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Title page

[(pF)Phe^4,Arg^{14},Lys^{15}]N/OFQ-NH$_2$ (UFP-102), a highly potent and selective agonist of the nociceptin/orphanin FQ receptor

[*]Giacomo Carrà, [*]Anna Rizzi, Remo Guerrini, Timothy A. Barnes, John McDonald, Christopher P. Hebbes, Flora Mela, Velga A. Kenigs, Giuliano Marzola, Daniela Rizzi, Elaine Gavioli, Silvia Zucchini, Domenico Regoli, Michele Morari, Severo Salvadori, David J Rowbotham, David G Lambert, Daniel R Kapusta, and Girolamo Calo'

Department of Experimental and Clinical Medicine, Section of Pharmacology and Neuroscience Center (G.C., A.R., F.M., G.M., D.R., E.G., S.Z., D.R., M.M., G.C.) and Department of Pharmaceutical Sciences and Biotechnology Center (R.G., S.S.), University of Ferrara, 44100 Ferrara, Italy. Department of Cardiovascular Sciences, Pharmacology and Therapeutics Group (T.A.B., J.McD., C.P.H., D.G.L.) and Department of Health Sciences, Division of Anaesthesia, Critical Care and Pain Management (D.J.R.), Leicester Royal Infirmary, Leicester, LE1 5WW, UK. Department of Pharmacology (V.A.K., D.R.K.), Louisiana State University, Health Sciences Center, New Orleans, Louisiana, U.S.

Running title page

Running Title: UFP-102, a novel NOP receptor agonist

Corresponding author:

Girolamo Calo', MD, PhD
Department of Experimental and Clinical Medicine,
Section of Pharmacology
via Fossato di Mortara 19, 44100 Ferrara, Italy
ph: +39-0532-291 221 fax: +39-0532-291 205
e-mail: g.calo@unife.it

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Abbreviations

CHO, Chinese hamster ovary; N/OFQ, nociceptin/orphanin FQ; i.c.v., intracerebroventricular; UFP-102, [(pF)Phe^4,Arg^{14},Lys^{15}]N/OFQ-NH_2; UFP-101, [Nphe^1,Arg^{14},Lys^{15}]N/OFQ-NH_2; J-113397, (±)trans-1-[1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one; ANOVA, analysis of variance.

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Abstract

A novel ligand for the nociceptin/orphanin FQ (N/OFQ) receptor (NOP), [(pF)Phe^4,Arg^{14},Lys^{15}]N/OFQ-NH_2 (UFP-102) has been generated by combining in the N/OFQ-NH_2 sequence two chemical modifications, [Arg^{14},Lys^{15}] and [(pF)Phe^4], that have been previously demonstrated to increase potency. In vitro, UFP-102 bound with high affinity to the human NOP receptor, showed at least 200-fold selectivity over classical opioid receptors and mimicked N/OFQ effects in CHO_{hNOP} cells, isolated tissues from various species, and mouse cortical synaptosomes releasing 5-HT. UFP-102 showed similar maximal effects but higher potency (2 - 48 fold) relative to N/OFQ. The effects of UFP-102 were sensitive to NOP selective antagonists (J-113397, pA_2 7.75 – 8.12 and UFP-101 pA_2 6.91 – 7.33) but not to naloxone, and no longer observed in tissues taken from NOP receptor knockout mice ($NOP^{-/-}$). In vivo, UFP-102 (0.01 – 0.3 nmol, i.c.v.) mimicked the pronociceptive action of N/OFQ (0.1 – 10 nmol, i.c.v.) in the mouse tail withdrawal assay, displaying higher potency and longer lasting effects. The action of UFP-102 was not apparent in $NOP^{-/-}$ mice. Similar results were obtained measuring locomotor activity in mice. In conscious rats, UFP-102 (0.05 nmol, i.c.v.) produced a marked and sustained decrease in heart rate, mean arterial pressure, and urinary sodium excretion and a profound increase in urine flow rate. These effects were comparable to those evoked by N/OFQ at 5 nmol. Collectively, these findings demonstrate that UFP-102 behaves as a highly potent and selective NOP receptor agonist which produces long lasting effects in vivo.

Introduction

Nociceptin /orphanin FQ (N/OFQ) (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995) selectively activates a G-protein coupled receptor named N/OFQ peptide receptor (NOP, Cox *et al.*, 2000). This novel peptide / receptor system is considered "a non opioid branch of the opioid family" of peptides and receptors (Cox *et al.*, 2000); this lineage is based on close structural and transductional similarities but contrasting with the pharmacological and functional differences between the N/OFQ – NOP and the classical opioid systems (Calo *et al.*, 2000b; Mogil *et al.*, 2001). Via NOP receptor activation N/OFQ modulates several biological functions including pain transmission, stress and anxiety, learning and memory, locomotor activity, food intake, and the motivational properties of drugs of abuse. N/OFQ also affects the functions of peripheral systems such as the cardiovascular, gastrointestinal, renal, genitourinary and respiratory (Calo *et al.*, 2000b; Mogil *et al.*, 2001).

