Published in final edited form as:

Neuropharmacology. 2016 June ; 105: 434–442. doi:10.1016/j.neuropharm.2016.02.003.

Beta-arrestin 2 rather than G protein efficacy determines the anxiolytic-versus antidepressant-like effects of nociceptin/orphanin FQ receptor ligands

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Abstract

Background and purpose—Nociceptin/orphanin FQ (N/OFQ) receptor (NOP) agonists produce anxiolytic-like effects in rodents while antagonists promote antidepressant-like effects. The aim of this study was to investigate the effect on anxiety and depression of NOP receptor partial agonists such as the peptides [F/G]N/OFQ(1–13)NH_2 and UFP-113 and the non-peptide AT-090.

Experimental approach—*In vitro* AT-090, UFP-113, and [F/G]N/OFQ(1–13)NH_2 were tested for their ability to promote NOP/G-protein and NOP/β-arrestin 2 interaction, using a bioluminescence resonance energy transfer assay. *In vivo*, they were tested in mice in the elevated plus maze (EPM) and in the forced swim (FST) tests. NOP partial agonists effects were systematically compared to those of full agonists (N/OFQ and Ro 65–6570) and antagonists (UFP-101 and SB-612111).

Key results—*In vitro*, AT-090, UFP-113, and [F/G]N/OFQ(1–13)NH^2 promoted NOP/G protein interaction, with maximal effects lower than those evoked by N/OFQ and Ro 65–6570. AT-090 behaved as a NOP partial agonist also in inducing β-arrestin 2 recruitment, while UFP-113 and [F/G]N/OFQ(1–13)NH^2 were inactive in this assay. *In vivo*, AT-090 induced anxiolytic-like effects

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Authorship contribution statement

LA, CR, ECG, and GC designed research; LA, CR, DM, and IUM performed the experiments; NTZ and RG contributed unpublished reagents/analytic tools; LA, CR, and DM analyzed data; LA, CR, ECG and GC wrote the paper.

Conflicts of interest

All other authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2016.02.003.

in the EPM but was inactive in the FST. Opposite results were obtained with UFP-113 and [F/G]N/OFQ(1–13)NH^2.

Conclusions and implications—NOP ligands producing similar effects on NOP/G protein interaction (partial agonism) but showing different effects on β-arrestin 2 recruitment (partial agonism vs antagonism) elicited different actions on anxiety and mood. These results suggest that the action of a NOP ligand on emotional states is better predicted based on its β-arrestin 2 rather than G-protein efficacy.

Keywords

N/OFQ; Anxiety; Depression; G protein; β-arrestin; NOP receptor partial agonist; BRET; Elevated plus maze; Forced swim test; Mouse

1. Introduction

The heptadecapeptide nociceptin/orphanin FQ (N/OFQ) was identified as the endogenous ligand of the N/OFQ peptide (NOP) receptor (Meunier et al., 1995; Reinscheid et al., 1995). The NOP receptor is widely expressed in the central nervous system (Mollereau and Mouledous, 2000; Neal et al., 1999). *In vivo* N/OFQ modulates a variety of biological functions (Lambert, 2008) including anxiety and depression (Gavioli and Calo', 2006, 2013; Toll et al., in press). Pivotal preclinical studies have suggested that NOP agonists, the peptide N/OFQ and the non-peptide Ro64–6198, produce anxiolytic-like effects in rodents (Jenck et al., 1997, 2000). These anxiolytic actions of the non peptide NOP agonist have been confirmed by a large number of laboratories as revised by Shoblock (2007). Moreover, other chemically unrelated non peptide NOP agonists have also been reported to induce anxiolytic properties (Varty et al., 2008; Hirao et al., 2008; Hayashi et al., 2009; Lu et al., 2011). However, the role of N/OFQ in regulating anxiety is complex and some laboratories reported an anxiogenic-like effect of N/OFQ (Kamei et al., 2004; Fernandez et al., 2004; Green et al., 2007). On the other hand, the blockade of N/OFQ signaling (both pharmacological with chemically distinct NOP selective antagonists and genetic with NOP knockout (NOP(−/−)) animals) promotes antidepressant-like effects (Gavioli et al., 2003, 2004; Goeldner et al., 2010; Redrobe et al., 2002; Rizzi et al., 2011; Vitale et al., 2009; Medeiros et al., 2015). Given that compounds with opposing NOP efficacies (NOP full agonists *vs* NOP antagonists) modulate anxiety and depression respectively, this study aimed to investigate the action of NOP partial agonists in models of anxiety and depression. We investigated both small-molecule and peptidic NOP partial agonists such as the non-peptide AT-090 (Ferrari et al., 2015), and the peptides UFP-113 (Arduin et al., 2007; Camarda et al., 2009) and [$Phe^1\psi(CH_2–NH)Gly^2$]N/OFQ(1–13)-NH_2 ([F/G]N/OFQ(1–13)NH_2; Guerrini et al., 1998). In order to compare their *in vitro* potency and efficacy, these NOP partial agonists were evaluated in parallel experiments for their ability to promote NOP/G-protein and NOP/β-arrestin 2 interaction using a bioluminescence resonance energy transfer (BRET) assay recently set up in our laboratories (Malfacini et al., 2015). *In vivo*, AT-090, UFP-113, and [F/G]N/OFQ(1–13)NH_2 were tested in mice in the elevated plus maze (EPM) and in forced swim test (FST) for assessing their putative anxiolytic- and antidepressive-like effects respectively. The *in vitro* and *in vivo* actions of these compounds were compared with those

of the standard NOP full agonists N/OFQ and Ro 65–6570 (Röover et al., 2000; Wichmann et al., 1999) and standard antagonists UFP-101 (Calo et al., 2002) and SB-612111 (Zaratin et al., 2004).

2. Methods

2.1. Drugs and reagents

NOP peptide ligands and Ro 65–6570 were synthesized in the Guerrini laboratory. AT-090 (1-(1-(cis-4-isopropylcyclohexyl) piperidin-4-yl)indoline-2,3-dione) was synthesized in the Zaveri laboratory (Astraea Therapeutics). All tissues culture media and supplements were from Invitrogen (Paisley, UK), while reagents were purchased from Sigma Chemical Co. (Poole, UK) or E. Merck (Darmstadt, Germany). For the *in vitro* studies stock solutions (1 mM) of peptides were made in ultrapure water, while stock solutions (10 mM) of Ro 65–6570 and AT-090 were prepared in DMSO. Stock solutions were kept at − 20 °C until use. For *in vivo* studies peptides and nortriptyline were dissolved in saline while Ro 65–6570 and SB-612111 (Tocris Bioscience) in 1% DMSO, and diazepam in 0.5% Tween 80 (Sigma Chemical Co). AT-090 was dissolved in 1% DMSO and 0.5% (2-hydroxypropyl)-β-cyclodextrin (Sigma Chemical Co). Peptides were injected intracerebroventricularly (i.c.v.) 5 min prior to the test. Diazepam (Sigma Chemical Co), nortriptyline (Tocris Bioscience), Ro 65–6570, and AT-090 were injected intraperitoneally (i.p.) 30 min prior the test. SB-612111 was given i.p. 30 min before the test when tested alone and 30 min before Ro 65–6570 or vehicle in antagonism experiments.

