Behavioral/Systems/Cognitive

Blockade of Nociceptin/Orphanin FQ Receptor Signaling in Rat Substantia Nigra Pars Reticulata Stimulates Nigrostriatal Dopaminergic Transmission and Motor Behavior

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A multidisciplinary approach was followed to investigate whether the opioid-like peptide nociceptin/orphanin FQ (N/OFQ) regulates the nigrostriatal dopaminergic pathway and motor behavior. Nigrostriatal dopaminergic cells, which express N/OFQ peptide (NOP) receptors, are located in the substantia nigra pars compacta and extend their dendrites in the substantia nigra pars reticulata, thereby modulating the basal ganglia output neurons. *In vitro* electrophysiological recordings demonstrated that N/OFQ hyperpolarized the dopaminergic cells of the substantia nigra pars compacta and inhibited their firing activity. *In vivo* dual-probe microdialysis showed that N/OFQ perfused in the substantia nigra pars reticulata reduced dopamine release in the ipsilateral striatum, whereas UFP-101 ([Nphe^1,Arg^{14},Lys^{15}]N/OFQ(1–13)-NH$_2$) (a selective NOP receptor peptide antagonist) stimulated it. N/OFQ microinjected in the substantia nigra pars reticulata impaired rat performance on a rotarod apparatus, whereas UFP-101 enhanced it. Electromyography revealed that N/OFQ and UFP-101 oppositely affected muscle tone, inducing relaxation and contraction of triceps, respectively. The selective NOP receptor nonpeptide antagonist J-113397 (1-[3*R*,4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H benzimidazol-2-one), either injected intranigrally or given systemically, also elevated striatal dopamine release and facilitated motor activity, confirming that these effects were caused by blockade of endogenous N/OFQ signaling. The inhibitory role played by endogenous N/OFQ on motor activity was additionally strengthened by the finding that mice lacking the NOP receptor gene outperformed wild-type mice on the rotarod. We conclude that NOP receptors in the substantia nigra pars reticulata, activated by endogenous N/OFQ, drive a physiologically inhibitory control on motor behavior, possibly via modulation of the nigrostriatal dopaminergic pathway.

Key words: **dopamine release; J-113397; substantia nigra; motor activity; UFP-101; microdialysis; nociceptin/orphanin FQ; N/OFQ**

Introduction

The opioid-like peptide nociceptin/orphanin FQ (N/OFQ) (Meunier et al., 1995; Reinscheid et al., 1995) modulates pain perception, mood, reward, learning and memory, food intake, and locomotion via activation of the N/OFQ peptide (NOP) receptor (Cox et al., 2000). N/OFQ and its receptor are diffusely expressed in the brain, and evidence for heterogeneity of N/OFQ sites, possibly representing splice variants or posttranslational modifications of the NOP receptor, has been presented (Calò et al., 2000; Mogil and Pasternak, 2001). The pharmacology of the N/OFQ–NOP receptor system has been extensively characterized by means of selective NOP receptor ligands (Zaveri, 2003). Conversely, less is known on the physiology of endogenous N/OFQ, possibly because of the lack of potent and selective antagonists, which only recently have been developed: the peptide UFP-101 ([Nphe^1,Arg^{14},Lys^{15}]N/OFQ(1–13)-NH$_2$) (Calò et al., 2002) and the nonpeptide J-113397 (1-[3*R*,4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H benzimidazol-2-one) (Kawamoto et al., 1999; Ozaki et al., 2000). Thus, although supraspinal administration of high doses of N/OFQ consistently impaired motor activity in rodents, endogenous N/OFQ does not tonically regulate spontaneous locomotion because selective NOP receptor antagonists (Noda et al., 1998; Calò et al., 2000, 2002; Kuzmin et al., 2004) or deletion of the NOP receptor gene (NOP$^{-/-}$ mice) failed to affect motor phenotype (Nishi et al., 1997; Murphy et al., 2002; Gavioli et al., 2003; Koizumi et al., 2004). However, daily intracerebroventricular injections of an antisense oligonucleotide to proN/OFQ induced hyperlocomotion in rats (Candeletti and Ferri, 2000), challenging this view. Moreover, endogenous N/OFQ regulation of locomotion under dynamic conditions (e.g., during motor tasks) was not investigated.

Consistent with NOP receptor expression onto mesencephalic dopaminergic (DAergic) neurons (Maidment et al., 2002;

Received March 17, 2004; revised May 25, 2004; accepted May 25, 2004.

This work was supported by Grant Cofin 2002 (C.B.) from the Italian Ministry of the University and by grants from the University of Ferrara (ex-60%) and Cassa di Risparmio di Ferrara Foundation (M. Morari).

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DOI:10.1523/JNEUROSCI.0987-04.2004

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Norton et al., 2002), motor-depressant actions of N/OFQ have been related to inhibition of the mesoaccumbal DAergic pathway (Murphy et al., 1996; Murphy and Maidment, 1999). However, N/OFQ may also inhibit the nigrostriatal DAergic pathway because it reduced striatal ^{3}H-DA release *in vitro* (Flau et al., 2002), striatal DOPAC content *in vivo* (Shieh and Pan, 1998), and, when perfused into the substantia nigra (SN) pars reticulata (SNr), facilitated glutamate release via D_2 receptor-mediated mechanisms (Marti et al., 2002a). It is known that the opioid system regulates motor activity, at least partly, via the nigrostriatal pathway: μ and δ receptor agonists stimulate locomotion and the nigrostriatal transmission, whereas κ receptor agonists inhibit both (Mansour et al., 1995). Thus, the N/OFQergic modulation of the nigrostriatal neurons may be behaviorally relevant.

