 0.3 and 1 g was applied to the mouse and rat vas deferens respectively. Tissues were continuously stimulated with two platinum ring electrodes delivering supramaximal rectangular pulses of 1 ms duration and 0.05 Hz frequency. The electrically evoked contractions (twitches) were measured isotonically with a strain gauge transducer (Basile 7006; UgoBasile s.r.l., Varese, Italy) and recorded with the PC-based acquisition system Power Lab (ADInstrument, Sydney, Australia). Following an equilibration period of 60 min, the contractions induced by electrical field stimulation were stable. At this time, cumulative concentration–response curves to N/OFQ were performed (0.5 log unit steps) in the absence or the presence of the ligand under investigation (15 min pre-incubation time).

In vivo *studies*

Reserpine treatment in mice. Reserpine was administered subcutaneously at a dose found to cause submaximal akinesia (1 mg·kg^{-1}; Volta *et al.*, 2010a). Motor impairment and its reversal by NOP receptor antagonists was evaluated 24 h after reserpine administration.

6-OHDA lesion in rats. Unilateral lesion of dopamine (DA) neurons (Marti *et al.*, 2005) was induced in isoflurane-anaesthetized male Sprague–Dawley rats (150 g; Harlan Italy, S. Pietro al Natisone, Italy) by stereotaxically injecting 8 µg of 6-OHDA (in 4 µL of saline containing 0.02% ascorbic acid) in the medial forebrain bundle according to the following coordinates from bregma: antero-posterior –4.4 mm, medio-lateral –1.2 mm, dorso-ventral –7.8 below dura (Paxinos and Watson, 1986). Two weeks after surgery, rats were injected with amphetamine (5 mg·kg^{-1} i.p.) and only those rats performing >7 ipsilateral turns/min were enrolled in the study (Marti *et al.*, 2002; 2007).

Behavioural analysis. Motor activity was evaluated by means of three behavioural tests specific for different motor abilities: the bar, drag and rotarod test (Marti *et al.*, 2005; 2007). The three tests were repeated in a fixed sequence (bar, drag and rotarod) before and after drug administration. Animals were trained for approximately 10 days to the specific motor tasks until their performance became reproducible.

Bar test. Originally developed to quantify morphine-induced catalepsy (Kuschinsky and Hornykiewicz, 1972), this test measures the ability of the animal to respond to an externally imposed static posture. The rat was placed gently on a table and forepaws were placed alternatively on blocks of increasing heights (1.5, 3 and 4.5 cm for mice; 3, 6 and 9 cm for rats). The time (in seconds) that each paw spent on the blocks (i.e. the immobility time) was recorded (cut-off time of 20 s).

Drag test. Animals were lifted from the tail (allowing the forepaws to rest on the table) and dragged backwards at a constant speed (~20 cm·s^{-1}) for a fixed distance (100 cm). The number of steps made by each forepaw was counted by two separate observers.

Rotarod test. The fixed-speed rotarod test (Rozas *et al.*, 1997) measures different motor parameters such as motor coordination, gait ability, balance, muscle tone and motivation to run. It was employed in 6-OHDA hemilesioned rats according to a previously described protocol (Marti *et al.*, 2004). Briefly, rats were tested at four increasing speeds (usually 15, 20, 25 and 30 r.p.m.; 180 s each), causing a progressive decrement of performance to ~40% of the maximal response.

Data presentation

In vitro data have been expressed as means ± SEM. For potency values, 95% confidence limits were indicated. Ca^{2+} mobilization has been expressed as fluorescence intensity units as percent over baseline. Data from isolated tissues have been expressed as percent of the control twitch. Agonist potencies were given as pEC_{50}, that is, the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect of that agonist. E_{max} is the maximal effect that an agonist can elicit in a given preparation. Concentration–response curve to agonists was fitted with the following equation:

$$\text{Effect} = \text{baseline} + (E_{max} - \text{baseline})/(1 + 10\text{^}((\log EC_{50} - X) \times n))$$

where X is the agonist concentration and n is the Hill coefficient of the concentration–response curve to the agonist.

Antagonist potencies derived from inhibition response curves have been expressed as pK_B calculated from the following equation:

$$pK_B = \log\left(IC_{50} \Big/ \left(\left[2 + ([A]/EC_{50})^n\right]^{1/n} - 1\right)\right)$$

where IC_{50} is the concentration of antagonist that produces 50% inhibition of the agonist response, [A] is the concentration of agonist, EC_{50} is the concentration of agonist producing a 50% maximal response, and n is the Hill coefficient of the concentration–response curve to the agonist (Kenakin, 2004).

The NOP antagonist properties of NiK-21273 were also assessed using the classical Schild protocol. In this case, antagonist potency was expressed in terms of pA_2 which is the negative logarithm to base 10 of the antagonist molar concentration that makes it necessary to double the agonist concentration to elicit the original response (Schild, 1973). Curve fitting was performed using PRISM 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Motor performance has been calculated as immobility time (in seconds, bar test), number of steps (drag test) and time spent on rod (in seconds, rotarod test). The effect of drug

(or vehicle) administration has been expressed in absolute values or as percent of control (pre-drug baseline) values. Statistical analysis has been performed by one-way repeated measures ANOVA followed by contrast analysis and the sequentially rejective Bonferroni test (implemented on Excel spreadsheet) to determine specific differences (i.e. at the single time point level) between groups. Drug interaction was studied experimentally according to a 2 × 2 factorial design with conventional two-way ANOVA, factor 1 being L-DOPA and factor 2 being SB-612111 or NiK-21273. *P*-values <0.05 were considered to be statistically significant.

Materials

Amphetamine, benserazide, L-DOPA, reserpine and 6-OHDA were purchased from Sigma Chemical Company (St Louis, MO, USA); dermorphin, DPDPE, dynorphin and naloxone were from Tocris Bioscience (Bristol, UK). N/OFQ was prepared and purified as previously described (Guerrini *et al.*, 1997). NiK-21273 was synthesized in the laboratories of NiKem Research S.r.l. (Milano, Italy). SB-612111 was provided by Dr NT Zaveri. NiK-21273 was freshly dissolved in 0.1 HCl eqM and 5% glucose solution, SB-612111 in 0.66% (0.1 mg·kg^{-1}) or 6.6% (1 mg·kg^{-1}) DMSO/saline solution. Reserpine was dissolved in 10% acetic acid saline solution, with pH adjusted to 4.5 with NaOH. L-DOPA was always administered in combination with benserazide (12 mg·kg^{-1}).

Results

In vitro *experiments*

Ca^{2+} mobilization studies. N/OFQ evoked a concentration-dependent stimulation of Ca^{2+} signal in CHO_{NOP} cells displaying high potency (pEC_{50} 9.19; $CL_{95\%}$ 8.85–9.53) and maximal effects of 200 ± 12% over baseline. NiK-21273 inhibited the stimulatory effect of N/OFQ (10 nM) in a concentration-dependent manner with a pK_B of 7.38, whereas naloxone was ineffective (Table 1). SB-612111 also inhibited N/OFQ effect in a concentration-dependent manner, with a pK_B of 8.18 (not shown). NiK-21273 selectivity was then evaluated in CHO cells stably expressing classical opioid receptors (CHO_{MOP}, CHO_{KOP} and CHO_{DOP} cells). In these cell lines, the MOP, KOP and DOP receptor selective agonists dermorphin, dynorphin A and DPDPE evoked concentration-dependent Ca^{2+} release showing different potencies (pEC_{50} 8.26, 9.19 and 8.36, respectively) and efficacies (~189, ~226 and ~102% over baseline respectively; not shown). Naloxone antagonized the stimulation evoked by 30 nM dermorphin (pK_B 8.73), 30 nM dynorphin A (pK_B 7.00) and 30 nM DPDPE (pK_B 6.80) in a concentration-dependent fashion, whereas NiK-21273 was ineffective (Table 1). In order to assess the nature of NiK-21273 antagonism, a classical Schild analysis was performed. NiK-21273 produced a concentration-dependent rightward shift of the N/OFQ curve without modifying its maximal effect (Figure 1A). The corresponding Schild plot (Figure 1A inset) was linear, yielding a pA_2 of 7.77. SB-612111 (10 nM–1 μM) replicated this behaviour (Figure 1B), the corresponding Schild plot (Figure 1B inset) yielding a pA_2 of 7.74.

Bioassay studies. NiK-21273 was also assessed in the electrically stimulated mouse and rat vas deferens, in which N/OFQ produces inhibitory effects via selective NOP receptor activation (Calo *et al.*, 2000). N/OFQ inhibited the twitch response in a concentration-dependent manner, both in the mouse (pEC_{50} 7.70; E_{max} −84 ± 3%; Figure 2A) and rat (pEC_{50} 7.48; E_{max} −86 ± 2%; Figure 2B) vas deferens. NiK-21273 alone was ineffective up to 1 μM in both preparations, but produced a concentration-dependent rightward shift of the N/OFQ curve without affecting its maximal effect (Figure 2). Schild analysis (Figure 2 insets) yielded a pA_2 value of 7.74 and 7.75 in the mouse and rat tissues respectively. SB-612111 (100 nM) replicated this behaviour with pK_B values of 8.77 (mouse) and 8.05 (rat).