2.2. In vitro studies

2.2.1. Cell and membrane preparation—Human Embryonic Kidney (HEK293) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin G, and 100 ng/ml streptomycin sulfate in a humidified atmosphere of 5% CO_2 at 37 °C.

Cell lines permanently co-expressing the different pairs of fusion proteins, i.e. NOP-RLuc/$G\beta_1$-RGFP and NOP-RLuc/β-arrestin 2-RGFP, were prepared as described previously (Molinari et al., 2008). For G-protein experiments enriched plasma membrane aliquots from transfected cells were prepared as previously described in details (Malfacini et al., 2015). The protein concentration in membranes was determined using the QPRO – BCA kit (Cyanagen Srl, Bologna, IT) and the spectrophotometer Beckman DU 520 (Brea, CA, USA).

2.2.2. Receptor-transducer interaction—These assays were carried out essentially as previously described by Malfacini et al. (2015). Briefly, in whole cells, luminescence was recorded in 96-well sterile poly-D-lysine-coated white opaque microplates, while in membranes it was recorded in 96-well untreated white opaque microplates (PerkinElmer, Waltham, MA, USA) using the luminometer Victor 2030 (PerkinElmer, Waltham, MA, USA). For the determination of NOP/β-arrestin 2 interaction, cells were plated 24 h before the experiment (100,000 cells/well). For the determination of NOP/G-protein interaction, membranes (3 μg of protein) were added to wells in DPBS. Coelenterazine (Synchem UG & Co. KG) at a final concentration of 5 μM was always injected 10 min prior reading the cell

plate. The receptor/G-protein interaction was measured in cell membranes to exclude the involvement of other cellular processes (i.e. arrestin recruitment, internalization). Different concentrations of ligands in 20 μL of PBS – BSA 0.01% (Sigma Chemical Co. Poole, UK) were added and incubated for an additional 5 min before reading luminescence. All the experiments were performed at room temperature.

2.3. In vivo studies

In vivo studies have been reported according to ARRIVE guidelines (Kilkenny et al., 2010) and comply with the European Communities Council directives (2010/63/E), Brazilian Law (No. 11.714/2008) and Italian regulations (D.Lgs, 26/2014). Protocols were approved by Ethic Committees for Animal Use of Federal University of Rio Grande do Norte (Protocol No. 21/2013) and of the University of Ferrara and the Italian Ministry of Health (Protocol No. 316/2013-B). Male CD-1 (Harlan, Udine, Italy) or Swiss mice (bred at the Federal University of Rio Grande do Norte, Brazil) were used in this study together with NOP(+/+) and NOP(−/−) mice (bred at the Department of Medical Sciences of the University of Ferrara, Italy). Details about the generation of mutant mice have been published previously (Bertorelli et al., 2002; Nishi et al., 1997), moreover NOP(+/+) and NOP(−/−) mice have been backcrossed on the CD-1 strain. All mice were 8–12 weeks old. Mice were housed in 425 × 266 × 155 mm cages, 5 mice/cage, under standard conditions (22 °C, 55% humidity, 12 h light–dark cycle, lights on 7.00 am) with food and water *ad libitum*. A total number of 652 mice have been used for this research.

2.3.1. Surgery—Mice were anesthetized with ketamine and xylazine (100 and 10 mg/kg, i.p., respectively) and placed in a stereotaxic apparatus. An 8 mm stainless steel guide cannula (25 × 0.7 mm) was implanted into the lateral ventricle and was fixed with dental cement. Coordinates toward the bregma were ML −1.1 mm, AP −0.6 mm, DV −1.0 mm. To prevent occlusion, a dummy cannula was inserted into the guide cannula. After surgery, the animals were allowed to recover for 5 days. For the i.c.v. injection, awake mice were gently restrained and the drug solution (2 μl/mouse)was injected (2 μL/min) with a cannula protruding 1 mm from the guide cannula. After completion of testing, mice were i.c.v. injected with trypan blue dye (2 μl). Ten min after, brains were removed to verify the placement of the guide cannula. Only the data from those animals with dispersion of the dye throughout the ventricles (>95% of the animals) were used.

2.3.2. Elevated plus maze—The EPM assay was carried out as described by Pellow et al. (1985). The apparatus (Hamilton–Kinder, Poway, CA, USA) consists of two open arms, which are facing two opposite enclosed arms connected by a central platform elevated 50 cm from the floor. A red light was focused on the central platform (~100 lux). Animals were placed at the center of the maze, with the head facing an open arm. The number of entries and the time spent in both closed and open arms were recorded during a 5 min period by an experienced observer. An entry was scored when the animal placed all four limbs into any given arm. Data are shown as percentage of time spent in the open arms and percentage of entries in the open arms calculated as follows: % of time spent in open arms = time spent in the open arms/(time spent in open arms + time spent in closed arms)*100; % of entries in the

open arms = number of entries in open arms/(number of entries in open arms + number of entries in closed arms)*100. Mice were randomly assigned to experimental groups.

2.3.3. Forced swim test—The FST was performed as described by Porsolt et al. (1977). Mice were placed individually in polyethylene cylinders, containing water at 25 ± 1 °C, for two swim sessions: an initial 15 min training session on day 1, and 24 h later (day 2), by a 5 min test session. Results were relative to the 5 min test session. The immobility time (i.e. the time spent floating in the water without struggling) was recorded by an experienced observer. Mice were randomly assigned to experimental groups.

2.4. Data analysis and terminology

Receptor-transducer interactions were calculated as BRET ratio, i.e. the ratio of CPS recorded through the 510(10) and 460(25) emission filters (PerkinElmer, Waltham, MA, USA). All data are expressed as agonist-induced change of BRET ratio by subtracting the baseline (i.e. the value obtained in the absence of ligand) from all the other values. Agonist potencies are given as pEC_{50} ($CL_{95\%}$). Maximal agonist effects (E_{max}) were expressed as fraction of the N/OFQ E_{max}, which was determined in every assay plate. Concentration-response curves to agonists were analyzed with the four-parameter logistic nonlinear regression model. Curves fitting was performed using PRISM 5.0 (Graph Pad Software Inc., San Diego, USA). Data are expressed as mean ± sem of n experiments and were analyzed statistically using one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons.