Understanding of the roles that the N/OFQ-NOP receptor system plays in physiology and pathology is to a major extent dependent upon the identification of highly potent and selective ligands. Currently available ligands for the NOP receptor and their therapeutic potential have been recently reviewed by Zaveri (2003); these are i) non peptide ligands generally discovered via high throughput screening in industrial laboratories (e.g. the NOP selective antagonists J-113397 (Ozaki *et al.*, 2000) and SB-612111 (Zaratin *et al.*, 2004); ii) small peptides identified by screening of synthetic peptide combinatorial libraries (e.g. the NOP selective partial agonist Ac-RYYRWK-NH_2 (Dooley *et al.*, 1997) or the non-selective NOP ligand peptide III-BTD (Becker *et al.*, 1999)); iii) N/OFQ related peptides identified by classical structure activity relationship studies (see for a review Guerrini *et al.*, 2000) including the full agonists N/OFQ(1-13)-NH_2 and N/OFQ-NH_2 (Calo *et al.*, 1996), the partial agonist [$Phe^1\Psi(CH_2\text{-}NH)Gly^2$]N/OFQ(1-13)-$NH_2$ (Guerrini *et al.*, 1998), and the pure antagonist [$Nphe^1$]N/OFQ(1-13)-NH_2 (Calo *et al.*, 2000a).

In addition to these ligands, two highly potent NOP agonists have been identified by alternative strategies. In the first case, substitution of Phe^4 with $(pF)Phe^4$ was employed to generate $[(pF)Phe^4]$N/OFQ(1-13)-NH_2 (Bigoni *et al.*, 2002; Rizzi *et al.*, 2002b). (pF)Phe is a non coded amino acid in which a hydrogen atom in the para position of the phenyl ring is substituted with the fluorine atom. In the second case, an extra pair of the basic residues, Arg and Lys, were introduced into position 14 and 15 of N/OFQ to generate $[Arg^{14},Lys^{15}]$N/OFQ (Okada *et al.*, 2000). Considering these approaches, we have combined in the N/OFQ-NH_2 sequence the chemical modifications which reduce ($[Phe^1\Psi(CH_2\text{-}NH)Gly^2]$) or eliminate ($[Nphe^1]$) agonist efficacy, with those which increase agonist potency ($[(pF)Phe^4]$ and $[Arg^{14}Lys^{15}]$). Among the novel NOP ligands that were synthetized, we identified the selective antagonist $[Nphe^1,Arg^{14}Lys^{15}]$N/OFQ-$NH_2$ (UFP-101) and the selective agonist $[(pF)Phe^4,Arg^{14},Lys^{15}]$ N/OFQ-NH_2 (UFP-102). In previous studies we have evaluated the pharmacological properties of UFP-101 in various in vitro and in vivo assays (Calo *et al.*, 2002; Gavioli *et al.*, 2003; Marti *et al.*, 2003; McDonald *et al.*, 2003b; Mela *et al.*, 2004).

In the present study we investigated the pharmacological profile of UFP-102 in vitro using cells expressing the human recombinant NOP receptor, various isolated tissues, and cerebral cortex synaptosomes which release 5-HT, and in vivo in the tail withdrawal and locomotor activity assays in mice and in cardiovascular and renal function studies performed in rats.

Methods

In vitro studies

Receptor binding and functional (GTPγ[^{35}S] binding and cAMP) assays on CHO cells expressing the human NOP receptor

CHO_{hNOP} cells were maintained in Dulbecco's MEM:Hams F12 (1:1) supplemented with 5% FCS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and fungizone (2.5 µg/ml). Media for stock (non experimental) cultures were further supplemented with hygromycin B (200 µg/ml) and geneticin (G418) (200 µg/ml). CHO cells expressing the human recombinant KOP (kappa), DOP (delta) and MOP (mu) opioid receptors were cultured in Hams F12 with 10% foetal calf serum penicillin (100 IU/ml), streptomycin (100 µg/ml) and fungizone (2.5 µg/ml). Stock cultures additionally contained geneticin (200 µg/ml) for maintainence of the receptor plasmid. Cell cultures were kept at 37°C in 5% CO_2 / humidified air and used for experimentation when confluent (3–4 days). For GTPγ[^{35}S] and [*leucyl*-^{3}H]N/OFQ competition binding studies membrane fragments obtained through homogenization of cell suspensions followed by centrifugation (20,000g, for 10 min at 4°C) in buffer consisting either Tris (50 mM), with or without $MgSO_4$ (5 mM) (competition binding for NOP or classical opioid respectively) or Tris (50 mM) EGTA (0.2 mM) (GTPγ[^{35}S] binding) were used. Membrane fragments were further homogenised and centrifuged for a total of three cycles. Whole cells, in Krebs HEPES buffer were used for studies of the inhibition of forskolin stimulated cAMP formation (Okawa *et al.,* 1999).

Competition binding assay

10 µg of CHO_{hNOP} membrane protein was incubated in 0.5 ml of homogenisation buffer containing 0.5% BSA, 10 µM of a cocktail of peptidase inhibitors (captopril, amastatin, bestatin and phosphoramidon), ~ 0.2 nM [^{3}H]N/OFQ and increasing concentrations (10^{-12} –

10^{-5} M) of the non-radiolabelled peptides under study. Non specific binding was defined in the presence of 1 μM N/OFQ. For opioid receptor selectivity studies of UFP-102 the ability of increasing concentrations to displace the binding of [^{3}H]diprenorphine was measured at classical human recombinant opioid receptors. Between 30-45 μg of $CHO_{hKOP/DOP/MOP}$ homogenated was incubated in homogenization buffer containing 10 μM peptidase inhibitors (as above) and ~0.4 nM[^{3}H]diprenorphine. Non-specific binding was defined in the presence of 10 μM naloxone. All competition binding studies were incubated for 1 hour at room temperature and bound and free radioactivity was separated by vacuum filtration using a Brandel cell harvester using WhatmanGF/B filters soaked in polyethylenimine (0.5%) to reduce non-specific binding.