In functional washout experiments performed in the electrically stimulated mouse vas deferens, the reversibility of NiK-21273 and SB-612111 action at equieffective concentrations (1 and 0.1 μM, respectively) was evaluated. The concentration–response curves to N/OFQ performed 1, 2 and 3 h after washing were superimposable to the control curve. The antagonist effect of 1 μM NiK-21273 (CR 30, where CR is the ratio between the EC50 of the agonist in the presence and in the absence of the antagonist) was easily, although not completely, reversed by washing the tissues for 1 h (CR 3). On

Table 1

In vitro antagonist potencies of NiK-21273 and naloxone

	NOP N/OFQ 10 nM	MOP Dermorphin 30 nM	KOP Dynorphin A 30 nM	DOP DPDPE 30 nM
NiK-21273 (10 pM–10 μM)	7.38 (7.06–7.70)	Inactive	Inactive	Inactive
Naloxone (10 pM–10 μM)	Inactive	8.73 (8.38–9.08)	7.00 (6.68–7.32)	6.80 (6.09–7.51)

Data were obtained from Ca^{2+} mobilization experiments performed in CHO cells expressing NOP or classical opioid receptors and Gα chimeric proteins. Data are mean ($CL_{95\%}$) of at least three separate experiments performed in duplicate.
Data indicate pK_B values derived from inhibition response curves.
Inactive means pK_B values <6.

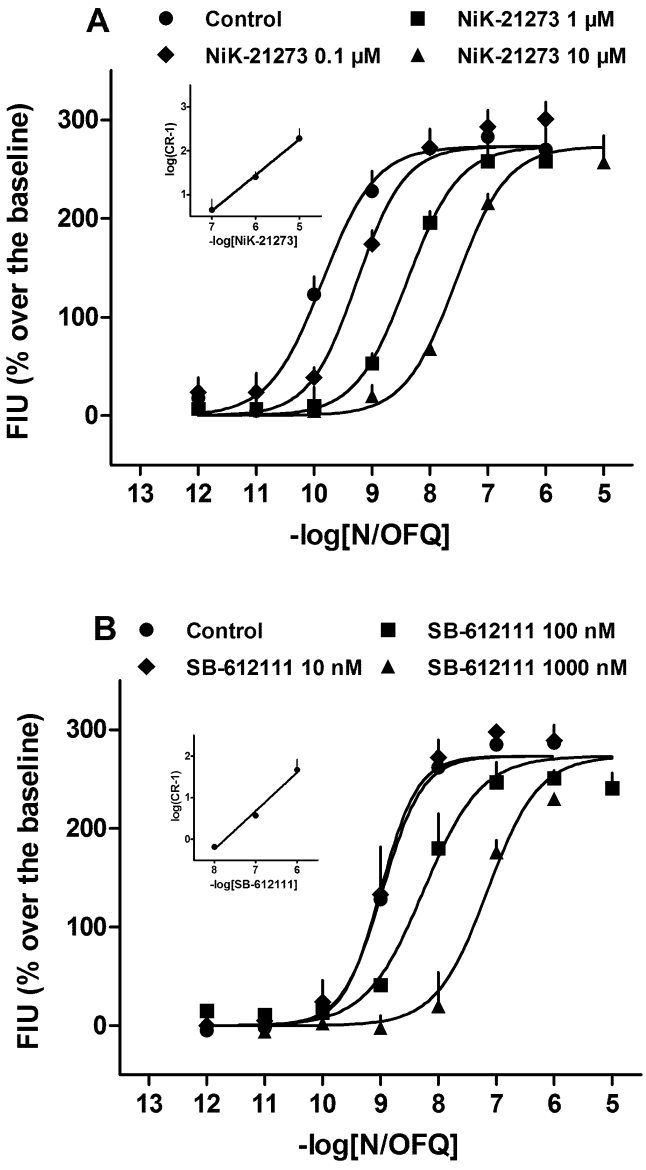


Figure 1

Concentration–response curve to N/OFQ obtained in the absence (control) and in presence of increasing concentrations of NiK-21273 (A) and SB-612111 (B). The corresponding Schild plots are shown in insets. Data are the mean of at least four separate experiments performed in duplicate.

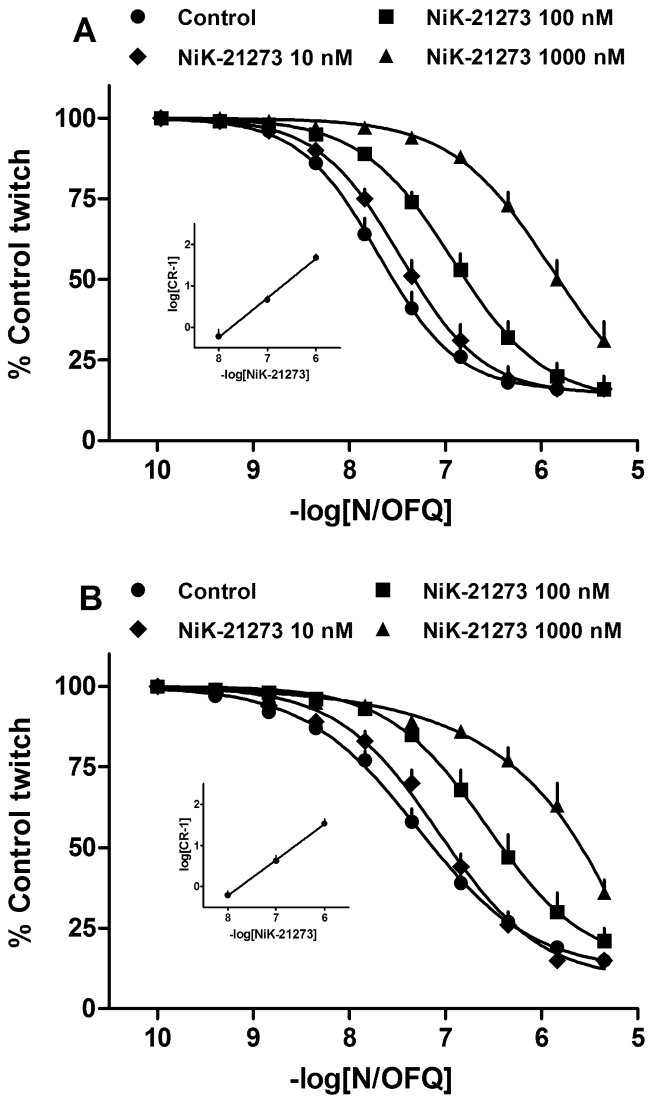


Figure 2

Concentration–response curve to N/OFQ obtained in the absence (control) and in the presence of increasing concentrations of NiK-21273 in the electrically stimulated mouse (A) and rat (B) vas deferens. The corresponding Schild plots are shown in insets. Data are the mean of at least four separate experiments.

the contrary, the antagonist effect exerted by 0.1 μM SB-612111 (CR 30) could not be reversed even after 3 h of washing (CR 10).

In vivo *experiments*

Acute effects of NiK-21273 and SB-612111 in reserpinized mice. As a preliminary investigation of the antiparkinsonian potential of SB-612111 and NiK-21273, and to determine the effective doses to be used in 6-OHDA hemilesioned rats, a dose-finding study was performed in reserpinized mice (Figure 3). Immobility time (bar test) of naïve mice before reserpine administration was 0.3 ± 0.2 s ($n = 46$) whereas the number of steps (drag test) was 14.3 ± 0.6 ($n = 47$). Reserpine caused dramatic elevation of immobility time in the bar test (Figure 3A, C) and reduction of the number of steps in the drag test (Figure 3B, D). NiK-21273 did not affect immobility time (Figure 3A) but almost doubled stepping activity at 0.1 and 1 mg·kg^{-1}, being ineffective at 10 mg·kg^{-1} (Figure 3B). Different from NiK-21273, SB-612111 (0.01–1 mg·kg^{-1}) dose dependently attenuated motor deficits in both motor tasks. SB-612111 was ineffective at 0.01 mg·kg^{-1} but almost halved the reserpine-induced akinesia at 0.1 and 1 mg·kg^{-1} (Figure 3C). SB-612111 caused a prolonged increase in stepping activity at 0.01, 0.1 and 1 mg·kg^{-1}, a maximal ~3-fold increase being observed at the highest dose (Figure 3D).