In vivo data are expressed as mean ± s.e.m. of n animals. Data were analyzed using one-way or two-way ANOVA followed by Dunnett's or Bonferroni's post hoc test, or using Kruskal–Wallis H test followed by the Dunn's post hoc test, as specified in figure legends. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. In vitro studies

3.1.1. BRET assay—N/OFQ promoted both NOP/G-protein and NOP/β-arrestin 2 interaction in a concentration-dependent manner and with similar potency (pEC_{50} of 8.91 ($CL_{95\%}$ 8.54–8.28) and 8.13 ($CL_{95\%}$ 7.78–8.47), respectively) (Fig. 1A). Ro 65–6570 mimicked the stimulatory effect of N/OFQ producing maximal effect similar to those induced by the natural peptide but being 10-fold less potent in inducing NOP/G-protein interaction (pEC_{50} of 7.90 ($CL_{95\%}$ 7.42–8.39)) and 100-fold less potent in the β-arrestin 2 assay (pEC_{50} ~ 6.3) (Fig. 1B). Of note, in the β-arrestin 2 assay, the concentration–response curve to Ro 65–6570 was not completed precluding an experimentally verified assessment of the asymptotic plateau, thus the pEC_{50} and E_{max} values obtained for this compound were extrapolated from the fitting routine. On the contrary, UFP-101 was inactive in evoking both NOP/G-protein and NOP/β-arrestin 2 interaction (Fig. 1C). In the G protein assay, AT-090 exhibited maximal effects that were significantly lower than that of N/OFQ ($\alpha = 0.64 \pm 0.04$) with potency value of 7.59 ($CL_{95\%}$ 7.23–7.95). A similar activity was displayed by this ligand in the β-arrestin 2 assay ($\alpha = 0.56 \pm 0.02$; pEC_{50} of 7.33 ($CL_{95\%}$ 7.08–8.57))

(Fig. 1D). The peptides [F/G]N/OFQ(1–13)NH_2 and UFP-113 induced NOP/G protein interaction with maximal effects lower than those of N/OFQ (α = 0.68 ± 0.02 and 0.52 ± 0.02, respectively) and with pEC_{50} of 8.45 ($CL_{95\%}$ 8.19–8.71) and of 9.79 ($CL_{95\%}$ 9.52–10.51), respectively. Both compounds produced a negligible stimulation of NOP/β-arrestin 2 interaction (Fig. 1E and F).

3.2. In vivo studies

3.2.1. Elevated plus maze—In the EPM test, control mice spent ~40 s (~25% of time) in and made ~ 5 entries (~30% of total entries) in the open arms. Diazepam (1 mg/kg, i.p.) significantly increased both the time spent in and the number of entries in the open arms. Ro 65–6570 (0.01–0.1 mg/kg, i.p.) increased in a dose-dependent manner the time spent in and the number of entries in the open arms, mimicking the action of diazepam (Fig. 2A and B). Of note, at the doses used in this study, Ro 65–6570 did not produce any motor impairment in the rotarod test (Fig. S1). The selective NOP receptor antagonist SB-612111 (10 mg/kg, i.p.), at a dose able to completely block the anxiolytic-like effects of Ro 65–6570, did not modify *per se* the mouse behavior in the EPM test (Fig. 2C and D). No differences were recorded between the behavior of NOP(+/+) and NOP(−/−) mice in the EPM test. Importantly, the anxiolytic-like effect of Ro 65–6570 was evident in NOP(+/+) but not NOP(−/−) mice (Fig. 2E and F). AT-090 (0.001–0.3 mg/kg, i.p.) significantly increased in a dose-dependent manner the time spent by animals in the open arms and their number of entries (Fig. 3A and B). At the higher dose tested AT-090 did not evoke any motor impairment in the rotarod test (Fig. S1). To evaluate the involvement of the NOP receptor in the anxiolytic-like action of AT-090, the compound was tested in NOP(+/+) and NOP(−/−) mice. AT-090 0.01 mg/kg produced anxiolytic-like effects in NOP(+/+) mice but was completely inactive in NOP(−/−) mice (Fig. 3C and D).

Saline icv injected mice spent ~56 s (~20% of time) in and made ~ 7 entries (~30% of total entries) in the open arms of the EPM. N/OFQ (0.1 nmol, i.c.v.) increased the time spent in but did not change the number of entries in the open arms (Fig. S2). On the other hand, UFP-113 (0.01–0.1 nmol, i.c.v.) and [F/G]N/OFQ(1–13) NH_2 (0.1–1 nmol, i.c.v.) did not modify animal behavior in the EPM (Fig. S2). At the doses used in this study, N/OFQ, UFP-113, and [F/G] N/OFQ(1–13)NH_2 did not produce any changes in the locomotor behavior assessed in the open field (Fig. S3).

3.2.2. Forced swim test—Control mice subjected to the FST spent ~150 s immobile. This immobility time was strongly decreased by the classical antidepressant nortriptyline (20 mg/kg, i.p.). A similar effect was produced by the selective NOP receptor antagonist SB-612111 (10 mg/kg, i.p., Fig. 4A). As expected, the selective NOP receptor agonist Ro 65–6570 (0.1 mg/kg, i.p.) (Fig. 4A) had no effect on the immobility time in the FST. The NOP partial agonist AT-090 (0.001–0.1 mg/kg, i.p., Fig. 4B) did not modify the immobility time of mice subjected to the FST. In a separate series of experiments AT-090 was injected i.c.v. (0.001 and 0.01 nmol, 10 min pretreatment) and did not change the behavior of mice in the FST (Fig. S4).

Similar to SB-612111, the selective NOP receptor peptide antagonist UFP-101 (10 nmol, i.c.v.) reduced the immobility time of mice (Fig. 4C). The action of UFP-101 was mimicked, in a dose-dependent manner, by peptidic NOP partial agonists UFP-113 (0.01–0.1 nmol, i.c.v., Fig. 4D) and [F/G]N/OFQ(1–13)NH_2 (0.1–1 nmol, i.c.v., Fig. 4E).

The *in vitro* and *in vivo* results obtained in this study are schematically summarized in Table 1.