GTPγ[^{35}S] binding assay

Experimentation was performed essentially as described by Berger *et al.,* (2000). Freshly prepared CHOhNOP membranes (20 μg) were incubated in 0.5 ml volumes of buffer consisting Tris (50 mM), EGTA (0.2 mM), GDP (100 μM), bacitracin (0.15 mM), BSA (1 mg/ml), peptidase inhibitors (amastatin, bestatin, captopril, phosphoramidon; 10 μM), GTPγ[35S] (~150 pM) and peptides in the concentration range of 10-12 – 10-5 M. Non-specific binding was determined in the presence of 10 μM unlabelled GTPγ[^{35}S]. Assays were incubated for 1h at 30°C with gentle shaking and bound and free radiolabel were separated by vacuum filtration onto Whatman GF/B filters. Polyethylenimine was not used. In all cases radioactivity was determined following filter extraction (8 hours, Optiphase Safe, Wallac) using liquid scintillation spectroscopy.

Cyclic AMP assay

Inhibition of forskolin stimulated cAMP was measured in whole CHO_{hNOP} cells. 0.3 ml volumes of cell suspension in Krebs/HEPES buffer were incubated in the presence of IBMX

(1 mM), forskolin (FSK) (1 μM) and varying concentrations of the peptides under study for 15 min at 37°C. Reactions were terminated by the addition of HCl (10 M) then neutralized with 10 M NaOH and 1 mM Tris, pH 7.4. The concentration of cAMP was measured using the protein binding method described by Brown *et al.*, (1971).

Bioassay on isolated tissues

Tissues were taken from male Swiss mice (30-35 g), albino guinea pigs (300-350 g), and Sprague Dawley rats (300-350 g). The mouse vas deferens and colon, the guinea pig ileum, and the rat vas deferens were prepared as previously described (Bigoni *et al.*, 1999; Rizzi *et al.*, 1999). The mouse and rat vas deferens, and the guinea pig ileum were continuously stimulated through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1 msec duration and 0.05 Hz frequency. The electrically evoked contractions (twitches) were measured isotonically with a strain gauge trasducer (Basile 7006) and recorded with the PC based acquisition system Autotrace 2.2 (RCS, Florence, Italy). After an equilibration period of about 60 min, the contractions induced by electrical field stimulation were stable; at this time, cumulative concentration-response curves to N/OFQ or UFP-102 were performed (0.5 log-unit step) in the absence or in presence of antagonists (UFP-101, J-113397, or naloxone, 15 min pre-incubation time). Contraction elicited by agonists was measured isometrically. The concentration-response curves to UFP-102 were performed consecutively, adding to the bath different concentrations of the peptide every 20 minutes followed by washing. Separate series of experiments were performed in tissues (colon and vas deferens) taken from CD1/C57BL6/J-129 mice with NOP receptor gene knock out ($NOP^{-/-}$) or wild type ($NOP^{+/+}$). These mice were genotyped by PCR. Details of the generation and breeding of mutant mice have been published previously (Nishi *et al.*, 1997; Gavioli *et al.*, 2003).

Neurochemical experiments on mouse cerebral cortex synaptosomes

Male Swiss, CD1/C57BL6/J-129 $NOP^{+/+}$ and $NOP^{-/-}$ mice (20-25 g) were used for these studies. On the morning of the experiment, mice were decapitated under light ether anaesthesia and the fronto-parietal cortex was isolated. Synaptosomes were prepared as previously described (Mela *et al*., 2004). Briefly, the cortex was homogenized in ice-cold 0.32 M sucrose buffer at pH 7.4 then centrifuged for 10 min at 1,000 g_{max} (4°C). The supernatant was then centrifuged for 20 min at 12,000 g_{max} (4°C) with the synaptosomal pellet being resuspended in oxygenated (95 % O_2, 5 % CO_2) Krebs solution (mM: NaCl 118.5, KCl 4.7, $CaCl_2$ 1.2, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10) containing ascorbic acid (0.05 mM) and disodium EDTA (0.03 mM). Synaptosomes were pre-loaded with [^{3}H]-5-HT by incubation (25 min) in medium containing 50 nM [^{3}H]-5-HT (specific activity of 27.8 Ci/mmol; NEN DuPont, Boston, MA, U.S.A). One milliliter aliquots of the suspension (protein concentration of about 0.35 mg protein/ml) were slowly injected into nylon syringe filters (outer diameter 13 mm, 0.45 µm pore size; internal volume approximately 100 µl, Phenomenex, MA, USA) connected to a peristaltic pump. Filters were maintained at 36.5 °C in a thermostatic bath and superfused at a flow rate of 0.4 ml/min with a pre-oxygenated Krebs solution. Sample collection (every 3 min) was initiated after a 20 min period of filter washout. K^+ stimulation (1 min pulse) was applied at the 38th min. Under these experimental conditions, previous studies demonstrated that perfusion with tetrodotoxin or omission of Ca^{2+} from the superfusion medium reduced to about 50% or virtually abolished, respectively the 10 mM K^+-evoked [^{3}H]-5-HT overflow (Mela *et al.*, 2004). N/OFQ and UFP-102 were added to the superfusion medium 9 min before the K^+ pulse and maintained until the end of the experiment. At the end of the experiment, radioactivity retained in the superfusate samples and filters (dissolved with 1 ml of 1 M NaOH followed by 1 ml of 1 M HCl) was determined by liquid scintillation spectrophotometry using a Beckman LS 1800 β-spectrometer and Ultima Gold XR scintillation fluid (Packard Instruments B.V., Groningen, The Netherlands).

In vivo studies

Animals.