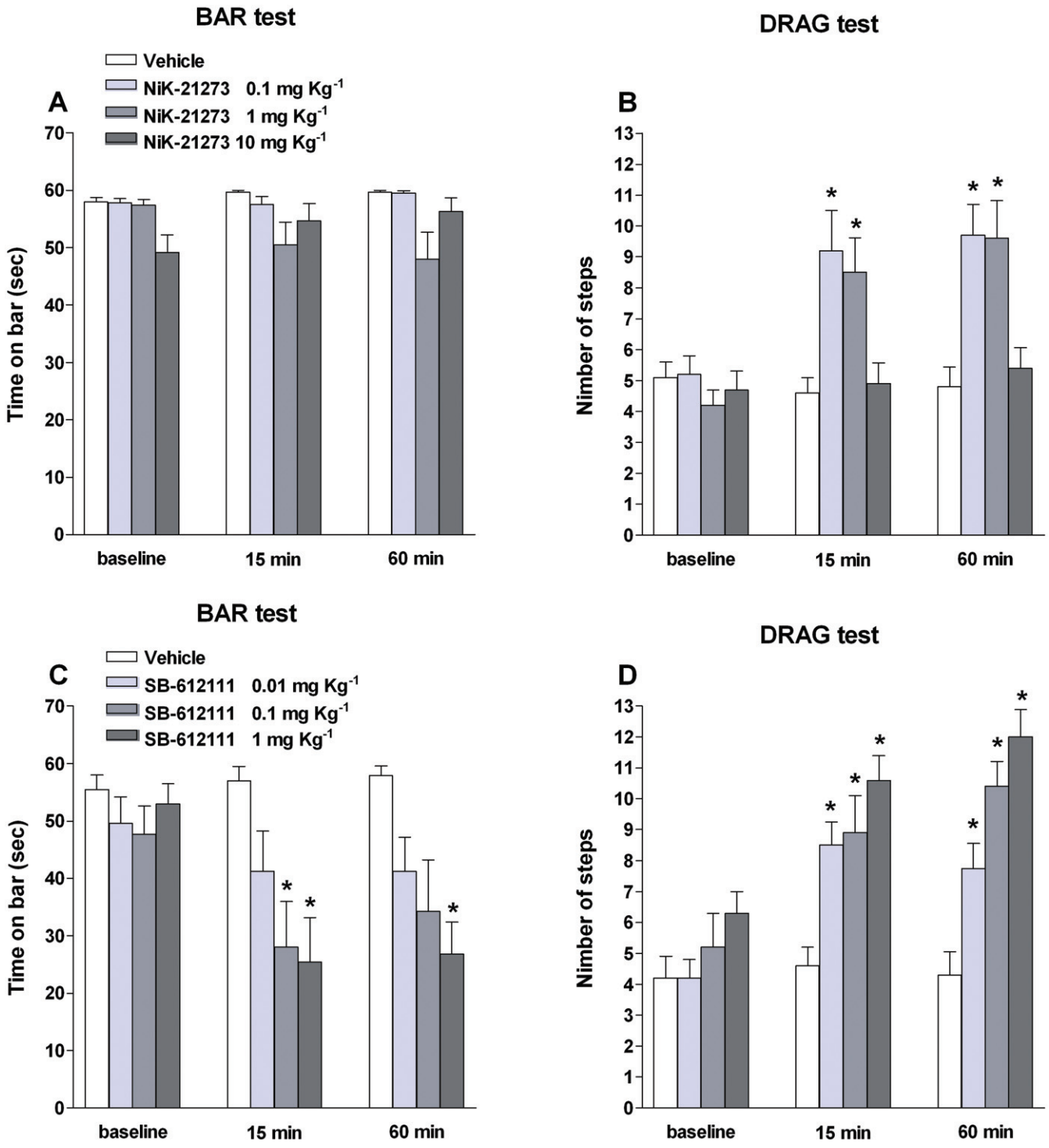


Figure 3

Acute administration of NiK-21273 and SB-612111 attenuated motor impairment in reserpinized mice. Reserpine (1 mg·kg^{-1}) was administered s.c. and behavioural test (bar and drag tests) was performed 24 h after administration. Each experiment consisted of three different sessions: a control session followed by two other sessions performed 15 and 60 min after vehicle NiK-21273 (0.1–10 mg·kg^{-1}) or SB-612111 (0.01–1 mg·kg^{-1}) administration. Data are expressed in absolute values (immobility time in seconds; A,C) or number of steps (B,D), and are means ± SEM of 6–8 mice per group. Statistical analysis was performed by one-way repeated measures ANOVA followed by contrast analysis and the sequentially rejective Bonferroni test. Relevant statistics results: (B) Significant effect of treatment ($F_{3,24}$ = 4.43, P = 0.013), time ($F_{2,64}$ = 25.52, P < 0.0001), and time × treatment interaction ($F_{6,64}$ = 8.40, P < 0.0001). (C) Significant effect of treatment ($F_{3,15}$ = 9.64, P = 0.0008), time ($F_{2,37}$ = 16.25, P < 0.0001), and time × treatment interaction ($F_{6,37}$ = 5.22, P < 0.0001). (D) Significant effect of treatment ($F_{3,15}$ = 14.01, P = 0.0001), time ($F_{2,37}$ = 55.66, P < 0.0001), and time × treatment interaction ($F_{6,37}$ = 8.29, P < 0.0001). *P < 0.05, significantly different from vehicle.

Acute effects of NiK-21273 and SB-612111 in 6-OHDA hemilesioned rats. Basal activity in naïve rats before 6-OHDA injection was: 5.0 ± 0.5 s in the bar test, 10.2 ± 0.4 steps in the drag test and 1033 ± 52 s in the rotarod test. 6-OHDA lesioning caused the appearance of motor deficits mainly affecting the contralateral paw. Indeed, the immobility time was about two times greater at the contralateral (41.4 ± 5.3 s) than ipsilateral (23.3 ± 4.9 s) paw whereas the number of steps was ~52% lower at the contralateral (4.0 ± 0.6) than ipsilateral (8.3 ± 0.6) paw. Rotarod performance was also ~55% reduced in hemiparkinsonian rats (461 ± 48 s). Systemic administration of NiK-21273 (0.5–15 mg·kg^{-1}; i.p.) reduced the immobility time, and elevated the number of steps and rotarod performance, showing a bell-shaped dose–response curve (Figure 4A–C). In the bar test (Figure 4A), NiK-21273 caused a ~45% reduction of akinesia selectively at the contralateral paw at the dose of 5 mg·kg^{-1}, being ineffective at lower and higher doses. The antiakinetic effect was also observed after 90 min. In the drag test (Figure 4B), NiK-21273 increased stepping at the contralateral paw both at 1.5 and 5 mg·kg^{-1} (~81 and ~162%, respectively), being ineffective at 15 mg·kg^{-1}. This effect lasted up to 90 min, when a small improvement (~16%, $P < 0.01$; data not shown) was also observed at the ipsilateral paw [significant effect of treatment ($F_{4,24} = 4.92$, $P = 0.0048$) and time ($F_{1,34} = 5.19$, $P = 0.0291$) but not a time × treatment interaction ($F_{4,34} = 0.49$, $P = 0.74$)]. Finally, NiK-21273 increased rotarod performance at 1.5 and 5 mg·kg^{-1} (~27 and ~54%, respectively), but not higher doses (Figure 4C). At 90 min after injection, only the effect of 5 mg·kg^{-1} NiK-21273 was observed.

Systemic administration of SB-612111 (0.01–1 mg·kg^{-1}; i.p.) provided dose-dependent attenuation of motor deficit in 6-OHDA hemilesioned rats (Figure 4D–F). SB-612111 at 1 mg·kg^{-1} provided long-lasting reduction of akinesia at the contralateral (~45%) paw (Figure 5D). Reduction of immobility time was also observed at the ipsilateral paw [significant effect of treatment ($F_{3,18} = 13.33$, $P < 0.0001$), time ($F_{1,24} = 7.69$, $P = 0.0102$), and time × treatment interaction ($F_{3,24} = 3.07$, $P = 0.0475$)] which was significant at 0.01, 0.1 and 1 mg·kg^{-1} (~38, ~44 and ~51% respectively; data not shown). In the drag test, SB-612111 (0.1 and 1 mg·kg^{-1}) caused a long-lasting increase of the number of steps selectively at the contralateral paw (~61 and ~102% respectively; Figure 4E). Likewise, SB-612111 (0.1 and 1 mg·kg^{-1}) improved rotarod performance at 30 min (~42 and ~69%, respectively) and 90 min (~32 and ~79%, respectively) after administration (Figure 4F).