The present study was aimed to investigate whether (1) exogenous N/OFQ inhibits the nigrostriatal DAergic pathway and motor behavior and (2) endogenous N/OFQ tonically activates SNr NOP receptors. Electrophysiological recordings were performed to study nigral DAergic cell activity *in vitro*, whereas dual probe microdialysis, a rotarod apparatus, and electromyography (EMG) were used to monitor striatal DA release, activity-induced locomotion, and muscle tone *in vivo*, respectively. The role of endogenous N/OFQ was investigated by using UFP-101 and J-113397 and by evaluating motor activity of NOP $^{-/-}$ mice.

Parts of this work has been published previously in abstract form (Marti et al., 2003a).

Materials and Methods

Male Sprague Dawley rats (300–350 gm; Stefano Morini, Reggio Emilia, Italy) were used in the study. The experimental protocols were approved by Ethical Committee of the University of Ferrara, and adequate measures were taken to minimize animal pain and discomfort.

Electrophysiology. Horizontal slices of ventral midbrain (300 μm thick) were prepared and maintained as described previously (Mercuri et al., 1995). Borosilicate microelectrodes filled with 2 M NaCl (5–10 MΩ) were used to record single DA neurons extracellularly (see Fig. 1*A*) and with 2 M KCl (40–60 MΩ) to record intracellularly (see Fig. 1*B*) in the SN pars compacta (SNc). Neurons were identified as DAergic using well established electrophysiological and pharmacological criteria (Mercuri et al., 1995; Pucak and Grace 1996; Shepard and Connelly, 1999). The voltage signals were obtained by an amplifier (Axoclamp-2 B; Axon Instruments, Foster City, CA), digitized using a Digidata 1322A (Axon Instruments) analog-to-digital interface and Axoscope software (Axon Instruments) running on an IBM-compatible computer, and saved for off-line analysis.

Microinjection technique. A guide-injection cannula (outer diameter, 0.55 mm) was stereotaxically implanted under isoflurane anesthesia 0.50 mm above the right SNr (anteroposterior, 5.5; mediolateral, 2.2; ventrodorsal, 7.3 from bregma) (Paxinos and Watson, 1982). Seven days after surgery, compounds were injected (0.5 μl volume) through a stainless-steel injector (outer diameter, 0.30 mm) protruding 1 mm beyond the cannula tip. At the end of each experiment, the placement of the probes was verified by microscopic examination.

Microdialysis technique. Two concentric probes were stereotaxically implanted under isoflurane anesthesia in the right dorsolateral striatum (DLS) (3 mm length) and ipsilateral SNr (1 mm length), as described previously (Marti et al., 2002b). Forty-eight hours after surgery, the microdialysis probes were perfused at a flow rate of 3 μl/min with a modified Ringer's solution (in mM: 1.2 $CaCl_2$, 2.7 KCl, 148 NaCl, and 0.85 $MgCl_2$), and samples were collected every 15 min, starting 6 hr after the onset of probe perfusion. *In vitro* DA recovery for the 3 mm probe was 12 ± 2%.

Endogenous DA analysis. DA was measured by means of reversed-phase HPLC coupled to electrochemical detection. Briefly, 27 μl samples were injected onto a 5-C18 Chromsep analytical column perfused at a flow rate of 0.4 ml/min (Beckman 118 pump; Beckman Instruments, Fullerton, CA) with a mobile phase containing 75 mM NaH_2PO_4, 20 μM EDTA, 0.01% triethylamine, 1.5 mM SDS, 10% methanol, and 16% acetonitrile, adjusted to pH 5.6 with NaOH. DA was detected by means of an electrochemical detector (Coulochem II model 5200; ESA, Chelmsford, MA) set at +175 mV. The limit of detection for DA was 10 fmol/sample.

Table 1. Rotarod training tasks in rats

	First speed	Second speed	Third speed	Fourth speed
Session 1 (t_0)	94.2 ± 1.8%	81.0 ± 3.7%	64.2 ± 4.4%	41.7 ± 4.2%
Session 2 (t_{50})	91.6 ± 2.6%	80.1 ± 3.6%	64.4 ± 4.2%	43.3 ± 4.3%
Session 3 (t_{100})	92.5 ± 2.4%	81.9 ± 3.5%	61.4 ± 4.4%	43.1 ± 4.1%

Naive, drug-free rats were trained to perform on the rotarod at four increasing speeds until their motor performance was reproducible along three consecutive sessions, performed at t_0 (session 1 or control session) or after 50 min (t_{50}, session 2) and 100 min (t_{100}, session 3). Speeds were selected to obtain progressive inhibition of rat performance down to ~40% of maximum performance (usually 25, 30, 35, and 40 rpm; cutoff time of 180 sec). Data are expressed as percentage means ± SEM of maximum response (45–50 determinations).

Studies on motor behavior. The fixed-speed rotarod (FSRR) test (Rozas et al., 1997; Rustay et al., 2003) was used to investigate the effects of NOP receptor ligands on physiologically stimulated motor activity. Rats were handled for 1 week by the same operator to reduce stress and trained for additional 10 d on a rotating spindle (7.6 cm diameter) until their motor performance became reproducible. To detect both facilitatory and inhibitory effects on motor activity (Rustay et al., 2003), a specific protocol was developed: rats were tested (t_0) at four increasing speeds (usually 25, 30, 35, and 40 rpm; 180 sec each), causing a progressive decrement of performance to ~40% of the maximal response (i.e., the experimental cutoff time) (Table 1). A similar response could be reproduced by applying this protocol 50 and 100 min later (t_{50} and t_{100}) (Table 1). Thus, to quantify drug effect on motor behavior, drugs were administered 10 min before t_{50}, and rotarod performance (total time spent on the rotarod) was calculated at t_{50} and t_{100} (i.e., 10 and 60 min after injection) as a percentage of control (t_0) performance.

Whenever pharmacological treatment was associated with contralateral turning, rotational behavior was specifically measured. Rats were left to habituate in circular bowls for 20 min before the beginning of the test. Contralateral turns (i.e., turns in direction opposite to the injection side) were counted every 5 min, from 15 min before to 90 min after drug injection.