4. Discussion & conclusions

Several studies have demonstrated that the N/OFQ-NOP system shows an unique pattern of actions on emotional states with its activation producing anxiolysis (Shoblock, 2007; Witkin et al., 2014) and its blockade producing antidepressant-like effects (Gavioli and Calo', 2013). Partial agonists are ligands able to bind and activate a given receptor but with reduced efficacy compared to full agonists. Theoretically, partial agonists can display agonist-like actions when a given system is silent while they display antagonist-like actions when the system is activated. Therefore, this study investigated the effects of different NOP receptor partial agonists for their ability to induce NOP/G protein and NOP/β-arrestin 2 interaction, and for their ability to modify the behavior of mice in the EPM and the FST.

GPCRs may signal not only by interacting with G-proteins but also with other effectors, including arrestins (Lefkowitz, 2013). This applies also to the NOP receptor (Chang et al., 2015; Mittal et al., 2013; Zhang et al., 2012). Recently, we established and validated using a large panel of NOP ligands a BRET assay to investigate the ability of ligands to induce NOP/G protein and NOP/β-arrestin 2 interaction (Malfacini et al., 2015). We used this assay to compare the actions of NOP partial agonists UFP-113, [F/G]N/OFQ(1–13)NH_2, and AT-090 as well as that of standard NOP ligands. NOP full agonists N/OFQ and Ro 65–6570, and partial agonists UFP-113, [F/G]N/OFQ(1–13)NH_2, and AT-090 stimulated, in a concentration-dependent manner, NOP/G-protein interaction. However, UFP-113, [F/G]N/OFQ(1–13)NH_2, and AT-090 showed lower maximal effects compared to N/OFQ and Ro 65–6570. In other words these compounds behaved as partial agonists at NOP/G protein. The following rank order of potency was calculated: UFP-113 > N/OFQ > [F/G]N/OFQ(1–13)NH_2 > Ro 65–6570 > AT-090, which is in line with previous studies (Malfacini et al., 2015; Ferrari et al., 2015). As expected, NOP antagonist UFP-101 did not induce NOP/G protein interaction and similar findings were previously obtained with SB-612111 (Malfacini et al., 2015). However both compounds antagonized N/OFQ effects showing a competitive type of interaction and the expected rank order of antagonist potency, i.e. SB-612111 > UFP-101 (Camarda et al., 2009; Malfacini et al., 2015). In the β-arrestin 2 assay, only N/OFQ, Ro 65–6570, and AT-090 stimulated NOP/β-arrestin 2 interaction. Similar to NOP/G protein experiments, AT-090 also behaved as partial agonist in stimulating NOP/β-arrestin 2 interaction. UFP-113, [F/G]N/OFQ(1–13)NH_2, and UFP-101 were inactive in this assay. These compounds as well as SB-612111 were able to antagonize N/OFQ stimulated NOP/β-arrestin 2 interaction (Malfacini et al., 2015); moreover UFP-101 and SB-61211 displayed similar pA_2 values in the two assays. UFP-113 and [F/G]N/OFQ(1–13)NH_2 showed, in the β-arrestin assay, pA_2 values close to their pEC_{50} values obtained in the G protein assay (Malfacini et al., 2015). To summarize, AT-090, UFP-113

and [F/G]N/OFQ(1–13)NH_2 displayed the same pharmacological activity in the NOP/G protein assay, i.e. partial agonism and similar efficacy, but showed clearly different activities in the NOP/β-arrestin 2 assay with AT-090 still behaving as a partial agonist while UFP-113 and [F/G]N/OFQ(1–13)NH_2 as antagonists.

In the EPM and FST, the results obtained in the present research using the standard NOP ligands are largely in agreement with previous studies. Both N/OFQ and Ro 65–6570 produced anxiolytic-like effects in the EPM test, confirming the pivotal studies by Jenck et al. (1997, 2000) and the large evidence later collected in different laboratories (Shoblock, 2007; Witkin et al., 2014). The anxiolytic-like effect of Ro 65–6570 was blocked by the selective NOP antagonist SB-612111 and no longer present in NOP(−/−) mice. These results demonstrate that the anxiolytic-like action of Ro 65–6570 is solely due to the activation of the NOP receptor. Of note, SB-612111 at a dose able to block the anxiolytic-like effect of Ro 65–6570, was totally inactive in the EPM test. This, together with the lack of phenotype of NOP(−/−) mice in the EPM, suggests that N/OFQergic signaling does not tonically control anxiety-like behavior at least under these experimental conditions.

Similar to supraspinal N/OFQ (Redrobe et al., 2002), Ro 65–6570, at the dose active in the EPM test, did not change the mouse behavior in the FST. On the contrary, UFP-101 and SB-612111 produced robust antidepressant-like effects in the same assay. This finding is in agreement with studies showing that the selective blockade of the NOP receptor using chemically different NOP antagonists provide antidepressant-like effects both in mice and rats reviewed in (Gavioli and Calo', 2013). Genetic studies corroborated this evidence; in fact, NOP(−/−) mice (Gavioli et al., 2003) and rats (Rizzi et al., 2011) display a robust antidepressant phenotype in despair tests. Thus, these data support the hypothesis that endogenous N/OFQ may be released under highly stressful situations and may contribute to depressive states. Importantly, very recently it has been demonstrated that a novel NOP selective antagonist named LY2940094 elicits robust antidepressant effects in animals as well as in patients (Post et al. in press).

The results presented here show that the non-peptide AT-090 induced anxiolytic-like effect in the EPM but was inactive in FST. Thus, *in vivo* AT-090 mimicked the action of NOP full agonists. Importantly, AT-090 did not produce anxiolytic-like effects in NOP(−/−) mice. Thus the *in vivo* activity of this compound in the EPM can be ascribed to the selective activation of the NOP receptor. This is in line with the fairly good profile of selectivity (>100 fold) previously reported for this compound (Ferrari et al., 2015). Notably, AT-090 was given i.p. and pharmacokinetic issues should be taken into account when discussing *in vivo* effects. In particular, the inactivity of AT-090 in the FST could be due to its inability to reach, via systemic administration, those brain areas important for the antidepressant-like effects of NOP antagonists. In order to investigate this possibility, AT-090 was injected i.c.v. in mice submitted to FST. However AT-090 was found to be inactive even after i.c.v. administration, suggesting that the lack of effect of AT-090 in the FST is due to pharmacodynamic rather than pharmacokinetic reasons.

The peptides UFP-113 and [F/G]N/OFQ(1–13)NH_2 mimicked the action of NOP antagonists *in vivo*, inducing robust antidepressant-like effects in the FST and being inactive

in EPM. Importantly, UFP-113 and [F/G]N/OFQ(1–13)NH_2 in the FST displayed the same order of potency (UFP-113 > [F/G]N/OFQ(1–13)NH_2) reported *in vitro* in the present and in previous studies (Arduin et al., 2007; Camarda et al., 2009).