Male Swiss albino and CD1/C57BL6/J-129 $NOP^{+/+}$ and $NOP^{-/-}$ mice weighing 20-25 g were used. Mice were handled according to guidelines published in the European Communities Council directives (86/609/EEC). They were housed in 425 x 266 x 155 mm cages (Tecniplast, MN, Italy), 8 animals / cage, under standard conditions (22°C, 55% humidity, 12-h light-dark cycle, light on at 7.00 AM) with food (MIL, standard diet, Morini, RE, Italy) and water *ad libitum* for at least 2 days before experiments began. Each mouse was used once. Mice were intracerebroventricularly (i.c.v.) injected (injection volume 2 µl) under light ether anaesthesia (i.e. just sufficient for losing the righting reflex) using the 'free hand' technique described by Laursen and Belknap (1986). In brief, a 27 gauge needle attached via a polyethylene tube to a 10 µl Hamilton syringe was used for the injection at approximate 45° angle, at 2 mm lateral to the bregma midline. Each mouse only received one i.c.v. injection.

Male Sprague-Dawley rats (Harlan Inc. Indianapolis, IN) weighing 275-300 g were used for the cardiovascular and renal function studies. Rats were housed in groups of five or fewer under a 12-h light/dark cycle (light on at 7.00 AM) until the day of the experiments. All rats were fed with normal sodium diets (sodium content, 163 mEq/kg) and were allowed tap water ad libitum. All the procedures were conducted in accordance with the Louisiana State University Health Sciences Center and the National Institutes of health guidelines for the care and use of animals.

Tail-withdrawal assay

All experiments were started at 10.00 a.m. and performed according to the procedure previously described in details (Calo *et al.*, 1998). Briefly, the mice were placed in a holder and the distal half of the tail was immersed in water at 48 °C. Withdrawal latency time was

measured by an experienced observer blind to drug treatment. A cut off time of 20 s was chosen to avoid tissue damage. For each series of experiments at least 12 mice were randomly assigned to each treatment. Tail-withdrawal latency was determined immediately before and 5, 15, 30, 60 and 90 min after i.c.v. injection of 2 µl of saline (control) or N/OFQ, or UFP-102.

Locomotor activity assay

Experiments were carried out between 14.00 and 18.00, following the procedure described by Rizzi *et al.* (2001a). Briefly, the mice were routinely tested 3 min after i.c.v. injection. Locomotor activity was assessed using Basile activity cages, which consist of a four-channel resistance detector circuit which converts the bridges "broken" by the animal paws into pulses that are summed by an electronic counter every 5 min. Total number of impulses were recorded every 5 min for 30 min. Mice were not accustomed to the cages before drug treatment and the experiment was performed in a quiet and dimly illuminated room. For each series of experiments at least 12 mice were randomly assigned to each treatment.

Cardiovascular and renal function studies

Five to six days before experimentation, rats were anesthetized with ketamine (40 mg/kg, i.m., ketamine; Vedco Inc., St. Joseph, MO) in combination with xylazine (5 mg/kg, i.m.; Butler, Columbus, OH) and chronically implanted with a stainless steel guide cannula in the lateral cerebroventricle for i.c.v. injection of drug/vehicle. On the day of the study, rats were anesthetized with sodium methohexital (75 mg/kg, i.p., and supplemented with 10 mg/kg, i.v. as needed; King Pharmaceuticals, Bristol, TN) and instrumented with left femoral artery (blood pressure measurement), vein (isotonic saline infusion) and urinary bladder (urine collection) catheters and a recording electrode to measure renal sympathetic nerve

activity, using standard techniques previously described (Kapusta et al., 1997; Kapusta and Kenigs, 1999). Rats were then placed in a rat holder (a chamber with Plexiglas ends connected by stainless steel rods) which permits forward and backward movement of the rat, allows for collection of urine and protects the renal nerve recording preparation. An i.v. infusion of isotonic saline (55 ml/min) was then started and continued throughout the experiment. The experimental protocol commenced after rats regained full consciousness and cardiovascular and renal excretory function stabilized. After collection of baseline control measurements for each parameter, UFP-102 (0.055 nmol; n=6), or isotonic saline vehicle (5 μl; n=8) was injected i.c.v. Immediately after central administration, experimental urine samples (10 min consecutive periods) were collected for 90 min. Data depicted in Figure 7 for N/OFQ is from previously published studies (Kapusta *et al.*, 1999), and is superimposed for comparison to findings with UFP-102.

Drugs

The peptides used in this study were prepared and purified as previously described (Guerrini *et al.*, 1997). J-113397 was prepared as a racemic mixture, according to De Risi *et al.*, (2001). Captopril, amastatin, bestatin, phosphoramidon, naloxone, cAMP, 3-isobutyl-1-methylxanthine (IBMX), bovine serum albumin (BSA), guanosine 5'-O-(3-thiotri-phosphate) (GTPγS), GDP, bacitracin and forskolin (FSK) were from Sigma (Poole, UK). All tissue culture media and supplements were from Invitrogen (Paisley, U.K.). [2,8-^{3}H]-cAMP (28.4 Ci mmol^{-1}), GTPγ^{35}S (1250 Ci mmol^{-1}) and [15,16-^{3}H]diprenorphine (1.85-2.59 Ci mmol^{-1}) were from Perkin Elmer Life Sciences and [*leucyl*-^{3}H]N/OFQ was from Amersham Biosciences. For in vitro experiments, the compounds were solubilized in H_2O and stock solutions (2 mM) were stored at –70 °C until use; for in vivo studies, the substances were solubilized in physiological medium just before performing the experiment.