EMG recordings. Bipolar electrodes (Teflon-coated stainless-steel wire; Clark Electromedical Instruments, Pangbourne, UK) were bilaterally implanted in the triceps muscles under ketamine anesthesia (100 mg/kg). The distal end of the electrodes (with ~300 μm of the insulation removed) were sutured to the belly of the muscle. The proximal end of the wire was joined to a five pin socket and secured with dental cement on the skull of the animals. Experiments were performed 7 d after surgery in unrestrained awake animals placed in a cage. EMG activity was recorded bilaterally (20 sec), before (t_0) or 10 (t_{10}) and 60 (t_{60}) min after intranigral injection of NOP receptor ligands. The EMG signals were amplified (P5 amplifier; Grass Instruments, Quincy, MA), filtered (bandpass, 30 Hz to 30 kHz), monitored on a storage oscilloscope (model 5113; Tektronix, Wilsonville, OR), and acquired by analog-to-digital interface for off-line analysis (CED 1401 and Spike2; Cambridge Electronic Design, Cambridge, UK). Then, the EMGs were rectified, and the area of the first 10 sec of activity was determined. The area-under-the-curve (AUC) value of the rectified EMG provided better estimate of the electrical activity of muscle fibers than the peak-to-peak amplitude (Buchthal and Kuhl, 1979). AUC values were normalized and expressed as a percentage of baseline t_0 values (see Fig. 6).

Motor behavior in NOP$^{+/+}$ and NOP$^{-/-}$ mice. NOP $^{+/+}$ and NOP $^{-/-}$ mice (25–30 gm) were generated on a mixed C57BL/6J and 129 genetic background (Nishi et al., 1997) and backcrossed with CD1 mice (Bertorelli et al., 2002) for nine generations, which would guarantee that >95% of their genetic background is of the CD1 type. Animals were genotyped by PCR. Spontaneous locomotor activity was measured in nonhabituated male mice by using Ugo Basile (Comerio, Italy) activity cages (Rizzi et al., 2001). The total number of impulses were recorded every 5 min for 30 min. The FSRR test was used to investigate the motor performance of nonhabituated mice and the adaptive changes occurring

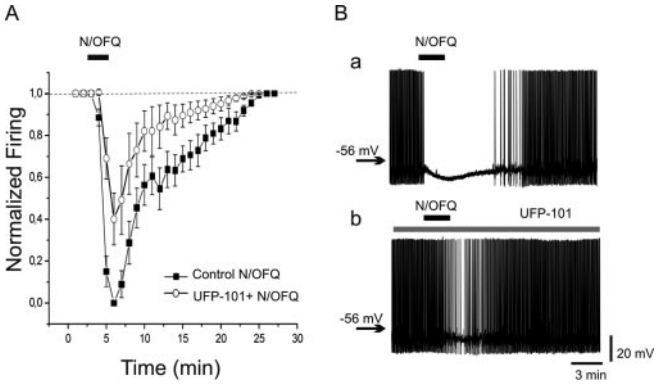

Figure 1. Effects of N/OFQ and UFP-101 on activity of SNc DAergic neurons. *A*, Extracellular recordings of SNc DAergic neurons. N/OFQ (100 nM; 3 min; black bar) reduced the firing rate of SNc DAergic neurons, and UFP-101 (1 μM) antagonized its effect. In the presence of UFP-101, not only the amplitude but also the duration of the firing inhibition was reduced. Perfusion with UFP-101 started 7–10 min before N/OFQ and continued until the end of experiment. The firing rates of neurons after drug application were normalized to the control firing rate in the same neuron. Points represent means ± SEM of seven experiments. *B*, Intracellular recordings of a DAergic neuron. N/OFQ (100 nM; 3 min; black bar) hyperpolarized the membrane and caused firing inhibition (*a*). N/OFQ-induced effects were clearly depressed by UFP-101 (1 μM) (*b*). Perfusion with UFP-101 started 7–10 min before N/OFQ and continued until the end of experiment (gray bar).

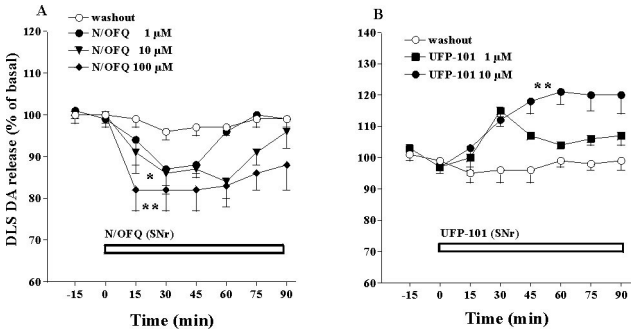

Figure 2. Effect of N/OFQ and UFP-101 on striatal DA release. *A*, Effect of local perfusion with N/OFQ (1–100 μM; 90 min; open bar) in the SNr of awake rats on DA release in the ipsilateral DLS. Basal DA (in nanomolar) and AUC (in arbitrary units) values were, respectively, as follows: 1.70 ± 0.31, 7283 ± 137.3 (washout; $n = 9$), 1.03 ± 0.26, 6887 ± 69.5 (1 μM N/OFQ; $n = 6$), 1.88 ± 0.31, 6599 ± 152.1 (10 μM N/OFQ; $n = 7$), 1.96 ± 0.33, and 6219 ± 328.5 (100 μM N/OFQ; $n = 5$). *B*, Effect of local perfusion with UFP-101 (1 and 10 μM; 90 min; open bar) in the SNr of awake rats on DA release in the ipsilateral DLS. Basal DA (in nanomolar) and AUC (in arbitrary units) values were, respectively, as follows: 1.59 ± 0.31, 7343 ± 187.1 (washout; $n = 6$); 1.35 ± 0.15, 7701 ± 266.8 (1 μM UFP-101; $n = 4$), 1.43 ± 0.30, and 8728 ± 219.8 (10 μM UFP-101; $n = 6$). Data are expressed as percentages ± SEM of basal pretreatment levels (calculated as the mean of the 2 samples before the treatment). *$p < 0.05$; **$p < 0.01$, significantly different from washout.

after repeated motor tasks. Each mouse was tested on the rotarod at a wide range of increasing speeds (5–55 rpm), and the time spent on the rod was calculated (180 sec cutoff time). This protocol was performed daily (from 9:00 A.M. to 12:00 P.M.) for 4 consecutive days.