The analysis of the present findings suggests that the action of a NOP ligand on emotional states is better predicted based on its β-arrestin 2 rather than G-protein efficacy. In fact comparing their *in vitro* and *in vivo* actions, it appears that NOP ligands able to promote NOP/β-arrestin 2 interaction (N/OFQ, Ro 65–6570, and AT-090) are also able to induce anxiolytic-like effects in EPM. On the other hand, compounds that inhibited the NOP/β-arrestin 2 interaction (UFP-101, SB-612111, UFP-113, and [F/G]N/OFQ(1–13)NH_2) produced antidepressant-like effects in the FST. The scaffolding proteins β-arrestin 1 and 2 have been traditionally associated with GPCR desensitization and internalization (Attramadal et al., 1992; Ferguson et al., 1996). However, recent findings suggest that β-arrestins can also act as G protein-independent effectors of GPCR signaling (Lefkowitz and Shenoy, 2005; Luttrell et al., 1999, 2001). At present there are no information about the involvement of β-arrestins in NOP mediated biological actions with the only exception of locomotor activity (Mittal et al., 2013). However, several studies suggest a role for β-arrestins in the control of emotional states (Avissar et al., 2004; Beaulieu et al., 2005; David et al., 2009; Golan et al., 2010). For example, chronic treatment with fluoxetine failed to induce anxiolytic-like effects in β-arrestin 2(−/−) mice in various assays (David et al., 2009). Beaulieu et al. (2008) demonstrated that lithium reduces the latency to cross in wild type but not β-arrestin 2(−/−) mice subjected to the light/dark test. Moreover, β-arrestin 2 knockout mice displayed an anxiety-like phenotype in the open field and in the novelty suppressed feeding tests (David et al., 2009). These data support the hypothesis that NOP/β-arrestin 2 signaling is important for eliciting anxiolytic-like effects and explain the inactivity of UFP-113 and [F/G]N/OFQ(1–13)NH_2 in the EPM test, in spite of their efficacy in promoting NOP/G protein interaction.

In assays of anti-depressant activity, it has been demonstrated that fluoxetine and lithium did not evoke antidepressant effects in β-arrestin 2(−/−) mice in the tail suspension test (Beaulieu et al., 2008; David et al., 2009) and that β-arrestin 2 signaling mediates lithium action on behavior through Akt signaling and consequent inhibition of glycogen synthase kinase 3 β (Beaulieu et al., 2008), which is considered a putative mechanism of action of mood stabilizers (for review see Beaulieu et al., 2009). Another main signaling pathway implicated in the pathophysiology of mood disorders is that of MAP kinases (Tanis and Duman, 2007). It is known that β-arrestin 2 forms complexes with individual members of a particular MAP kinase cassette, thus keeping the activated MAP kinase in the cytoplasm (DeFea et al., 2000; Luttrell et al., 2001). Different antidepressant drugs reduce β-arrestin 2 protein levels in the cytosol via ubiquitinylation and degradation (Golan et al., 2010) and promote MAP kinase translocation to the nucleus. These findings support the involvement of β-arrestin 2 in mood disorders and the putative role of its blockade in the action of antidepressants. Thus, it can be hypothesized that NOP antagonists induce antidepressant effects by blocking the recruitment of β-arrestin 2.

However we emphasize that these are speculative hypotheses that need to be experimentally validated in future studies, by testing strongly biased NOP agonists both toward G-protein

and β-arrestin in relevant models of anxiety and depression, and by investigating in the same assays the phenotype of β-arrestin 2(−/−) mice in response to NOP selective ligands.

In conclusion, this study investigated and compared the actions of a series of selective NOP ligands encompassing full and partial agonists and pure antagonists *in vitro* for their ability to stimulate receptor interaction with G protein and β-arrestin and *in vivo* in mice for their ability to evoke anxiolytic- and antidepressant-like effects. Partial agonists showing the same efficacy in promoting NOP/G protein interaction produced different effects *in vivo*; these effects seem to be related to their ability to stimulate or block the NOP/β-arrestin 2 interaction. Thus the present findings suggest that the action of a NOP ligand on emotional states is better predicted from its β-arrestin 2 rather than G-protein efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

GC and RG are among the inventors of the patent WO2006087340 that includes UFP-113 and founders of the University of Ferrara spin off company UFPeptides s.r.l., the assignee of such patent. NTZ is a founder and employee of Astraea Therapeutics.

This study was funded by CNPq, Brazil (Science without Borders grant 401289/2014-1 to ECG), CAPES Foundation, Brazil (PhD fellowship- Process no 4785-14-2 to LA), and the University of Ferrara, Italy (FAR grant to GC). Funding from NIH grant R43NS070664 (NTZ) is also gratefully acknowledged. CR holds a fellowship from the Italian Society for Pharmacology (SIF).

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Abbreviations

N/OFQ nociceptin/orphanin FQ

NOP	N/OFQ peptide receptor
GPCR	G protein-coupled receptor
HEK293	Human Embryonic Kidney
EPM	elevated plus maze
FST	forced swim test
BRET	bioluminescence resonance energy transfer.