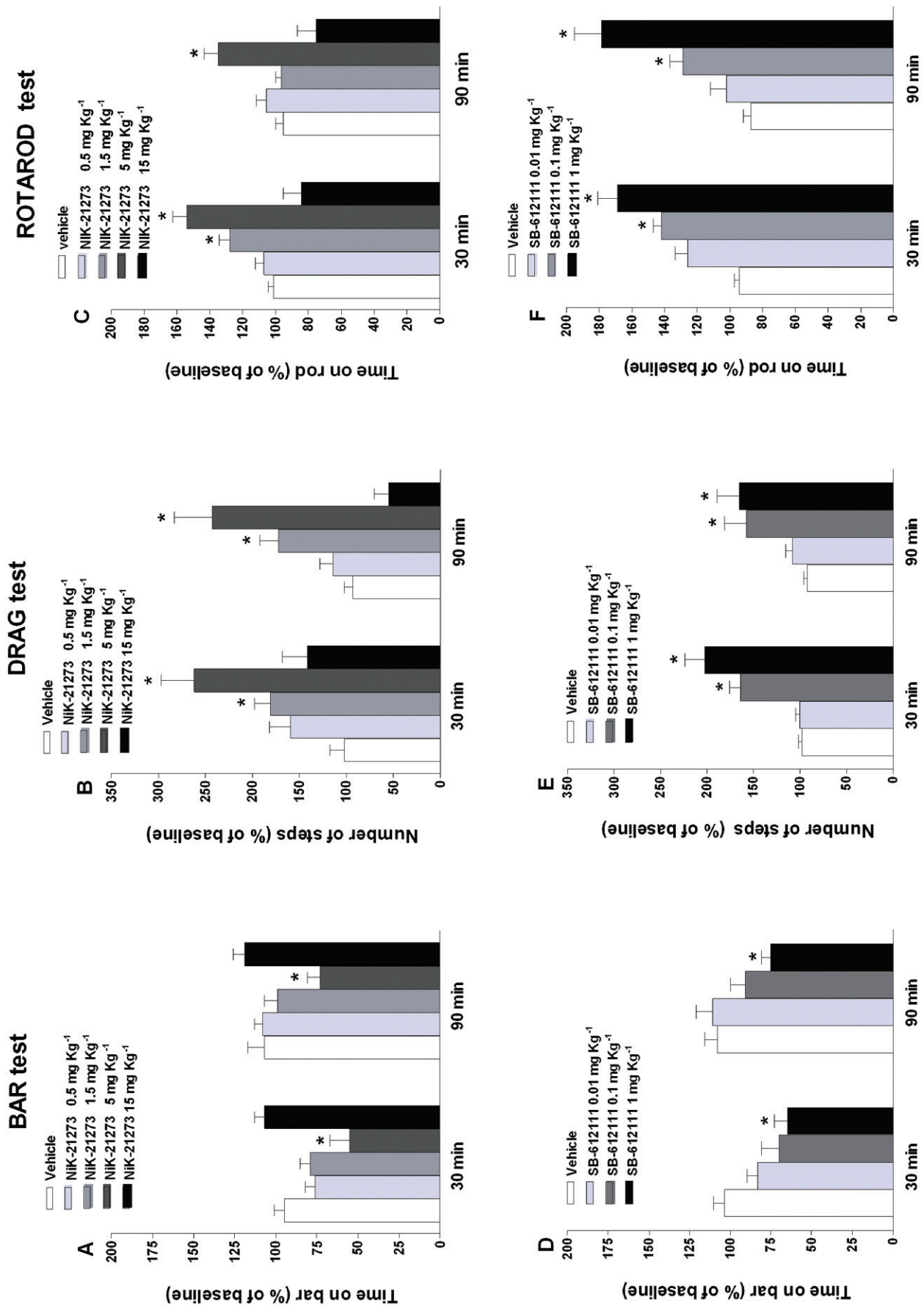
Co-administration of NiK-21273 or SB-612111 with L-DOPA. We previously reported (Marti *et al.*, 2007; 2008) that NOP receptor antagonists J-113397 and Trap-101 produced synergistic or additive antiparkinsonian effects in hemiparkinsonian rats when co-administered with L-DOPA. We therefore tested whether NiK-21273 and SB-612111 could synergize with L-DOPA. Based on the dose–response curve previously performed in hemiparkinsonian rats (Marti *et al.*, 2007), we selected a subthreshold (0.1 mg·kg^{-1}) and a submaximal (1 mg·kg^{-1}) dose of L-DOPA for combination experiments. NiK-21273 (0.5 mg·kg^{-1}) and L-DOPA (0.1 mg·kg^{-1}; Figure 5) were ineffective alone in the three tests, but caused a significant reduction of akinesia (Figure 5A), stimulation of stepping (Figure 5B) and rotarod performance (Figure 5C) when given in combination.

Likewise, subthreshold doses of SB-612111 (0.01 mg·kg^{-1}) synergized with L-DOPA (0.1 mg·kg^{-1}) in reducing (~58%) the immobility time at the contralateral paw (Figure 6A), and producing remarkable stimulation of stepping (Figure 6B) and rotarod performance (Figure 6C) (~108 and ~59%, respectively) when given in combination.

We then tested the interaction between submaximal doses of L-DOPA (1 mg·kg^{-1}) and maximal doses of SB-612111 (1 mg·kg^{-1}). At this dose, both compounds reduced the immobility time at the contralateral paw in the bar test (~52 and ~29% respectively; Figure 6D), the combination resulting in no greater inhibition (~44%). A similar result was observed at the ipsilateral paw. Likewise, both L-DOPA and SB-612111 elevated stepping activity at the contralateral paw (~64 and ~107% respectively; Figure 6E); their combination being similarly effective (~76%). L-DOPA and SB-612111 also elevated rotarod performance at 30 min after administration (~40 and ~73% respectively; Figure 6F). However, in this test the combination resulted in a milder facilitation than that induced by SB-612111 alone.

Chronic effects of NiK-21273 and SB-612111 in hemiparkinsonian rats. We then investigated whether the acute antiparkinsonian effects of NiK-21273 and SB-612111 could be maintained over chronic administration. Rats were given one injection of NOP receptor antagonist every day for 16 days, and motor activity was evaluated before (baseline) and 30 min after drug administration both at the contralateral and ipsilateral paws. Chronic treatment with NiK-21273 resulted in a significant reduction of basal immobility time at the contralateral paw in the bar test (Figure 7A). The reduction was similar (~44%) after 16 days of treatment with both the doses tested (0.5 and 1.5 mg·kg^{-1}). A smaller but still significant ($P < 0.01$) reduction (~25–31%) was observed at the ipsilateral paw for the higher dose, starting 8 days after administration [significant effect of time ($F_{4,84} = 14.48$, $P = 0.0004$) but not treatment ($F_{2,14} = 3.13$, $P = 0.07$), or time × treatment interaction ($F_{8,84} = 1.17$, $P = 0.32$)]. As expected (see Figure 4), no acute effect was detected after administration of NiK-21273 in the bar test (Figure 7B). In the drag test, prolonged administration of NiK-21273 (1.5 mg·kg^{-1}) was associated with delayed increase of basal stepping activity at the contralateral paw (Figure 7C), the ipsilateral one being unaffected (not shown). The improvement was observed after 12 days (~60%) and 16 days (~47%) treatment. In parallel, the acute response to NiK-21273 vanished over time, since NiK-21273 improved stepping activity at the contralateral paw after the first (~70%) but not subsequent challenges (Figure 7D). No acute effect was observed at the ipsilateral paw (not shown). Finally, chronic treatment with NiK-21273 (0.5 and 1.5 mg·kg^{-1}) did not modify the rotarod performance at baseline (Figure 7E). As for the drag test, NiK-21273 produced an acute increase in rotarod performance at 1.5 mg·kg^{-1} only in the first (~40%) but not later treatment sessions (Figure 7F).

Different from NiK-21273, repeated administration of SB-612111 (0.01 and 1 mg·kg^{-1}; i.p.) failed to affect baseline motor activity in any of the three tests (Figure 8A,C,E). Nonetheless, SB-612111 (1 mg·kg^{-1}) attenuated akinesia in the bar


BAR test
DRAG test
ROTAROD test
A
B
C
D
E
F
Vehicle
NIK-21273 0.5 mg Kg-1
NIK-21273 1.5 mg Kg-1
NIK-21273 5 mg Kg-1
NIK-21273 15 mg Kg-1
SB-612111 0.01 mg Kg-1
SB-612111 0.1 mg Kg-1
SB-612111 1 mg Kg-1
Time on bar (% of baseline)
Number of steps (% of baseline)
Time on rod (% of baseline)
30 min
90 min

(~30%; Figure 8B), elevated stepping activity in the drag (~54%; Figure 8D) and improved gait abilities in the rotarod (~50%; Figure 6F) tests at first administration. These effects were maintained substantially unchanged along the course of treatment, with the exception of the antiakinetic effect in the bar test that was found not to be significant in the last session (16th day, Figure 8B). No changes in performance at the ipsilateral paw were observed (not shown).

Discussion and conclusions

SB-612111 is one of the most potent and selective NOP receptor antagonists identified thus far, showing high affinity for the NOP receptor (Ki 0.33 nM) and selectivity over MOP (Ki 58 nM), KOP (Ki 160 nM) and DOP (Ki 2109 nM) opioid receptors (Zaratin *et al.*, 2004). *In vitro*, SB-612111 prevented N/OFQ actions at native NOP receptors with high potency (pA_2 8.2–8.5; Zaratin *et al.*, 2004; Spagnolo *et al.*, 2007) as confirmed in the present study (pK_B 8.05 and 8.77). *In vivo*, SB-612111 counteracted the N/OFQ-induced hyperalgesia and food intake in the 0.1–1 mg·kg^{-1} dose range (Zaratin *et al.*, 2004; Rizzi *et al.*, 2007), and evoked antidepressant-like effects at higher doses (10 mg·kg^{-1}) without altering spontaneous activity or inducing gross behavioural changes (Rizzi *et al.*, 2007). In the present study, SB-612111 relieved akinesia/bradykinesia both in reserpinized mice and hemiparkinsonian rats at 0.1 mg·kg^{-1}, producing beneficial effects in some tests at even lower doses (0.01 mg·kg^{-1}). This confirms that motor activity under parkinsonian conditions is exquisitely sensitive to NOP receptor antagonism, possibly due to the greater tonic motor inhibition resulting from up-regulated N/OFQ expression and release in SNr. SB-612111 also synergized with subthreshold doses of L-DOPA, the antiparkinsonian reference compound. The synergism was particularly robust if compared to that produced by subthreshold doses of J-113397 and L-DOPA (0.1 mg·kg^{-1}) on rotarod performance (Marti *et al.*, 2007), or to the potentiation of a threshold dose of Trap-101 induced by L-DOPA (0.1 mg·kg^{-1}) in the bar and rotarod tests (Marti *et al.*, 2008). It is possible that the high affinity of SB-612111 for the NOP receptor and its good brain penetrability (brain : blood ratio ~10 at steady state; S. Ronzoni, pers. comm.) may provide a rapid and efficient blockade of the NOP receptor. To confirm this view, a clear additive effect was observed (Marti *et al.*, 2007) when challenging submaximal doses of J-113397 (1 mg·kg^{-1}) with L-DOPA (1 mg·kg^{-1}) whereas maximal (1 mg·kg^{-1}) doses of SB-612111 could not improve further the antiparkinsonian effect of L-DOPA (1 mg·kg^{-1}) in the bar and drag tests, indicating maximal beneficial (ceiling) effect. With regard to combined administration, a previous study in marmosets reported that J-113397 potentiated the effect of L-DOPA at the cost of inducing motor side effects such as dyskinesia (Visanji *et al.*, 2008). The present experiments were not designed to measure abnormal involuntary movements (the rodent correlate of dyskinesia) after administration of L-DOPA in combination with NiK21273 or SB-61211. Nonetheless, we exclude that combination of subthreshold doses was dyskinesiogenic since we observed that AIMs appearance in dyskinetic rats compromises rotarod performance (M. Marti and M. Morari, pers. comm.). For the