Data presentation and statistical analysis. DA release has been expressed as percentage ± SEM of basal values (calculated as mean of the two samples before the treatment). Motor performance of rats has been presented as percentage ± SEM of the control session, whereas motor performance of mice has been presented in absolute values (in seconds). Statistical analysis was performed (Prism software; GraphPad Software, San Diego, CA) on AUC values (expressed in arbitrary units) by ANOVA, followed by the Newman–Keuls test for multiple comparisons. p values <0.05 were considered to be statistically significant.

Materials. N/OFQ and UFP-101 were prepared as described previously (Guerrini et al., 2000). J-113397 was synthesized in our laboratories as a racemic mixture (De Risi et al., 2001). All drugs were freshly dissolved in Ringer's or isosmotic saline solution.

Results

Effects of NOP receptor ligands on the activity of SNc DAergic neurons

To test whether nigral NOP receptors modulate the activity of SNc DAergic neurons, the effects of NOP receptor ligands were first examined using intracellular recordings *in vitro* (Fig. 1). DAergic cells were spontaneously firing at a rate of 1.3 Hz (range, 0.5–3 Hz) and had a spike width of >1.2 msec. N/OFQ (100 nM) caused membrane hyperpolarization (6.5 ± 1.2 mV; $n = 5$) that resulted in depression of action potential discharge. The firing rate recovered to control conditions within 15–20 min from drug washout (Fig. 1*A*). The specificity of N/OFQ action was investigated by using UFP-101. Perfusion with UFP-101 (1 μM) did not change the rate and pattern of firing discharge. However, it reduced by 60 ± 12% ($p < 0.03$; paired t test; $n = 5$) the extent and time course of (100 nM) N/OFQ-induced firing inhibition and membrane hyperpolarization (Fig. 1*A*,*B*). UFP-101 did not counteract inhibition induced by higher (300 nM) N/OFQ concentrations. However, when the agonist was discontinued, the inhibitory action of N/OFQ washed faster in the presence of UFP-101 than in control conditions ($p < 0.05$; $n = 5$ as measured at 4, 8, and 12 min wash; data not shown).

Effect of SNr perfusion with NOP receptor ligands on DA release in the DLS

To test whether nigral NOP receptors modulate the nigrostriatal dopaminergic transmission, NOP receptor ligands were perfused (90 min) through a microdialysis probe implanted in the SNr, and DA was recovered via another probe implanted in the ipsilateral DLS. Basal extracellular DA levels in the DLS were 1.65 ± 0.35 nM ($n = 16$) and were inhibited by N/OFQ ($F_{(3,23)} = 6.79$; $p = 0.0019$) (Fig. 2*A*), perfused intranigrally at 10 ($p < 0.05$) and 100 ($p < 0.01$) μM (maximum reduction to ~84 and ~82% of basal levels, respectively). On the contrary, intranigral perfusion with UFP-101 increased striatal DA release ($F_{(2,13)} = 11.8$; $p = 0.0012$) (Fig. 2*B*) at 10 μM ($p < 0.01$). At this concentration, UFP-101 also prevented the inhibition brought about by 10 μM N/OFQ. Indeed, N/OFQ effect in the presence of UFP-101 (AUC, 7431 ± 369; $n = 5$) was not different from control (AUC, 7190 ± 169; $n = 6$; $p = 0.54$; data not shown).

Effect of SNr injections of NOP receptor ligands on motor behavior

In view of the ability of N/OFQ and UFP-101 to oppositely modulate striatal DA release, the effect of intranigral injections of NOP receptor ligands on the rotarod performance were assessed (Figs. 3, 4). N/OFQ depressed motor performance at 10 ($F_{(4,28)} = 107.5$; $p < 0.0001$) (Fig. 3*A*) and 60 ($F_{(4,19)} = 11.16$; $p < 0.0001$) min, postinjection time. At 10 min postinjection time, 0.1 nmol of N/OFQ produced a significant (~40%) inhibition, whereas 10 nmol of N/OFQ abolished motor activity, with rats showing marked impairment of motor coordination and flaccid muscle tone. UFP-101 alone (0.1–30 nmol) biphasically regulated motor performance ($F_{(4,28)} = 73.75$; $p < 0.0001$) (Fig. 3*B*). Significant increases were observed at 1 and 10 nmol UFP-101 (~47 and ~76%, respectively; $p < 0.05$), whereas marked impairment was observed at 30 nmol (~97%; $p < 0.01$). At 30 nmol, however, UFP-101 induced complex changes in motor behavior, such as