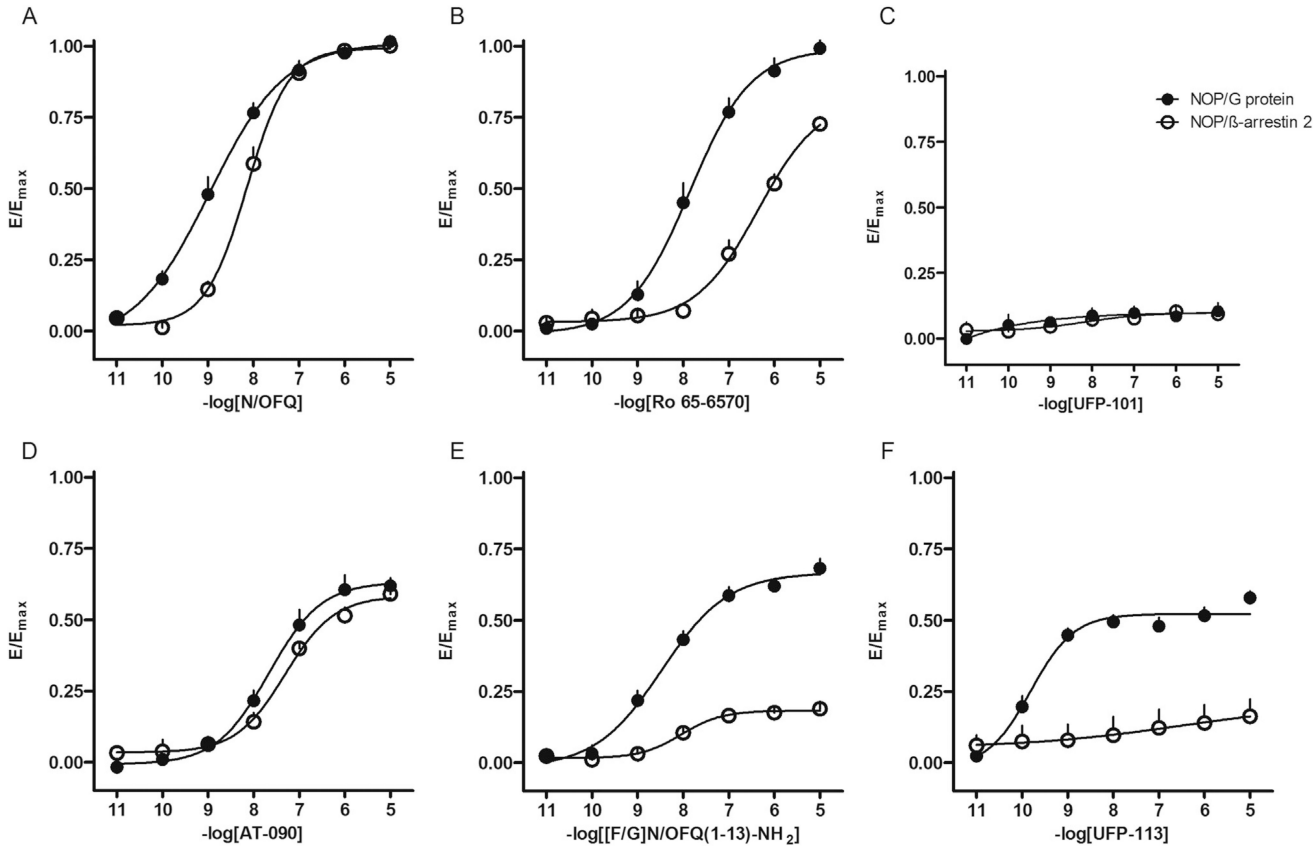

Fig. 1.

BRET assay. Effects of N/OFQ (A), Ro 65–6570 (B), UFP-101 (C), AT-090 (D), [F/G]N/OFQ(1–13)NH_2 (E), and UFP-113 (F) on NOP/G-protein and NOP/β-arrestin 2 interaction. Effects are expressed as E/E_{max}, where E is the effect evoked by the compound and E_{max} is the maximal effect evoked by N/OFQ. E_{max} corresponds to a stimulation of 0.33 ± 0.02 BRET ratio units over the baseline for the NOP/G-protein assay and of 0.11 ± 0.01 BRET ratio units over the baseline for the NOP/β–arrestin 2 assay. Data are shown as mean ± sem of 4 separate experiments performed in duplicate.

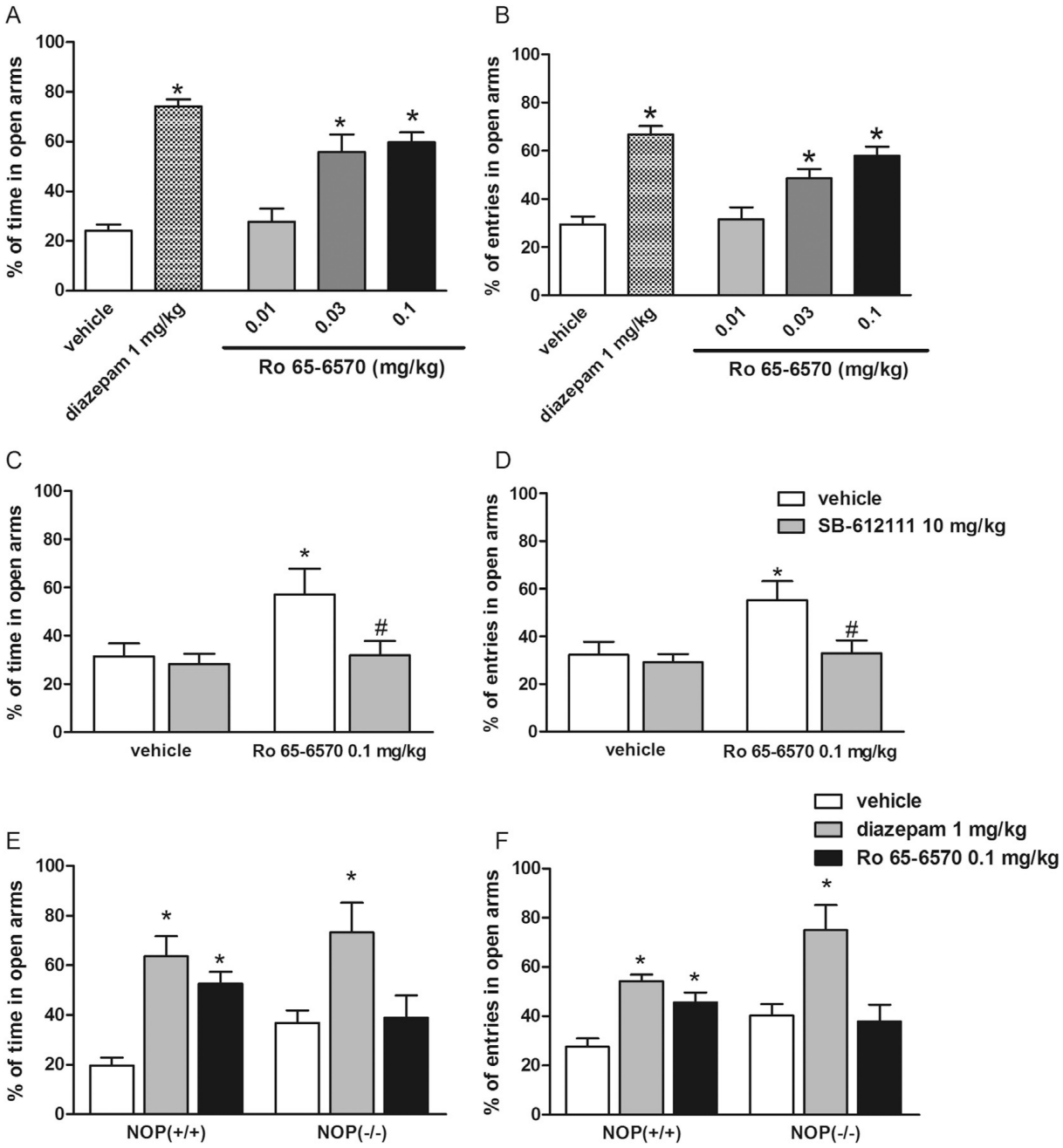

Fig. 2.