Figure 4
Acute administration of NiK-21273 and SB-612111 attenuated motor deficits in 6-OHDA hemilesioned rats. NiK-21273 (0.5–15 mg·kg^{-1}) and SB-612111 (0.01–1 mg·kg^{-1}) were administered systemically (i.p.) and motor activity was evaluated in the bar (A, D), drag (B, E) and rotarod (C, F) tests. Only motor activity at the contralateral paws has been shown. Information (including statistical values) on motor activity at the ipsilateral paws has been provided in text. Each experiment consisted of three different sessions: a control session followed by two other sessions performed 30 and 90 min after vehicle or drug administration (see Methods). Data are expressed as percentages of motor activity in the control session (baseline) and are means ± SEM of 7–8 rats per group. Basal motor activity values in the bar, drag and rotarod tests are given in text. Statistical analysis was performed by repeated measures ANOVA followed by contrast analysis and the sequentially rejective Bonferroni test. Statistics results: (A) Significant effect of treatment ($F_{4,24}$ = 7.18, P = 0.0005) and time ($F_{1,30}$ = 17.71, P = 0.0002) but not a time × treatment interaction ($F_{4,30}$ = 1.10, P = 0.37). (B) Significant effect of treatment ($F_{4,24}$ = 12.48, P < 0.00001) and time ($F_{1,34}$ = 6.04, P = 0.0192) but not a time × treatment interaction ($F_{4,34}$ = 1.07, P = 0.38). (C) Significant effect of treatment ($F_{4,24}$ = 15.95, P < 0.00001) and time ($F_{1,34}$ = 18.12, P = 0.0001) but not a time × treatment interaction ($F_{4,34}$ = 2.40, P = 0.07). (D) Significant effect of treatment ($F_{3,18}$ = 7.60, P = 0.0017) and time ($F_{1,24}$ = 7.14, P = 0.0133) but not a time × treatment interaction ($F_{3,24}$ = 0.82, P = 0.49). (E) Significant effect of treatment ($F_{3,18}$ = 10.61, P < 0.0001), but not time ($F_{1,24}$ = 1.42, P = 0.24) or time × treatment interaction ($F_{3,24}$ = 1.12, P = 0.35). (F) Significant effect of treatment ($F_{3,18}$ = 23.79, P < 0.0001), but not time ($F_{1,24}$ = 2.09, P = 0.16) or time × treatment interaction ($F_{3,24}$ = 1.42, P = 0.26). *P < 0.05, significantly different from vehicle.

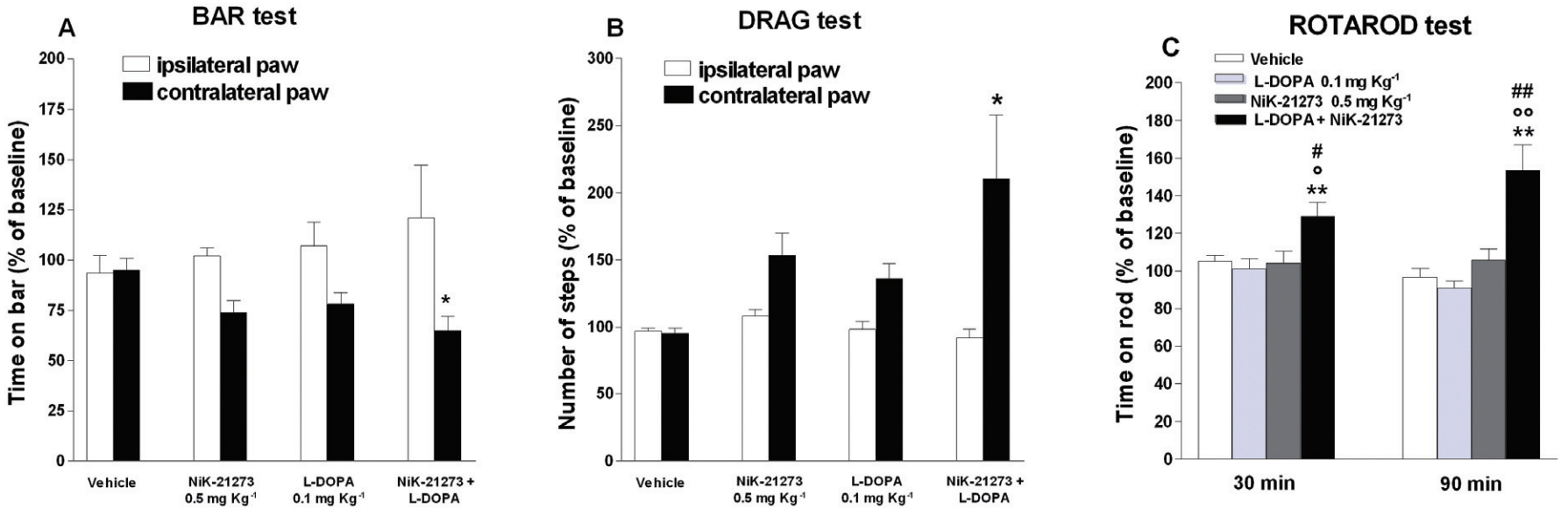


Figure 5

Acute co-administration of NiK-21273 and L-DOPA additively attenuated motor deficits in 6-OHDA hemilesioned rats. The effect of the combination of NiK-21273 (0.5 mg·kg^{-1}) and L-DOPA (0.1 mg·kg^{-1}) on motor performance in the bar (A), drag (B) and rotarod (C) tests was evaluated. In the bar and drag tests, the contralateral (A, C) and ipsilateral (B,D) paws were evaluated separately. Compounds were given i.p. at the same time. Each experiment consisted of three different sessions: a control session followed by two other sessions performed 30 and 90 min after vehicle or NiK-21273 administration (see Methods). For the bar and drag tests, only the effects at 30 min are shown. Data are expressed as percentages of motor activity in the control session (baseline) and are means ± SEM of 8 rats per group. Statistical analysis was performed by conventional two-way ANOVA followed by the Bonferroni test (A, B) or repeated measures ANOVA followed by contrast analysis and the sequentially rejective Bonferroni test (C). Relevant statistics results: (A) Contralateral paw, main effect of L-DOPA ($F_{1,28} = 7.32$, $P = 0.0151$) but not NiK-21273 ($F_{1,28} = 4.18$, $P = 0.05$), and a non-significant NiK-21273 × L-DOPA interaction ($F_{1,28} = 0.44$, $P = 0.51$). (B) Contralateral paw, main effect of NiK-21273 ($F_{1,28} = 9.19$, $P = 0.0052$) but not L-DOPA ($F_{1,28} = 3.66$, $P = 0.07$), and non-significant NiK-21273 × L-DOPA interaction ($F_{1,28} = 0.47$, $P = 0.53$). (C) Significant effect of treatment ($F_{3,21} = 12.05$, $P < 0.0001$) but not time ($F_{1,26} = 0.07$, $P = 0.78$), and a time × treatment interaction at the limit of significance ($F_{3,26} = 2.93$, $P = 0.05$). *$P < 0.05$, **$P < 0.05$, significantly different from vehicle. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different from L-DOPA alone. °$P < 0.05$, °°$P < 0.01$, significantly different from NiK-21273 alone.

Table 2

NOP receptor selective antagonist potencies at human recombinant and native NOP receptors in comparison with threshold effective doses in 6-OHDA hemilesioned rats

	Ca^{2+} mobilization	Isolated tissues		6-OHDA rats *in vivo*		
NOP antagonist	$CHO_{NOP+G\alpha qi5}$	Mouse vas deferens	Rat vas deferens	Bar ($mg \cdot kg^{-1}$)	Drag ($mg \cdot kg^{-1}$)	Rotarod ($mg \cdot kg^{-1}$)
NiK-21273	7.77	7.74	7.74	5	1.5	1.5
SB-612111	7.74	8.50[a]	8.20[a]	1*	0.1	0.1
J-113397	7.88[b]	7.53[a]	7.97[a]	0.1[c]	1[c]	1[c]
Trap-101	7.93[b]	7.75[d]	7.53[e]	10[f]	1[f]	10[f]
Compound 24	9.03[g]	8.44[g]	8.28[g]	0.1[h]	0.1[h]	0.1[h]
GF-4	7.27[e]	7.82[e]	7.30[e]	1[e]	0.1[e]	1[e]

In vitro studies were performed in CHO cells (Ca^{2+} mobilization assay) or mouse and rat vas deferens (electrically stimulated tissues). Data are expressed as pA_2 or pK_B (*in vitro*) or $mg \cdot kg^{-1}$ (*in vivo*). Bar and drag values are referred to the motor activity at the contralateral paw.
*SB-61211 was effective at the ipsilateral paw yet at 0.01 $mg \cdot kg^{-1}$.
[a]Spagnolo *et al.* (2007).
[b]Camarda *et al.* (2009).
[c]Marti *et al.* (2005).
[d]Trapella *et al.* (2006).
[e]Volta *et al.* (2010b).
[f]Marti *et al.* (2008).
[g]Fischetti *et al.* (2009).
[h]Volta *et al.* (2011).