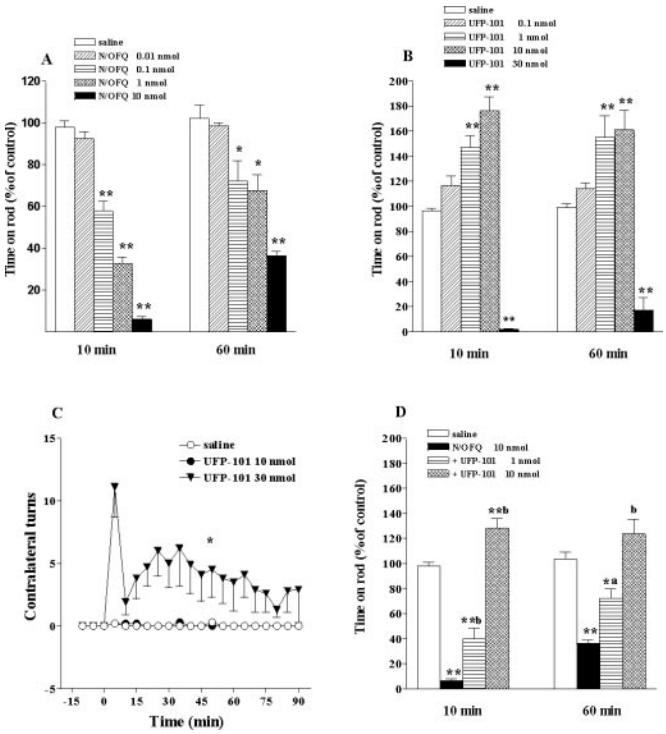

Figure 3. Effect of N/OFQ and UFP-101 on motor activity. Effect of N/OFQ (0.01–10 nmol) (*A*) and UFP-101 (0.1–30 nmol) (*B*) or the combination of both (*D*) in the SNr on the rotarod performance in rats. Each experiment consisted of three different sessions: a control session, followed by two other sessions performed 10 and 60 min after saline or NOP receptor ligand injections (see Materials and Methods). Data are expressed as percentages ± SEM of motor activity in the control session and are means of 6–12 determinations. In *C*, contralateral turning induced by UFP-101 is shown. Total number of turns in 90 min were 1.36 ± 0.15 ($n = 11$), 1.37 ± 0.18 ($n = 8$), and 69.8 ± 23.9 ($n = 16$) for saline and the 10 and 30 nmol UFP-101 groups, respectively. *$p < 0.05$; **$p < 0.01$, significantly different from saline. [a]$p < 0.05$; [b]$p < 0.01$, significantly different from N/OFQ.

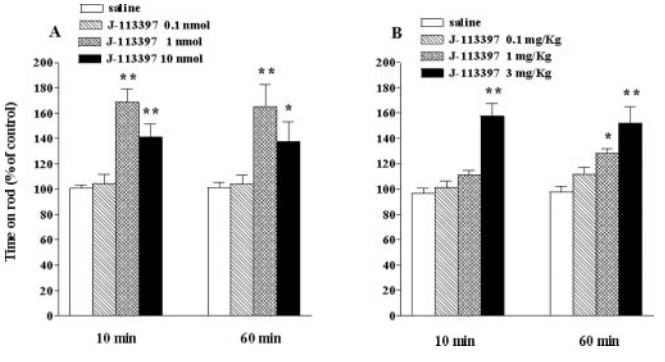

Figure 4. Effect of J-113397 on motor activity. Effect of J-113397 injected (0.1–10 nmol) in the SNr (*A*) or systemically administered (0.1–3 mg/kg, i.p.) (*B*) on the rotarod performance in rats. Each experiment consisted of three different sessions: a control session, followed by two other sessions performed 10 and 60 min after saline or UFP-101 injections (see Materials and Methods). Data are expressed as percentages ± SEM of motor activity in the control session and are means of 6–10 determinations. *$p < 0.05$; **$p < 0.01$, significantly different from saline.

spontaneous contralateral turning ($p < 0.05$) (Fig. 3*C*) and increase in tonic muscle activity (see Figs. 6, 7), which may have hampered correct execution of the task and caused dramatic motor incoordination (Rozas et al., 1997). UFP-101 motor effects were still evident 60 min after injection ($F_{(4,28)} = 32.28$; $p < 0.0001$). To investigate the selectivity of N/OFQ action, intranigral coinjections of N/OFQ and UFP-101 were performed. As

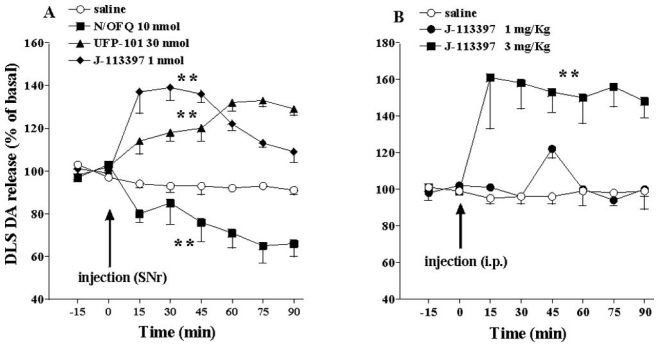

Figure 5. Effect of N/OFQ, UFP-101, and J-113397 on striatal DA release. Effects of N/OFQ (10 nmol), UFP-101 (30 nmol), and J-113397 (1 nmol) injection in the SNr (*A*) or J-113397 systemic administration (1–3 mg/kg, i.p.) (*B*) on spontaneous DA release in the DLS. *A*, Basal DA (in nanomolar) and AUC (in arbitrary units) values were: 1.52 ± 0.17, 7001 ± 112.8 (saline; $n = 10$), 1.48 ± 0.16, 5460 ± 374.4 (10 nmol of N/OFQ; $n = 5$), 2.13 ± 0.40, 9363 ± 252 (30 nmol of UFP-101; $n = 5$), 1.53 ± 0.12, and 9480 ± 173.5 (1 nmol of J-113397; $n = 5$). *B*, Basal DA (in nanomolar) and AUC (in arbitrary units) values were as follows: 1.48 ± 0.21, 7245 ± 195.6 (saline; $n = 5$), 1.25 ± 0.10, 7663 ± 219.9 (1 mg/kg J-113397; $n = 4$), 1.52 ± 0.32, and 11594 ± 956 (3 mg/kg J-113397; $n = 4$). Data are expressed as percentages ± SEM of basal pretreatment levels (calculated as the mean of the 2 samples before the treatment). **$p < 0.01$, significantly different from saline.

shown in Figure 3*D*, 1 nmol of UFP-101 attenuated the inhibitory effect of 10 nmol of N/OFQ, whereas 10 nmol of UFP-101 prevented it.