Effects of standard compounds in the EPM test. Panels A and B: effect of diazepam (1 mg/kg, i.p., 30 min prior the test) and Ro 65–6570 (0.01–0.1 mg/kg, i.p., 30 min prior the test) in CD-1 mice subjected to the EPM test. Data are mean ± s.e.m. of 12–16 mice per group. Kruskal–Wallis H test followed by the Dunn's post hoc test revealed an effect of treatment both for the percentage of time spent in the open arms ($H_{(4)} = 42.41$, A) and the percentage of entries in the open arms ($H_{(4)} = 37.42$, B). *$p < 0.05$ vs vehicle. Panels C and D: effect of SB-612111 (10 mg/kg, i.p., 30 min pre-treatment) *per se* and against Ro 65–6570 (0.1 mg/kg, i.p., 30 min before starting the test) in CD-1 mice subjected to the EPM

test. Data are mean ± s.e.m. of 10–12 mice per group. Two-way ANOVA followed by the Bonferroni's post hoc test revealed an effect of Ro 65–6570 and of the interaction Ro 65–6570 × SB-612111 both for the percentage of time spent in the open arms ($F_{(1,38)}$ = 5.18 for Ro 65–6570 and $F_{(1,38)}$ = 4.85 for the interaction, C) and for the percentage of entries in the open arms ($F_{(1,38)}$ = 5.39 for Ro 65–6570 and $F_{(1,38)}$ = 4.82 for the interaction, D). *p < 0.05 vs vehicle, #p < 0.05 vs Ro 65–6570. Panels E and F: effect of diazepam (1 mg/kg, i.p., 30 min before starting the test) and Ro 65–6570 (0.1 mg/kg, i.p., 30 min before starting the test) in NOP(+/+) and NOP(−/−) mice subjected to the EPM test. Data are mean ± s.e.m. of 8–10 mice per group. Two-way ANOVA followed by the Bonferroni's post hoc test revealed an effect of treatment and of the interaction treatment × genotype for the percentage of time spent in the open arms ($F_{(2,47)}$ = 17.68 for treatment and $F_{(2,47)}$ = 3.86 for the interaction, D) and an effect of treatment for the percentage of entries in the open arms ($F_{(2,47)}$ = 11.79, E). *p < 0.05 vs vehicle, #p < 0.05 vs NOP(+/+).

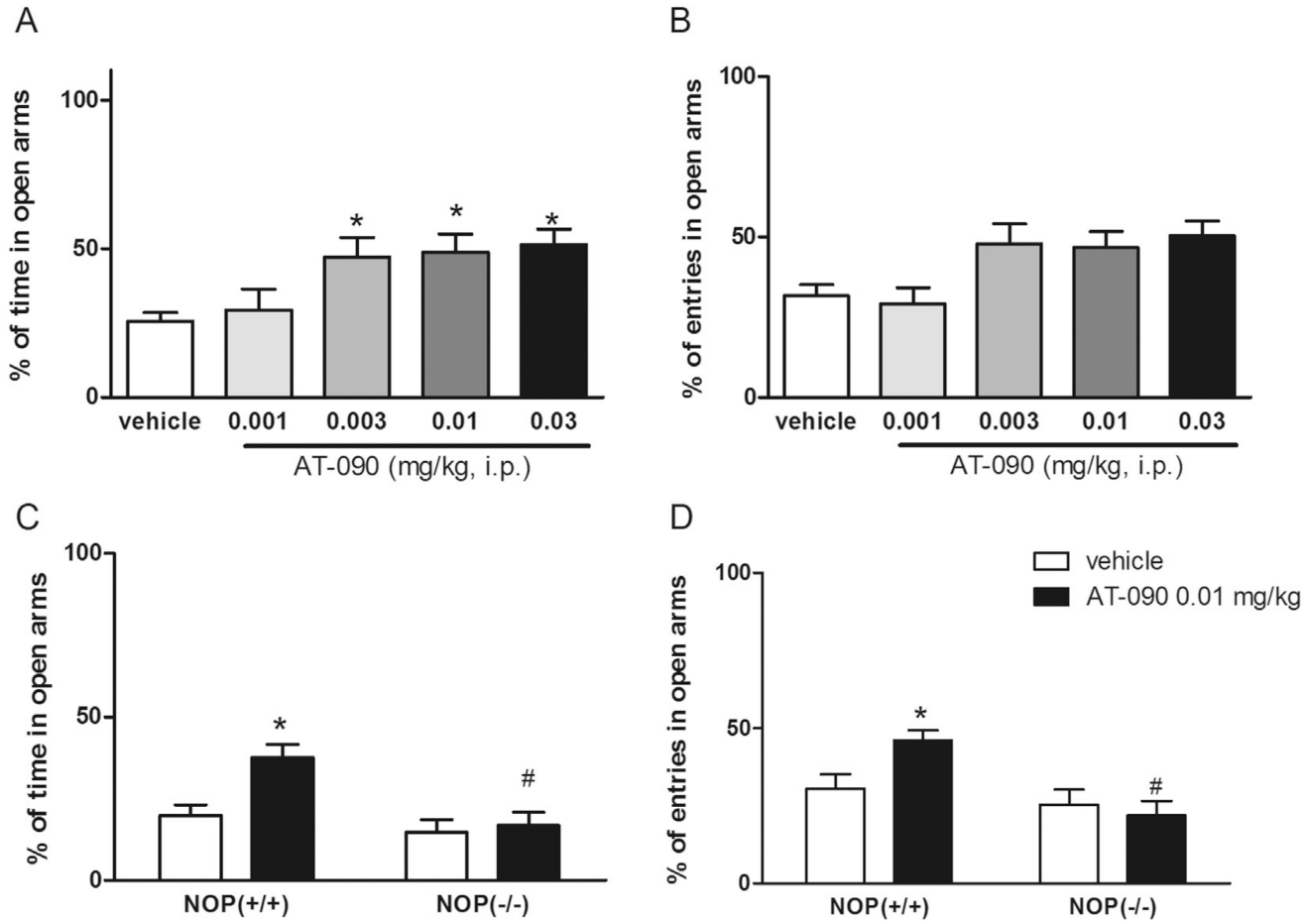

Fig. 3.