same reason, however, we cannot exclude that the reduction in rotarod performance observed following application of high NiK-21273 and L-DOPA doses may reflect motor incoordination due to dyskinesia appearance. The present study reveals for the first time the reproducibility of the antiparkinsonian effects of SB-612111 during chronic treatment. Indeed, no decrease in the stepping and rotarod promoting action of SB-612111 was observed over a 16-day administration protocol, and also its antiakinetic effect in the bar test was observed throughout but the last session. To the best of our knowledge, only two studies have investigated motor changes in response to repeated administration of a NOP receptor antagonist (Okabe and Murphy, 2004; Vitale *et al.*, 2009). In the former, a high dose (10 $mg \cdot kg^{-1}$) of J-113397 was administered systemically every other day for 5 days (three sessions) under a protocol of methamphetamine sensitization in mice. In the latter, the peptide antagonist UFP-101 was given i.c.v. (10 nmol) for 21 days to investigate its antidepressant activity in rats. Both studies monitored spontaneous locomotion (horizontal activity, rearings) and reported no baseline effects following chronic NOP receptor blockade, in line with that observed here for SB-612111.

Similar to SB-612111, the novel piperidine NiK-21273 behaves *in vitro* as a pure, fairly potent and selective NOP receptor antagonist. NiK-21273 antagonized the inhibitory action of N/OFQ at human recombinant NOP receptors with comparable potency to SB-612111 (pA_2 7.77), although it was less potent than SB-612111 at animal native NOP receptors (pA_2 7.74; Table 2). Consistently, NiK-21273 replicated the effects of SB-612111 in both reserpinized mice and 6-OHDA hemilesioned animals *in vivo* with similar efficacy but lower potency. Nonetheless, some differences in the behavioural profiles of SB-612111 and NiK-21273 were noticed. For instance, SB-612111 caused marked reduction of immobility time in the bar test in reserpinized mice and 6-OHDA hemilesioned rats, both at the ipsilateral and contralateral paws, while NiK-21273 was only effective in 6-OHDA hemilesioned rats at the contralateral paw. In the bar test, also the ipsilateral paw, which is operated by the hemisphere contralateral to the lesioned side, becomes akinetic after unilateral 6-OHDA lesioning, although less severely than the contralateral one. This is no surprise as also the primary motor cortex contralateral to the lesioned side is affected (reduced excitability; see Viaro *et al.*, 2011) by unilateral destruction of the nigrostriatal DA tract. Conversely, a clear motor asymmetry is observed in the stepping test, where motor impairment only affects the contralateral paw. This might indicate that the behavioural tests engage different brain areas/circuits and, consequently, different populations of NOP receptors. The low potency of NiK-21273 at the NOP receptor (see Table 2), the poor brain penetrance of NiK-21273 (brain : plasma ratio ~0.2 after bolus injection; S. Ronzoni, pers. comm.), and the fact that central NOP receptors are differentially saturated by endogenous N/OFQ (particularly after DA denervation) might explain the milder effect of NiK-21273 in the bar test.

Perhaps more relevant, NiK-21273 dose–response curves in mice and rats were bell-shaped, restraining drug action within a narrow dose range. Dual motor responses following administration of the NOP antagonists J-113397 (Viaro *et al.*, 2008), GF-4 (Volta *et al.*, 2010b) and Compound 24 (Volta *et al.*, 2011) were previously reported in hemiparkinsonian rats, low doses (0.01–1 $mg \cdot kg^{-1}$) improving and higher ones (30 $mg \cdot kg^{-1}$) inhibiting movement. However, the ratio between the first inhibitory and stimulatory doses (a sort of

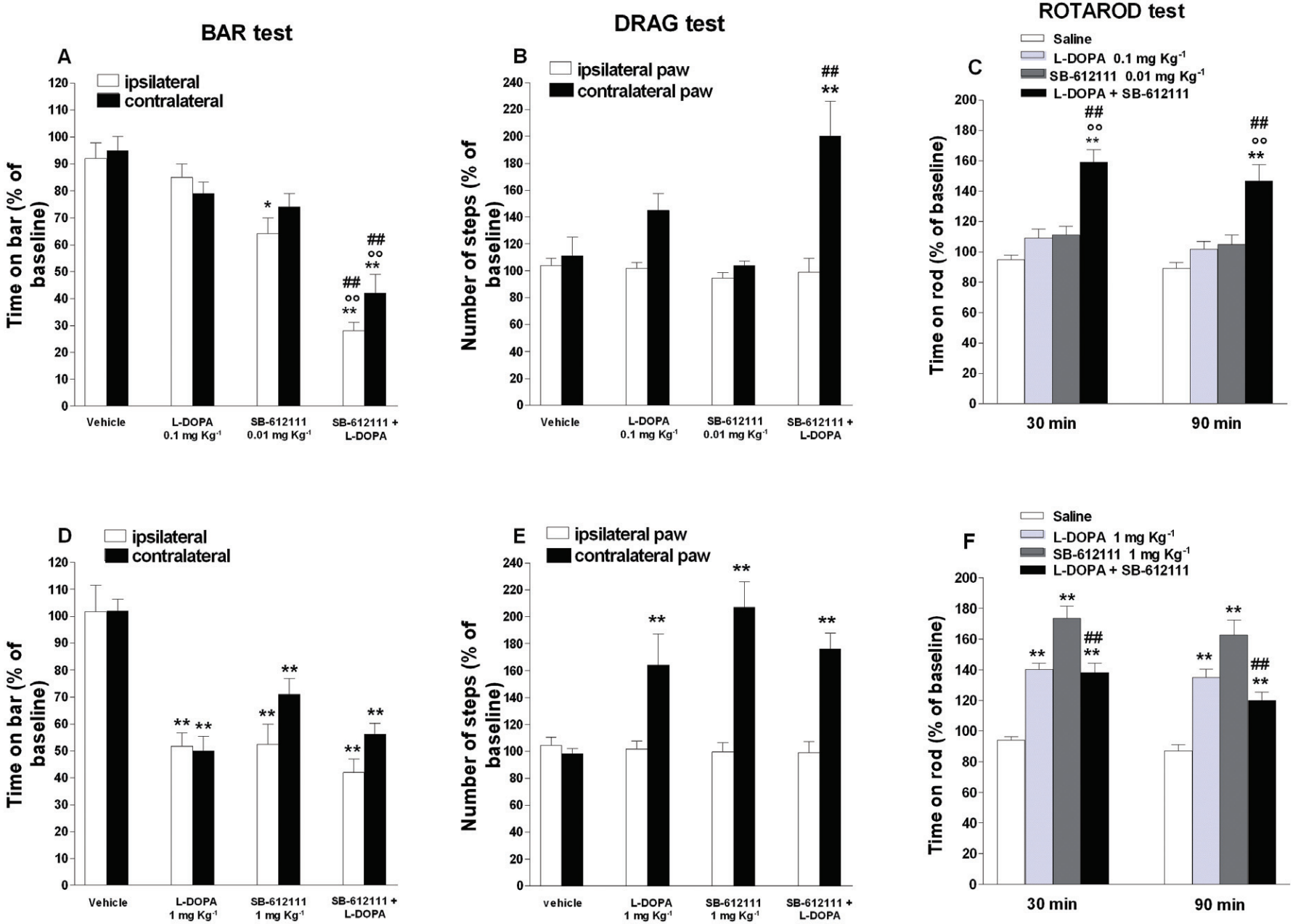

BAR test
A
ipsilateral
contralateral
Time on bar (% of baseline)
Vehicle
L-DOPA 0.1 mg Kg-1
SB-612111 0.01 mg Kg-1
SB-612111 + L-DOPA
DRAG test
B
ipsilateral paw
contralateral paw
Number of steps (% of baseline)
ROTAROD test
C
Saline
L-DOPA 0.1 mg Kg-1
SB-612111 0.01 mg Kg-1
L-DOPA + SB-612111
Time on rod (% of baseline)
30 min
90 min
D
E
F
L-DOPA 1 mg Kg-1
SB-612111 1 mg Kg-1
vehicle