Data analysis and terminology

All data are expressed as mean ± standard error of the mean (S.E.M.) and the number of separate experiments is reported for each series of data. Data have been analyzed statistically with the Student's *t*-test or one way ANOVA followed by the Dunnett test, as specified in table and figure legends; p values less than 0.05 were considered significant. Concentration of ligands producing 50% inhibition of specific binding (IC_{50}) was corrected for the competing mass of radioligand using the Cheng and Prusoff equation to yield K_i values. Curve fitting was performed using PRISM 3.0 (GraphPad Software In., San Diego, U.S.A.). Agonist potencies were expressed as pEC_{50}, which is the negative logarithm to base 10 of the agonist molar concentration that produces 50% of the maximal possible effect of that agonist. The E_{max} is the maximal effect that an agonist can elicit in a given tissue/preparation. In mouse vas deferens experiments, pA_2 of UFP-101 vs N/OFQ and UFP-102 was evaluated by Schild analysis, while in the other preparations pK_B values were calculated using the Gaddum Schild equation pK_B = -log((CR-1)/[Antagonist]), assuming a slope equal to unity. Spontaneous 5-HT release from synaptosomes was expressed as fractional release (i.e. tritium efflux expressed as percentage of the tritium content in the filter at the onset of the corresponding collection period) while K^+-evoked tritium overflow was calculated by subtracting the estimated spontaneous efflux (obtained by interpolation between the samples preceding and following the stimulation) from the total efflux observed in the stimulated sample.

In vivo data from studies in mice were analyzed as follows: raw data from tail withdrawal experiments were converted to the area under the curve (90 min); the AUC data were used for statistical analysis; locomotor activity data were analyzed statistically using the data expressed as cumulative impulses over the 30 min observation period. Cardiovascular and renal function data from rat studies were analyzed using repeated measures ANOVA.

Results

In vitro studies

Competition binding, GTPγ[^{35}S] binding stimulation and inhibition of cAMP accumulation at human NOP receptors

The ability of UFP-102 to bind to opioid receptors was evaluated using membranes of CHO cells expressing human recombinant NOP and classical opioid receptors (MOP, DOP, and KOP). In CHO_{hNOP} membranes, UFP-102 produced a concentration-dependent inhibition of [^{3}H]-N/OFQ binding with a pK_i value of 10.67, displaying an affinity for the NOP receptor 17-fold higher than that of N/OFQ (pK_i = 9.44). UFP-102 also binds to classical opioid receptors albeit with reduced affinity by 223-fold at KOP (pK_i = 8.32), by 3300-fold at MOP (pK_i = 7.15), and by > 17.000-fold at DOP (pK_i = 6.42) receptors.

In line with previous findings, in CHO_{hNOP} membranes N/OFQ stimulated binding GTPγ[^{35}S]binding, and in whole CHO_{hNOP} cells inhibited forskolin stimulated cAMP accumulation. In both these assays UFP-102 mimicked the effects of the natural ligand behaving as a full agonist; although UFP-102 was more potent than N/OFQ by 26-fold in the GTPγ[^{35}S], and by 2-fold in the cAMP assay (table 1).

Bioassays in isolated tissues

In the electrically stimulated mouse and rat vas deferens and guinea pig ileum UFP-102 concentration dependently inhibited twitches eliciting maximal effects similar to N/OFQ but being 20 - 48 fold more potent (table 1).

The kinetics of the inhibitory effects of N/OFQ and UFP-102 on the mouse vas deferens electrically induced twitch response can be seen in figure 1. The inhibitory effect (≈ 40 % inhibition) induced by 10 nM N/OFQ occurred immediately after adding the peptide to the bath and was rapidly (≈ 1 min) reversible after washing of the tissue. In contrast, an equiefficacious concentration of UFP-102 (i.e. 0.3 nM) induced a slower inhibitory effect

which reached a plateau only after ≈ 10 min; moreover, the recovery of the tissue to the control twitch took greater than 10 min.

The selective NOP receptor antagonist UFP-101 was used to probe the nature of the agonist response to both N/OFQ and UFP-102 in the mouse vas deferens. UFP-101 did not modify *per se* the electrically-induced twitches, but displaced to the right the concentration response curve to N/OFQ and UFP-102 in a concentration dependent manner (figure 2, top panels). Curves obtained in the presence of UFP-101 were parallel to the control, and reached similar maximal effects even in the presence of the highest concentration of antagonist (i.e. 10 μM). The corresponding Schild plots were linear with slopes not significantly different from unity, and yielded pA_2 values of 7.18 and 7.22 against N/OFQ and UFP-102, respectively (figure 2, bottom panels).

Single concentrations of the NOP selective antagonists (1 μM UFP-101 and 0.1 μM J-113397) and the non-selective opioid receptor antagonist naloxone (1 μM) were challenged against the inhibitory effects induced by UFP-102 in the rat and mouse vas deferens and in the guinea pig ileum. In all the tissues, the antagonists did not modify *per se* the control twitches and naloxone did not affect the concentration response curve to UFP-102. In contrast both UFP-101 and J-113397 displaced to the right the concentration response curve to UFP-102 without modifying its maximal effects. pK_B values calculated from these experiments are summarised in table 2 and compared to those obtained testing the same antagonists against N/OFQ.

The effects of N/OFQ, UFP-102 and the DOP selective agonist deltorphin-I were investigated in the electrically stimulated mouse vas deferens taken from wild type ($NOP^{+/+}$) and NOP receptor knockout ($NOP^{-/-}$) mice. In $NOP^{+/+}$ tissues UFP-102 mimicked the inhibitory effects of N/OFQ (E_{max} 91 ± 1%; pEC_{50} 7.62), showing similar maximal effects (86 ± 2%) but higher potencies (pEC_{50} 9.40). In tissues taken from $NOP^{-/-}$ mice, N/OFQ and UFP-102 were inactive up to 1 μM. In the same series of experiments, the DOP receptor selective

agonist deltorphin-I displayed similar high potency and efficacy in tissues from $NOP^{+/+}$ and $NOP^{-/-}$ mice (data not shown).