To confirm that NOP receptor blockade in the SNr resulted in facilitation of motor activity, the nonpeptide NOP receptor antagonist J-113397 was tested (Fig. 4). J-113397 injected into the SNr facilitated motor performance both at 10 ($F_{(3,18)} = 24.43$; $p < 0.0001$) and 60 ($F_{(3,17)} = 16.43$; $p < 0.0001$) min, postinjection time (Fig. 4*A*). The effect was significant for the 1 and 10 nmol doses ($p < 0.01$). Likewise, J-113397 systemically (intraperitoneally) administered elevated motor performance at 10 ($F_{(3,23)} = 20.22$; $p < 0.0001$) and 60 ($F_{(3,23)} = 11.21$; $p < 0.0001$) min, postinjection time (Fig. 4*B*). A significant effect was detected at 3 mg/kg ($p < 0.01$), although a delayed increase was produced by the 1 mg/kg dose ($p < 0.05$).

Effect of SNr injection of NOP receptor ligands on DA release in the DLS

To investigate whether changes of motor behavior were associated with changes of striatal DA release, NOP receptor ligands were injected into the SNr, and DA release was monitored in the ipsilateral DLS. Ten nanomoles of N/OFQ depressed DA release (maximum inhibition of ~35%), whereas 30 nmol of UFP-101 and 1 nmol of J-113397 facilitated it (maximum increase of ~32 and ~39%; $p < 0.01$) (Fig. 5*A*). Systemic J-113397 administration (Fig. 5*B*) also elevated striatal DA release ($F_{(2,9)} = 17.19$; $p = 0.0008$) (Fig. 5*B*) but only at 3 mg/kg (~61%; $p < 0.01$).

Effect of SNr injection of NOP receptor ligands on EMG activity

To quantify the apparent changes of muscle tone caused by intranigral injections of N/OFQ and UFP-101, bilateral EMGs of the rat triceps were recorded (Fig. 6). Ten nanomoles of N/OFQ long-lastingly depressed muscle tone in both triceps of the rat regardless of the side investigated ($F_{(3,40)} = 6.748$, $p = 0.0009$ and $F_{(3,55)} = 226.6$, $p < 0.0001$, for the ipsilateral and contralateral side, respectively). Muscle tone was depressed contralaterally to ~19 and ~26% of baseline, 10 and 60 min after injection, respectively (both, $p < 0.01$), whereas ipsilaterally the effect was less

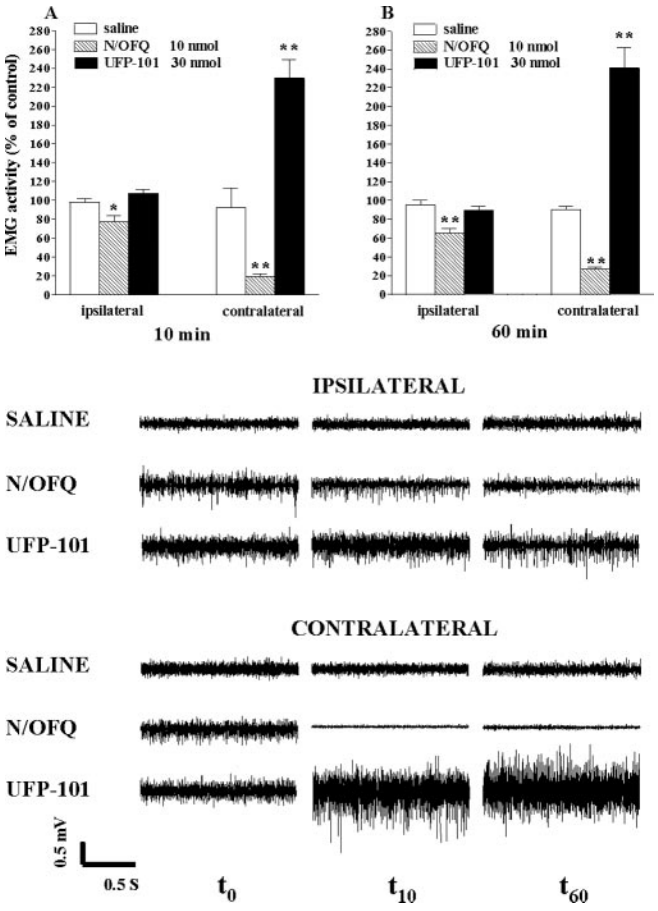

Figure 6. Effects of N/OFQ and UFP-101 on tonic muscle activity. Effects of N/OFQ (10 nmol) and UFP-101 (30 nmol) injection in the substantia nigra pars reticulata of awake rats on EMG activity of triceps muscles contralateral and ipsilateral to the injection side. EMG activity was recorded 10 (*A*) and 60 (*B*) min after drug or saline injection and is expressed as percentage means ± SEM of pretreatment control values (8–15 determinations). *C*, Representative EMG traces of triceps muscle activity after N/OFQ and UFP-101 injections. Triceps contralateral and ipsilateral to the injection side were recorded at time 0 (t_0) or 10 (t_{10}) and 60 (t_{60}) min after drug or saline injections. *$p < 0.05$; **$p < 0.01$, significantly different from saline.

marked (~77 and ~64% of baseline; $p < 0.05$ and $p < 0.01$, respectively). On the contrary, 30 nmol of UFP-101 increased EMG activity in the contralateral ($F_{(3,79)} = 16.94$; $p < 0.0001$) but not ipsilateral triceps. The effect was robust after 10 min and still unchanged 60 min after injection (~230 and ~240%, respectively; both, $p < 0.01$).