Figure 6
Acute co-administration of SB-612111 and L-DOPA synergistically attenuated motor deficits in 6-OHDA hemilesioned rats. The effects of two different drug combinations are shown: low doses of SB-612111 (0.01 mg·kg^{-1}) and L-DOPA (0.1 mg·kg^{-1}; i.p.), and high doses of both compounds (1 mg·kg^{-1} each). In the bar and drag tests, the contralateral (A, C) and ipsilateral (B, D) paws were evaluated separately. Compounds were given i.p. at the same time. Each experiment consisted of three different sessions: a control session followed by two other sessions performed 30 and 90 min after vehicle or SB-612111 administration (see Methods). For the bar and drag tests, only the effects at 30 min are shown. Data are expressed as percentages of motor activity in the control session (baseline) and are means ± SEM of 7–8 rats per group. Statistical analysis was performed by conventional two-way ANOVA followed by the Bonferroni test (A–B, D–E) or repeated measures ANOVA followed by contrast analysis and the sequentially rejective Bonferroni test (C, F). Relevant statistics results: (A) Contralateral paw, main effect of L-DOPA ($F_{1,32} = 27.23$, $P < 0.0001$) and SB-612111 ($F_{1,32} = 18.65$, $P = 0.0001$), non-significant interaction between the two ($F_{1,32} = 2.18$, $P = 0.15$). Ipsilateral paw, main effect of L-DOPA ($F_{1,32} = 19.53$, $P < 0.0001$) and SB-612111 ($F_{1,32} = 72.86$, $P = 0.0001$), significant interaction between the two ($F_{1,32} = 8.53$, $P = 0.0064$). (B) Contralateral paw, main effect of L-DOPA ($F_{1,31} = 16.92$, $P = 0.0003$) but not SB-612111 ($F_{1,31} = 3.65$, $P = 0.06$), significant interaction between the two ($F_{1,31} = 5.52$, $P = 0.0254$). (C) Significant effect of treatment ($F_{3,18} = 67.14$, $P < 0.0001$) but not time ($F_{1,24} = 0.88$, $P = 0.18$) or time × treatment interaction ($F_{3,24} = 0.06$, $P = 0.97$). (D) Contralateral paw, main effect of L-DOPA ($F_{1,36} = 23.56$, $P < 0.0001$) and SB-612111 ($F_{1,36} = 28.50$, $P < 0.0001$), but not a significant interaction between the two ($F_{1,36} = 2.14$, $P = 0.15$). Ipsilateral paw, main effect of L-DOPA ($F_{1,36} = 11.64$, $P = 0.0016$) and SB-612111 ($F_{1,36} = 10.75$, $P = 0.0023$), significant interaction between the two ($F_{1,36} = 5.16$, $P = 0.0292$). (E) Contralateral paw, main effect of SB612111 ($F_{1,36} = 19.72$, $P < 0.0001$) but not L-DOPA ($F_{1,36} = 2.36$, $P = 0.13$), and a significant interaction between the two ($F_{1,36} = 12.85$, $P = 0.0010$). (F) Significant effect of treatment ($F_{3,24} = 48.82$, $P < 0.0001$) and time ($F_{1,30} = 5.87$, $P = 0.0216$), non-significant time × treatment interaction ($F_{3,30} = 0.49$, $P = 0.69$). *$P < 0.05$, **$P < 0.01$ significantly different from vehicle. ##$P < 0.01$ significantly different from L-DOPA alone. °°$P < 0.01$, significantly different from SB-612111 alone.

'therapeutic window') was narrowest for NiK-21273 (~10) among these NOP antagonists, which might represent a clear limitation for its *in vivo* applicability. Another peculiarity in the behavioural profile of NiK-21273 emerged during chronic treatment. Indeed, tolerance to acute beneficial effects in the drag and rotarod tests was observed together with delayed improvement in baseline performance in the bar and drag tests. The acute and chronic changes were clearly not related since in the bar test only a baseline effect of NiK-21273 was found, wherein in the rotarod test only acute effects were recorded. Moreover, in the drag test, where both phenomena were observed, they were temporally dissociated. Therefore, the acute and chronic motor effects induced by NiK-21273 may be mediated by different neural mechanisms.

The different behavioural responses of SB-612111 and NiK-21273 during chronic treatment are difficult to be explained at present. A comparison between a series of selective small molecule NOP receptor non-peptide antagonists (Table 2) showed a good correlation between the potency at native rat peripheral NOP receptors (Compound 24 ≥ SB-612111 > J-113397 > NiK-21273 > Trap-101 > GF4) with *in vivo* antiparkinsonian potencies, expressed by threshold doses in the bar, drag and rotarod tests in 6-OHDA hemilesioned rats (Table 2). A remarkable exception was represented by GF-4 which was more potent *in vivo* than that expected on the basis of *in vitro* potency data. The lack of (comparable) pharmacokinetic data makes it impossible to understand whether the different *in vivo* behavioural profiles of NiK-21273 and SB-612111 (and more in general, NOP antagonists) are generated by different bioavailability or CNS penetration, rather than pharmacodynamic properties, such as dissociation from the NOP receptors or binding to splice variants of the NOP receptor. Indeed, NiK-21273 has a poorer brain penetrance than SB-612111. Moreover, *in vitro* data showed that SB-612111 dissociates very slowly from the NOP receptor (also see Spagnolo *et al.*, 2007), whereas NiK-21273 blockade is fairly short lived. This would rule out the possibility that the blockade of the NOP receptor exceeds the plasma half-life of NiK-21273, leading on one hand to amelioration of baseline activity, and on the other to extinction of acute effects. Finally, previous evidence of heterogeneity in the *in vitro* (Chiou *et al.*, 2004) and *in vivo* (Kuzmin *et al.*, 2004) responses to NOP receptor agonists has been reported and interpreted on the basis of the interaction with different splice variants of the NOP receptor (Mogil and Pasternak, 2001).

An explanation of the different profiles of NiK-21273 and SB-612111 may be attempted based on our previous studies on the mechanism of action of NOP antagonists. The antiparkinsonian effect of NOP antagonists was demonstrated to take place in SNr where NOP receptor blockade normalizes the motor imbalance between inhibitory and inhibitory inputs impinging on nigro-thalamic neurons which has been generated by DA depletion. Indeed, NOP antagonists enhance GABA and reduce glutamate release leading to over-inhibition of nigro-thalamic output neurons (Marti *et al.*, 2007; 2008). These neurochemical changes are likely due to blockade of the inhibitory N/OFQ influence over NOP-expressing GABA neurons, but also rely on D2 receptor availability since higher doses of NOP antagonists are required to elicit the same antiakinetic effect in the presence of a D2