In the isolated mouse colon, UFP-102 mimicked the contractile effect of N/OFQ showing similar maximal effects but 2 fold higher potency (table 1). The effects of UFP-102 were also investigated in colon tissues taken from $NOP^{+/+}$ and $NOP^{-/-}$ mice. 10 nM UFP-102 produced a contraction of colon tissues of $NOP^{+/+}$ mice amounting to 55 ± 6% of contraction induced by 10 μM of carbachol, while it was found inactive up to 1 μM in tissues taken from $NOP^{-/-}$ mice. Carbachol produced similar contractile effects in tissues from $NOP^{+/+}$ and $NOP^{-/-}$ mice (data not shown).

[^{3}H]-5-HT overflow in mouse cerebral cortex synaptosomes

As previously reported by Mela *et al.* (2004) 1 min pulse of KCl 10 mM evoked a [^{3}H]-5-HT overflow from mouse cerebral cortex synaptosomes that was inhibited in a concentration-dependent manner by N/OFQ (0.1-1000 nM). Analysis of the concentration-response curve yielded a pEC_{50} value of 8.52. Under the same experimental conditions, UFP-102 mimicked the effects of N/OFQ showing similar maximal effects but being 7 fold more potent than the natural ligand (table 1). To determine whether the effects of UFP-102 were dependent on activation of NOP receptors, maximally effective peptide concentrations were tested in synaptosomes obtained from $NOP^{-/-}$ mice. Basal and K^+-evoked [^{3}H]-5-HT overflow from $NOP^{+/+}$ and $NOP^{-/-}$ mice was not different from that measured in Swiss mice according to previously reported data (Mela *et al.*, 2004). In $NOP^{+/+}$ mice, 1 μM UFP-102 inhibited K^+-evoked tritium overflow approximately to the same extent as in Swiss mice (67 ± 4% of control values), while in $NOP^{-/-}$ mice the peptide was ineffective (101 ± 7% of control values).

In vivo studies

Tail withdrawal assay

I.c.v. injection of 0.01 nmol of UFP-102 in mice did not induce any effect on gross behavior. In contrast, mice treated with 0.1 and 0.3 nmol showed a decrease in locomotor activity, ataxia and loss of the righting reflex, in a similar manner to that which occurs after i.c.v. injection of high doses of N/OFQ (i.e. 10 nmol) (Calo *et al.*, 1998; Reinscheid *et al.*, 1995; Rizzi *et al.*, 2001a). However, whilst the N/OFQ effects appeared immediately after i.c.v. injection, those produced by UFP-102 were only evident after 30 min.

Results summarized in figure 3 show that the tail withdrawal latencies of saline injected mice were stable at 4-5 s over the time course of the experiment. UFP-102 at 0.01 nmol was inactive, whilst it induced statistically significant pronociceptive effects at 0.1 and 0.3 nmol which peaked at 30 – 60 min post-injection. The pronociceptive effects induced by UFP-102 were long lasting and were still evident 180 min after the i.c.v. injection (data not shown).

We compared the effects of high doses of N/OFQ (10 nmol) and UFP-102 (0.3 nmol) in $NOP^{+/+}$ and $NOP^{-/-}$ mice. As shown if figure 4 top panel, tail withdrawal latencies of saline injected $NOP^{+/+}$ mice were stable at 5-6 s over the time course of the experiment and were similar to those found in Swiss mice injected with saline. $NOP^{-/-}$ mice displayed basal tail withdrawal latencies similar to wild type mice, however, in these animals injection of saline produced a short lasting increase in tail withdrawal latencies. Both N/OFQ and UFP-102 induced a statistically significant pronociceptive effect in $NOP^{+/+}$ mice (figure 4, top panel), while they were completely inactive in mice lacking the NOP receptor gene (figure 4, bottom panel).

Locomotor activity assay

As shown in figure 5, mice treated with saline displayed a progressive reduction in spontaneous locomotor activity from 277 ± 40 impulses/5 min to 101 ± 22 impulses/5 min

during the 30 min experiment. Also in this assay, UFP-102 mimicked the effect of N/OFQ (Rizzi *et al.*, 2001a), eliciting a dose-dependent (0.01 – 0.3 nmol) reduction of locomotor activity. UFP-102 was inactive at 0.01 nmol, while at 0.1 and 0.3 nmol produced a statistically significant reduction in locomotor spontaneous activity compared to saline-injected mice. 1 nmol N/OFQ produced a rapid and short lasting inhibition of spontaneous locomotor activity (Rizzi *et al.*, 2001a), whilst the action of the equiefficacious dose of UFP-102 (0.1 nmol) was slow in onset yet produced a long lasting inhibitory response (figure 5). Indeed, when spontaneous locomotor activity was measured for 30 min 3h after injection, N/OFQ was completely inactive (saline 915 ± 88; 1 nmol N/OFQ 727 ± 94) while 0.1 nmol UFP-102 virtually abolished motor activity (192 ± 56).

The effects of 10 nmol N/OFQ and 0.3 nmol UFP-102 on locomotor spontaneous activity were investigated in $NOP^{+/+}$ and $NOP^{-/-}$ mice. As shown in figure 6, top panel, N/OFQ and UFP-102 essentially suppressed locomotor activity in $NOP^{+/+}$ mice whilst they were inactive in mice lacking the NOP receptor gene (figure 6, bottom panel).