Motor behavior in NOP $^{+/+}$ and NOP $^{-/-}$ mice

To additionally strengthen the view that endogenous N/OFQ physiologically controls motor behavior, spontaneous and activity-induced locomotion of NOP $^{+/+}$ and NOP $^{-/-}$ mice was tested. NOP $^{+/+}$ and NOP $^{-/-}$ mice showed comparable spontaneous locomotion (2334 ± 180 and 2300 ± 123 counts in 30 min, respectively) (Table 2) but performed differently on the rotarod (Fig. 7). Rotarod activity of NOP $^{+/+}$ mice on day 1 progressively decayed to zero in the 5–35 rpm speed range (Fig. 7*A*), and total time spent on the rod was 514 ± 38 sec (Fig. 7*C*). Motor ability improved with exercise ($F_{(7,71)} = 20.57$; $p < 0.0001$), as shown by rightward shift of the time × rpm curve and increase in performance in subsequent tests (Fig. 7*C*). Motor performance of NOP $^{-/-}$ mice on day 1 decayed to zero in a wider range of speeds (5–50 rpm) (Fig. 7*B*) and was significantly higher than in NOP $^{+/+}$ mice (700 ± 36 sec; $p < 0.05$) (Fig. 7*C*). A progressive improvement of motor ability was observed in the following tests (Fig. 7*B*), although maximum performance was significantly higher in NOP $^{-/-}$ (1229 ± 79 sec) compared with NOP $^{+/+}$ mice (896 ± 51 sec; $p < 0.05$) (Fig. 7*C*).

Discussion

N/OFQ application to nigral slices reduced the firing of SN DAergic neurons, whereas N/OFQ injection in the SNr *in vivo* reduced striatal DA release, rotarod performance, and muscle tone. Blockade of N/OFQ signaling *in vivo* (either pharmacologically or genetically) produced effects opposite than those of N/OFQ, overall suggesting that exogenous and endogenous N/OFQ inhibit the nigrostriatal DAergic pathway and motor behavior.

SN NOP receptors inhibit the nigrostriatal DAergic pathway

Previous studies have shown that intracerebroventricular N/OFQ reduced spontaneous (Murphy et al., 1996; Murphy and Maidment, 1999; Koizumi et al., 2004) and pharmacologically stimulated (Di Giannuario et al., 1999; Lutfy et al., 2001) DA release in the nucleus accumbens but not striatum (Di Giannuario and Pieretti 2000), ruling out an involvement of N/OFQ in the modulation of the nigrostriatal axis. The present finding that intranigral N/OFQ inhibited striatal DA release in a UFP-101-sensitive way contradicts this view. In particular, reduction of striatal DA levels after intranigral N/OFQ was comparable with that observed in the nucleus accumbens after intrategmental N/OFQ (Murphy and Maidment, 1999), although it was evident at lower N/OFQ concentrations (10 μM vs 1 mM), possibly because of the use of anesthesia in that study. Inhibition of SN DAergic cells, which express NOP receptors (Maidment et al., 2002; Norton et al., 2002), may underlie reduction of striatal DA levels by intranigral N/OFQ because, in line with that found in ventral tegmental area slices (Zheng et al., 2002), SN DAergic cells were inhibited by N/OFQ (via UFP-101-sensitive NOP receptors). Firing inhibition resulted from membrane hyperpolarization, very likely caused by K^+ channel opening (Zheng et al., 2002). Different from that reported for the mesoaccumbal pathway in the mouse (Koizumi et al., 2004), however, an N/OFQergic inhibitory tone on the nigrostriatal transmission was disclosed because NOP receptor antagonists given into the SNr facilitated striatal DA release. This facilitation was likely a result of NOP receptor blockade because it was consistently observed with chemically unrelated molecules, delivered to the SNr via different routes, at doses reported to selectively affect N/OFQ responses (for UFP-101, see Calò et al., 2002; Koizumi et al., 2004; Kuzmin et al., 2004; for J-113397 see, Ozaki et al., 2000; Ueda et al., 2000; McLeod et al., 2001; Lutfy et al., 2002). Tonic N/OFQergic control of the nigrostriatal DAergic transmission was disclosed only *in vivo* because UFP-101 did not affect firing activity in nigral slices. This is no surprise, because tonic inhibition of SN DAergic neurons mediated by GABA and DA (via $GABA_A$ and D_2 receptors, respectively) was also observed *in vivo* (Chiodo and Bunney, 1984; Paladini and Tepper, 1999) but not *in vitro* (Pinnock, 1984; Lacey et al., 1987; Mercuri et al., 1990).

SN NOP receptors inhibit motor behavior

Previous studies have shown that N/OFQ inhibited spontaneous (Reinscheid et al., 1995; Devine et al., 1996; Rizzi et al., 2001) cocaine-stimulated (Lutfy et al., 2001) and morphine-stimulated (Di Giannuario et al., 1999; Di Giannuario and Pieretti, 2000) locomotion, whereas Ro 64–6198 [(1*S*,3a*S*)-8-2,3,3a,4,5,6-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4.5]

decan-4-one] (a nonpeptide NOP receptor agonist) induced hypolocomotion (Kuzmin et al., 2004) and impaired rotarod performance (Jenck et al., 2000). The fact that intranigral N/OFQ inhibited rotarod performance and muscle tone extends these findings, indicating that SNr NOP receptors drive an inhibitory control on motor behavior. These receptors appear to be tonically activated by endogenous N/OFQ, especially under execution of a motor task, because both UFP-101 and J-113397 facilitated the rotarod performance, although only UFP-101 (at high doses) affected spontaneous locomotion. This indicates that exercise-induced activity is more sensitive to blockade of SNr N/OFQ signaling. It is plausible that N/OFQ-sensitive motor pathways come into play during exercise or that, during exercise, endogenous N/OFQ is released in greater amounts than at rest. Studies on NOP$^{+/+}$ and NOP$^{-/-}$ mice confirmed the specific involvement of endogenous N/OFQ in the modulation of physiologically stimulated activity. Indeed, although the two genotypes displayed similar spontaneous activity (Nishi et al., 1997; Murphy et al., 2002; Gavioli et al., 2003; Koizumi et al., 2004), NOP$^{-/-}$ mice outperformed NOP$^{+/+}$ mice on the rotarod. It is noteworthy that this difference was significant at the first rotarod challenge and maintained during training, suggesting that greater spatial learning ability and memory reported for NOP$^{-/-}$ mice (Manabe et al., 1998) were not involved. The mechanisms underlying the greater performance of NOP$^{-/-}$ mice on the rotarod are presently unknown. A previous study demonstrated that striatal DA release increased during rotarod performance (Bergquist et al., 2003), suggesting that the greater performance of NOP$^{-/-}$ is a result of greater DA release in the striatum. The finding that NOP$^{-/-}$ and NOP$^{+/+}$ mice did not display any difference in basal and heroin-stimulated striatal DA levels (Murphy et al., 2002; Koizumi et al., 2004), however, may suggest that other mechanisms are involved. In this respect, both DA-dependent and DA-independent mechanisms may underlie motor effects of NOP receptor ligands. Indeed, N/OFQ inhibited both DAergic and GABAergic mesencephalic neurons (Zheng et al., 2002) and reduced accumbal DA release (Murphy and Maidment, 1999) or increased SNr glutamate release (Marti et al., 2002a), partly via bicuculline-sensitive mechanisms.