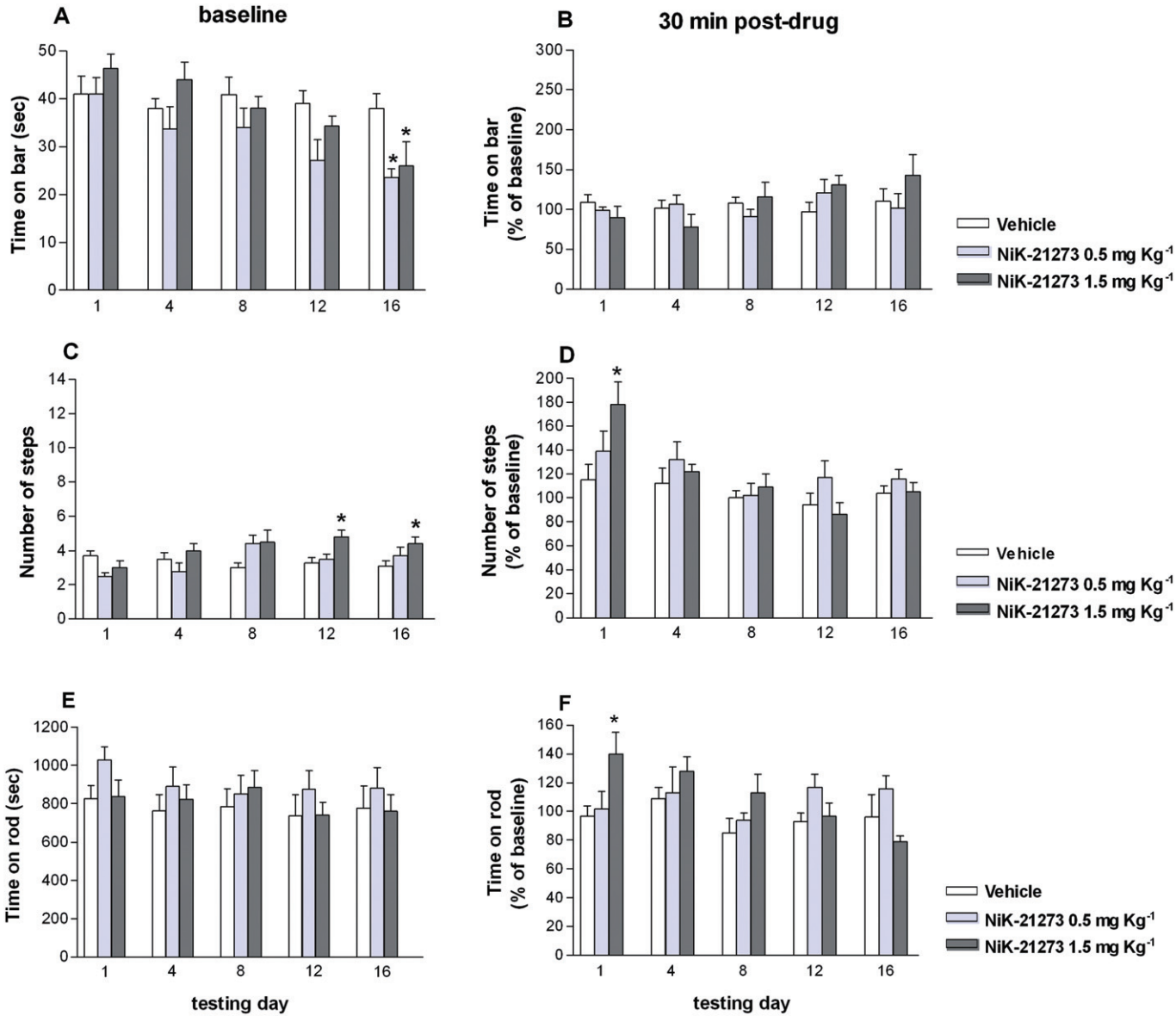


Figure 7

Chronic administration of NiK-21273 attenuated motor disabilities in 6-OHDA hemilesioned rats, inducing tolerance to its acute effects. NiK-21273 (0.5 and 1.5 mg·kg^{-1}; i.p.) was administered once daily for 16 days. Motor activity was evaluated in the bar (A–B), drag (C–D) and rotarod (E–F) tests before ('baseline') and 30 min after ('30 min post-drug') drug administration. In the bar and drag tests, motor activity at the contralateral paws has been shown. Information (including relevant statistical values) on motor activity at the ipsilateral paws has been provided in text. Data are expressed as immobility time (in seconds; A), number of steps (C), or time on rod (in seconds; E) or percentages of motor activity in the control session (baseline; B, D, F), and are means ± SEM of 8 rats per group. Statistical analysis was performed by repeated measures ANOVA followed by contrast analysis and the sequentially rejective Bonferroni test. Relevant statistics results: (A) Non-significant effect of treatment ($F_{2,14}$ = 2.14, P = 0.15), significant effect of time ($F_{4,84}$ = 11.97, P < 0.0001) and time × treatment interaction ($F_{8,84}$ = 2.86, P = 0.007). (C) Non-significant effect of treatment ($F_{2,14}$ = 2.70, P = 0.10), significant effect of time ($F_{4,84}$ = 4.97, P = 0.0012) and time × treatment interaction ($F_{8,84}$ = 4.34, P = 0.0002). (D) Non-significant effect of treatment ($F_{2,14}$ = 2.85, P = 0.90), significant effect of time ($F_{4,84}$ = 6.34, P = 0.0002) and time × treatment interaction ($F_{8,84}$ = 2.59, P = 0.0139). (F) Non-significant effect of treatment ($F_{2,14}$ = 2.85, P = 0.90), significant effect of time ($F_{4,84}$ = 6.34, P = 0.0002) and time × treatment interaction ($F_{8,84}$ = 2.59, P = 0.0139). *P < 0.05, significantly different from vehicle.

receptor blocker (Volta *et al.*, 2011). We can therefore speculate that NiK-21273 and SB-612111 differentially affect these pathways. Interestingly, the narrow therapeutic window of NiK-21273 may reflect a stronger impact of this drug on DA transmission. Indeed, the motor inhibition caused by high doses of NOP antagonists was found to be reversed by D2 receptor antagonist amisulpride, possibly involving D2 autoreceptors (Viaro *et al.*, 2010; Volta *et al.*, 2011).

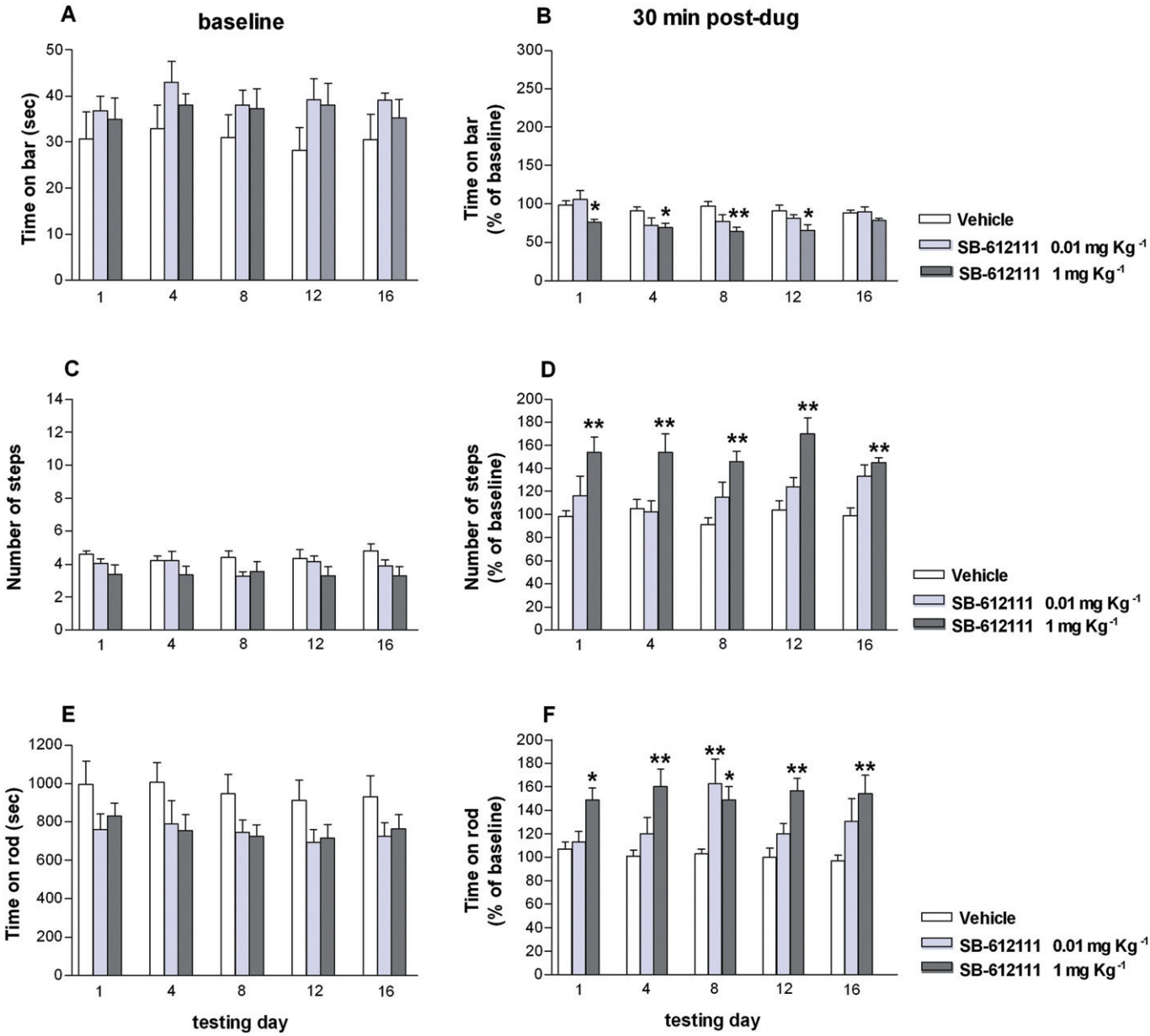


Figure 8

Chronic administration of SB-612111 attenuated motor disabilities in 6-OHDA hemilesioned rats, without inducing tolerance to its acute effects. SB-612111 (0.01 and 1 mg·kg^{-1}; i.p.) was administered once daily for 16 days. Motor activity was evaluated in the bar (A–B), drag (C–D) and rotarod (E–F) tests before ('baseline') and 30 min after ('30 min post-drug') drug administration. In the bar and drag tests, motor activity at the contralateral paws has been shown. Information (including relevant statistical values) on motor activity at the ipsilateral paws has been provided in text. Data are expressed as immobility time (in seconds; A), number of steps (C), or time on rod (in seconds; E) or percentages of motor activity in the control session (baseline; B, D, F), and are means ± SEM of 6–8 rats per group. Statistical analysis was performed by repeated measures ANOVA followed by contrast analysis and the sequentially rejective Bonferroni test. Relevant statistics results: (B) Significant effect of treatment ($F_{2,12}$ = 13.29, $P < 0.01$) and time ($F_{4,82} = 2.72$, $P = 0.035$) but not time × treatment interaction ($F_{8,82} = 1.32$, $P = 0.24$). (D) Significant effect of treatment ($F_{2,14} = 22.26$, $P < 0.01$) but not time ($F_{4,84} = 1.58$, $P = 0.18$) or time × treatment interaction ($F_{8,84} = 1.08$, $P = 0.38$). (F) Significant effect of treatment ($F_{2,14} = 9.93$, $P < 0.01$) but not time ($F_{4,84} = 0.71$, $P = 0.58$) or time × treatment interaction ($F_{8,84} = 1.67$, $P = 0.11$). *$P < 0.05$, **$P < 0.01$, significantly different from vehicle.

Concluding remarks

The antiparkinsonian properties of the novel NOP receptor antagonist NiK-21273 in comparison with SB-612111 were assessed in a functional (reserpinized mouse) and neurodegeneration (6-OHDA hemilesioned rat) model of parkinsonism. NiK-21273 and SB-612111 acutely attenuated parkinsonian-like motor disabilities with similar efficacy and

different potency, in line with their different affinity for native NOP receptors. Importantly, they both synergized with L-DOPA at subthreshold doses, confirming that a NOP antagonist might be used to reduce L-DOPA dosage and delay its side effects. Different from SB-612111, however, NiK-21273 showed a bell-shaped profile of action, and tolerance occurred to its acute effects over a prolonged treatment, possibly indicating hitherto unidentified differences in the pharmacokinetic/pharmacodynamic properties. Since other structurally unrelated NOP antagonists showed a bell-shaped profile in hemiparkinsonian rats, these data indicate that loss of efficacy (or overt motor inhibition) is a drug class property but the therapeutic window varies among molecules. More relevant from a clinical perspective, evidence that repeated NOP receptor blockade with SB-612111 induces reproducible beneficial effects on motor performance has been provided for the first time. This would suggest that chronic therapy with low doses of SB-612111, perhaps in combination with L-DOPA, may provide long-term symptomatic relief in parkinsonian patients (Marti *et al.*, 2005; 2007; Viaro *et al.*, 2010).

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Conflict of interest

None.

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