Cardiovascular and renal functions in conscious Sprague-Dawley rats.

The cardiovascular, renal excretory and renal nerve responses to i.c.v. injection of N/OFQ and UFP-102 are shown in figure 7. As depicted, i.c.v. UFP-102 produced a significant decrease in heart rate, mean arterial pressure, urinary sodium excretion, and renal sympathetic nerve activity, and an increase in urine flow rate. In general, the directional changes in cardiovascular and renal function produced by i.c.v. UFP-102 were the same as those elicited by i.c.v. N/OFQ (N/OFQ data from Kapusta et al., 1999 are shown in figure 7 for comparative purpose). There are, however, several differences in the responses produced by these two compounds. First, based on the dose tested in these studies, UFP-102 (0.055 nmol) appears to be approximately 100-fold more potent than N/OFQ (5.5 nmol). In addition, whilst both N/OFQ and UFP-102 produced a decrease in mean arterial pressure over the first

ten minutes following injection, the hypotensive response produced by central UFP-102, but not N/OFQ, was sustained for the duration of the 90 min study. Similarly, whilst each ligand produced a comparable onset and magnitude of increase in urine flow rate, the diuresis produced by i.c.v. UFP-102 was sustained for a substantially longer period of time (approximately 40-min) than that elicited by central N/OFQ. Finally, unlike N/OFQ which produced a gradual decrease in renal sympathetic nerve activity that was significant by 30 min, i.c.v. UFP-102 did not evoke a renal sympathoinhibitory effect until 60 min following drug administration.

Discussion

In vitro and in vivo obtained from the present study demonstrate that UFP-102 is a highly potent and selective full agonist for the NOP receptor. Indeed this is the most potent NOP ligand identified to date.

UFP-102 binds with high affinity to the recombinant human NOP receptor and produces maximal effects similar to those of N/OFQ in both the GTPγ[^{35}S] and cAMP assays performed in CHO_{hNOP} cells. The full agonist behavior of UFP-102 was confirmed in animal tissue preparations expressing NOP receptors from both peripheral (isolated tissues) and central (synaptosomes) sites of origin. Typically UFP-102 displayed increases in potency of > 10 fold relative to the natural peptide; the only exceptions were the cAMP assay and the mouse colon bioassay where UFP-102 was only 2 fold more potent than N/OFQ and in synaptosomes where the equieffective concentration ratio was equal to 7. It should be noted that the later preparations are characterized by very high stimulus / response coupling efficiencies (large receptor reserve) as demonstrated by the fact that the NOP partial agonist [Phe^{1}Ψ(CH_2-NH)Gly^2]N/OFQ(1-13)-NH_2 (Guerrini *et al.*, 1998) behaves as a full agonist in these preparations (Mela *et al.*, 2004; Okawa *et al.*, 1999; Rizzi *et al.*, 1999) whilst as a partial agonist or as an antagonist in the other preparations (Bigoni *et al.*, 1999; McDonald *et al.*, 2003a). Thus, in systems with large receptor reserve the difference in potency between UFP-102 and N/OFQ will tend to be underestimated.

In tissues, UFP-102 kinetics of action were slower and its effects more resistant to wash-out than those elicited by the natural ligand N/OFQ. Similar kinetics of action have been reported for other NOP ligands such as the peptide ZP120 (Rizzi *et al.*, 2002a) and the non peptide Ro 64-6198 (Rizzi *et al.*, 2001b). The reason(s) for the differences in kinetic behaviour of these NOP agonists compared to N/OFQ are at present unknown.

Both pharmacological and knockout findings in vitro converge indicating that UFP-102 is a highly selective ligand whose effects are exclusively due to NOP receptor activation.

First, UFP-102 binds with relatively low affinity to classical opioid receptors being at least 200-fold selective for NOP; second, in functional assays the actions of UFP-102 were resistant to naloxone but inhibited by the selective NOP antagonists UFP-101 (Calo *et al.*, 2002) and J-113397 (Bigoni *et al.*, 2000; Ozaki *et al.*, 2000) with pA_2/pK_B values similar to those obtained against N/OFQ; third, the effects of UFP-102 in addition to those of N/OFQ, were no longer evident in tissues (colon, vas deferens, cerebral cortex) taken from mice lacking the NOP receptor gene.

The high potency and selectivity of action of UFP-102 were confirmed in *in vivo* studies. UFP-102 mimicked the pronociceptive and locomotor inhibitory effects in mice and the cardiovascular and renal effects in rats produced by supraspinal administration of N/OFQ (Calo *et al.*, 1998; Kapusta *et al.*, 1997; Rizzi *et al.*, 2001a). In all these assays UFP-102 displayed higher potency than the natural peptide (between 10 and 100-fold) and produced slow onset but longer lasting effects. These kinetic features of UFP-102, also revealed by the *in vitro* experiments, may be attributed to a slower but stronger binding to the NOP receptor compared to N/OFQ; at least in part, they may due also to a decrease susceptibility of UFP-102 to degradation by peptidases, a feature that may be conferred to the molecule by the presence, towards the C-terminal, of the cationic residues Arg^{14},Lys^{15} (Rizzi *et al.*, 2002c).

The high selectivity of action of UFP-102 was also confirmed *in vivo* in mice. In fact, knockout studies, which represent the acid test for *in vivo* selectivity of action, clearly demonstrated that the biological effects of UFP-102 are solely due to NOP receptor activation. Indeed, no differences were recorded in $NOP^{-/-}$ mice injected with saline or UFP-102 in either the tail withdrawal or the locomotor activity assay.