The complex motor pattern of response (contralateral rotations, rigidity, and motor incoordination) induced by 30 nmol of UFP-101 may indicate that tonic N/OFQ regulation of spontaneous activity can be unveiled, provided a high degree of NOP receptor blockade, leading to asymmetric motor disinhibition, is reached into one SNr. Similar to UFP-101, unilateral injection of morphine into the SNr also induced contralateral turning, possibly via increased nigrostriatal DAergic transmission (Iwamoto and Way, 1977; Matsumoto et al., 1988; Bontempi and Sharp, 1997; but see Morelli and Di Chiara, 1985) and rigidity, possibly via disinhibition of nigrofugal GABAergic pathways (Turski et al., 1982, 1983). It is therefore possible that high doses of UFP-101, by acting on NOP receptors located on different neuronal subtypes and nigrofugal pathways, affect different parameters of motor behavior. On the other hand, the possibility that high doses of UFP-101 exert aspecific effects (i.e., beyond NOP receptor blockade) cannot be ruled out because UFP-101 has been reported to induce motor effects in NOP$^{-/-}$ mice (Koizumi et al., 2004). The different motor profile of J-113397 compared with UFP-101 may further support this view, although this difference may also be caused by interaction with NOP receptors bearing different pharmacological properties (Mogil and Pasternak, 2001; Marti et al., 2003b; Kuzmin et al., 2004).

Table 2. Spontaneous locomotor activity assay in wild-type (NOP$^{+/+}$) and NOP receptor knock-out (NOP$^{-/-}$) mice

	5 min	10 min	15 min	20 min	25 min	30 min
NOP$^{+/+}$ (sec)	477 ± 36	426 ± 34	397 ± 31	355 ± 32	344 ± 31	335 ± 30
NOP$^{-/-}$ (sec)	474 ± 26	414 ± 22	389 ± 24	356 ± 19	346 ± 21	318 ± 19

Animals ($n = 18$–20 for each genotype) were placed in a Ugo Basile cage, and spontaneous activity was recorded every 5 min for up to 30 min. Data (in seconds) are means ± SEM of at least three experiments.

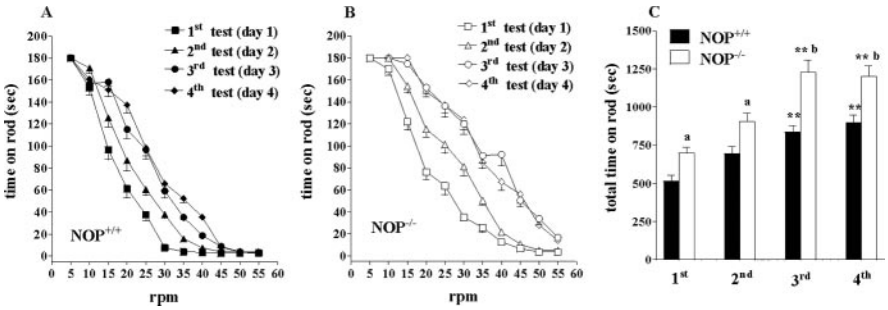

Figure 7. Rotarod performance in wild-type (NOP$^{+/+}$) and NOP receptor knock-out (NOP$^{-/-}$) mice. Motor performance of NOP$^{+/+}$ (*A*) and NOP$^{-/-}$ (*B*) mice in a 5–55 rpm speed range. NOP$^{+/+}$ and NOP$^{-/-}$ mice were tested daily on the rotarod for 4 consecutive days (see Materials and Methods). Data are expressed as number of seconds and are means ± SEM of 10 determinations for each speed. *C*, Total time spent on the rotarod, expressed as the sum of the performances recorded at each speed, as shown in *A* and *B*. $^{**}p < 0.01$, significantly different from the first session. $^{a}p < 0.05$; $^{b}p < 0.01$, significantly different from NOP$^{+/+}$ mice.

Concluding remarks

Pharmacological and genetic evidence demonstrated that endogenous N/OFQ inhibits the nigrostriatal DAergic pathway and activity-stimulated locomotion by activating SNr NOP receptors. These data extend previous studies indicating that endogenous N/OFQ tonically modulates neurosecretion (Marti et al., 2002a; Kawahara et al., 2004), firing activity (Albrecht et al., 2001), and, at least under certain conditions, pain (Calò et al., 2000; Ueda et al., 2000; Zaratin et al., 2004), mood (Redrobe et al., 2002; Gavioli et al., 2003), and food intake (Polidori et al., 2000). NOP receptor antagonists may thus be proven effective in relieving hypokinesia under conditions of enhanced nigral N/OFQergic tone. This may be the case in Parkinson's disease. Indeed, DA denervation induced by intranigral injection of 6-hydroxydopamine is associated with increased levels of nigral N/OFQ mRNA (Norton et al., 2002), suggesting that endogenous N/OFQ may contribute to hypokinesia induced by DA loss.

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