Effects of AT-090 in the EPM test. Panels A and B: dose–response curve to AT-090 (0.001–0.03 mg/kg, i.p., 30 min prior the test) in CD-1 mice subjected to the EPM test. Data are mean ± s.e.m. of 15–17 mice per group Kruskal–Wallis H test followed by the Dunn's post hoc test revealed an effect of treatment both for the percentage of time spent in the open arms ($H_{(4)} = 19.39$, A) and the percentage of entries in the open arms ($H_{(4)} = 14.44$, B). *$p < 0.05$ vs vehicle. Panels C and D: effect of AT-090 (0.01 mg/kg, i.p., 30 min prior the test) in NOP(+/+) and NOP(−/−) mice subjected to the EPM test. Data are mean ± s.e.m. of 10 mice per group. Two-way ANOVA followed by the Bonferroni's post hoc test revealed an effect of treatment and of the interaction treatment × genotype for the percentage of time spent in the open arms ($F_{(1,38)} = 10.31$ for treatment and $F_{(1,38)} = 6.03$ for the interaction, C) and for the percentage of entries. *$p < 0.05$ vs vehicle, #$p < 0.05$ vs NOP(+/+).

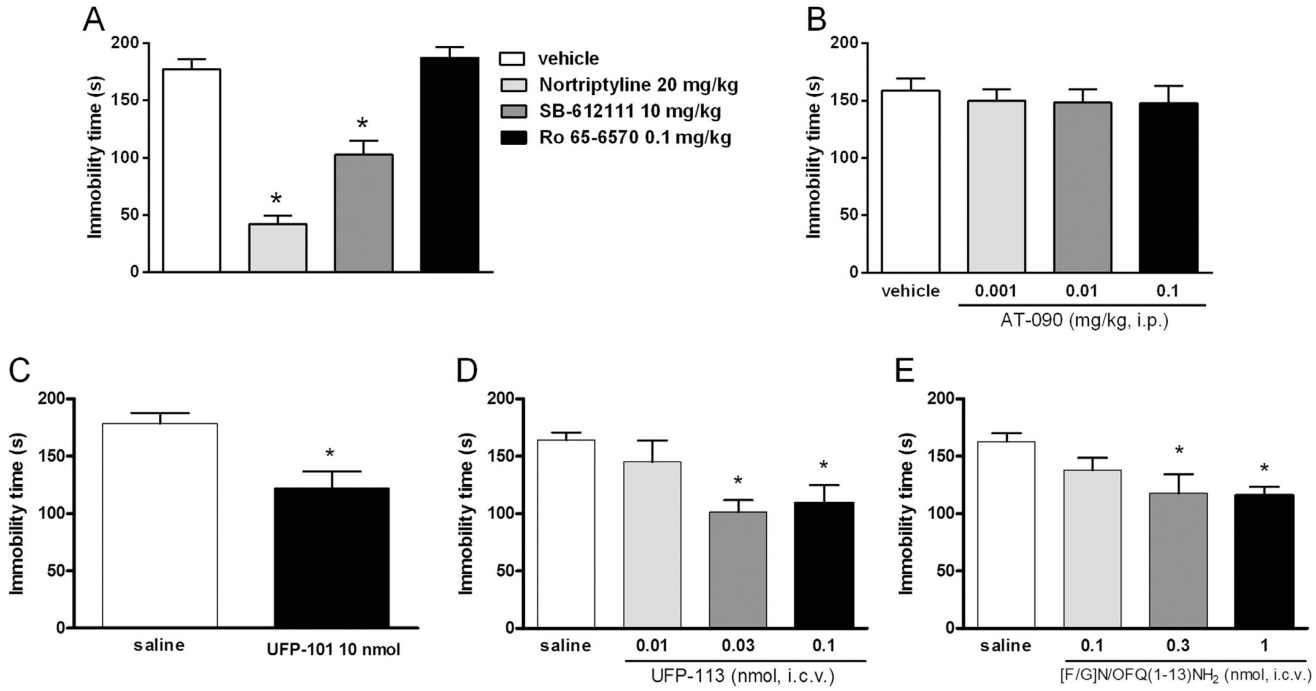

Fig. 4.

Effects of standard compounds and partial agonists of NOP receptor (AT-090, UFP-113, and [F/G]N/OFQ(1–13)NH_2) in the FST. Panel A: effects of nortriptyline (20 mg/kg, i.p., 30 min prior the test), SB-612111 (10 mg/kg, i.p., 30 min prior the test), and Ro 65–6570 (0.1 mg/kg, i.p., 30 min prior the test) on the immobility time of CD-1 mice subjected to the FST. Data are mean ± s.e.m. of 11–16 mice per group. One-way ANOVA followed by the Dunnett's post hoc test revealed an effect of treatment ($F_{(3,55)}$ = 45.38). *p < 0.05 vs vehicle. Panel B: dose–response curve to AT-090 (0.01–0.1 mg/kg, i.p., 30 min prior the test) on the immobility time of CD-1 mice subjected to the FST. Data are mean ± s.e.m. of 8–12 mice per group. Panel C: effect of UFP-101 (10 nmol, i.c.v., 5 min prior the test) on the immobility time of Swiss mice subjected to the FST. Data are mean ± s.e.m. of 16–17 mice per group. Unpaired Student t test revealed an effect of treatment ($t_{(31)}$ = 3.30). *p < 0.05 vs saline. Panel D: dose–response curve to UFP-113 (0.01–0.1 nmol, i.c.v., 5 min prior the test) in Swiss mice subjected to the FST. Data are mean ± s.e.m. of 10–13 mice per group. One-way ANOVA followed by the Dunnett's post hoc test revealed an effect of treatment ($F_{(3,47)}$ = 7.90). Panel E: dose–response curve to [F/G]N/OFQ(1–13)NH_2 (0.1–1 nmol, i.c.v., 5 min prior the test) in Swiss mice subjected to the FST. Data are mean ± s.e.m. of 13 mice per group. One-way ANOVA followed by the Dunnett's post hoc test revealed effect of treatment ($F_{(3,48)}$ = 6.50). *p < 0.05 vs saline.

Table 1

In vitro and *in vivo* pharmacological activity of NOP ligands.

	In vitro		***In vivo***	
	G protein	**β-arrestin**	**EPM**	**FST**
N/OFQ	full agonist	full agonist	anxiolytic	inactive
Ro 65–6570	full agonist	full agonist	anxiolytic	inactive
UFP-101	antagonist	antagonist	inactive	antidepressant
SB-612111	antagonist	antagonist	inactive	antidepressant
[F/G]N/OFQ(1–13)NH_2	partial agonist	antagonist	inactive	antidepressant
UFP-113	partial agonist	antagonist	inactive	antidepressant
AT-090	partial agonist	partial agonist	anxiolytic	inactive