In conclusion, an extensive range of experimental data obtained in a variety of *in vitro* preparations and *in vivo* studies in mice and rats studies demonstrate that UFP-102 is a highly potent and selective full agonist at NOP receptors. In comparison to the native ligand N/OFQ,

UFP-102 showed a gain of potency, a slow onset, and a relatively long duration of action, with these responses being observed in both *in vitro* and especially in *in vivo* assays. This unique pharmacological profile makes UFP-102 a very valuable pharmacological tool to elucidate the different biological functions regulated by the N/OFQ – NOP receptor system, particularly *in vivo* where prolonged duration of action and high potency are features that will reduce by several fold the biologically active dose of the drug.

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Footnotes

[*]Both authors equally contributed to the present work.

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Figure legends

Figure 1 Typical tracings showing the effect of equieffective concentrations of N/OFQ and UFP-102 in the electrically stimulated mouse vas deferens. Abscissa: time in min. Ordinate: control twitch.

Figure 2 Electrically stimulated mouse vas deferens. Top panels: concentration response curve to N/OFQ (left) and UFP-102 (right) obtained in the absence (control) and presence of increasing concentrations of UFP-101 (0.1, 1, and 10 μM). Corresponding Schild plots are shown in the bottom panels. Points indicate the means and vertical lines the S.E.M. of at least 4 experiments**.**

Figure 3 Mouse tail withdrawal assay. Dose response curve to UFP-102 (0.01 – 0.3 nmol i.c.v.) injected i.c.v. Points indicate the means and vertical lines the S.E.M. of 5 experiments. * $p < 0.05$ vs saline according to ANOVA followed by the Dunnett's test performed on AUC data (see methods).

Figure 4 Mouse tail withdrawal assay. Effects of i.c.v. injection of N/OFQ (10 nmol), and UFP-102 (0.3 nmol) in $NOP^{+/+}$ (top panel) and $NOP^{-/-}$ mice (bottom panel). Points indicate the means and vertical lines the S.E.M. of 4 experiments. * $p < 0.05$ vs saline according to ANOVA followed by the Dunnett's test performed on AUC data (see methods).

Figure 5 Mouse locomotor activity assay. Top panel: dose response curve to UFP-102 (0.01- 0.3 nmol) injected i.c.v. Points indicate the means and vertical lines the S.E.M. of 5 experiments. Bottom panel: cumulative impulses over the 30 min observation were used for statistical analysis. * $p < 0.05$ vs saline, according to ANOVA followed by the Dunnett's test.

Figure 6 Mouse locomotor activity assay. Effects of N/OFQ (10 nmol) and UFP-102 (0.3 nmol) on spontaneous locomotor activity in $NOP^{+/+}$ (top panels) and in $NOP^{-/-}$ (bottom panels) mice. Points indicate the means and vertical lines the S.E.M. of 4 experiments. Cumulative impulses over the 30 min observation were used for statistical analysis. * $p < 0.05$ vs saline, according to ANOVA followed by the Dunnett's test.

Figure 7 Cardiovascular, renal excretory, and renal nerve responses produced by i.c.v. N/OFQ (5.5 nmol), UFP-102 (0.055 nmol) and vehicle (5 μl) in conscious Sprague-Dawley rats. Values are means ± S.E.M. of at least 8 rats. Urine samples (consecutive 10-min periods) were collected during control (C, 20 min) and immediately after drug/vehicle injection for 70 (N/OFQ) or 90 min (UFP-102 and vehicle). Data for N/OFQ, from a previously published study (Kapusta et al., 1999), are superimposed for comparison to findings with UFP-102. HR, heart rate; MAP, mean arterial pressure; V, urine flow rate; UNaV, urinary sodium excretion; RSNA, renal sympathetic nerve activity. *$p < 0.05$, statistically different from corresponding control value, according to ANOVA followed by the Dunnett's test.

TABLE 1

In vitro effects of N/OFQ and UFP-102 at recombinant and native NOP receptors

	N/OFQ		**UFP-102**		**CR**
	pEC_{50} ($CL_{95\%}$)	E_{max}	pEC_{50} ($CL_{95\%}$)	E_{max}	
GTPγ[^{35}S] /CHO_{hNOP}	8.70 (8.51-8.89)	10.71 ± 0.73	10.12 (10.0-10.24)	12.26 ± 0.39	26
cAMP / CHO_{hNOP}	9.86 (9.43-10.28)	- 103 ± 1%	10.17 (9.93-10.41)	- 104 ± 1%	2
Mouse vas deferens	7.76 (7.65-7.87)	-88 ± 3%	9.44 (9.28-9.60)	-92 ± 3%	48
Guinea pig ileum	7.94 (7.70-8.18)	-69 ± 7%	9.24 (9.09-9.39)	-65 ± 7%	20
Rat vas deferens	7.24 (7.11-7.37)	-83 ± 3%	8.57 (8.40-8.74)	-85 ± 2%	21
Mouse colon	8.92 (8.71-9.13)	54 ± 4%	9.19 (8.84-9.54)	61 ± 7%	2
Mouse cerebral cortex synaptosomes	8.64 (8.35-8.68)	66 ± 3%	9.42 (9.22-9.50)	67 ±4%	7

Data are the mean ± S.E.M. of at least 4 experiments. CR: concentration ratio

Maximal effects were expressed as stimulation factor (binding of GTPγ[^{35}S] in the presence of ligand divided by that under basal conditions) in GTPγ[^{35}S] experiments, as % inhibition of forskolin-stimulated response in cAMP studies, as % inhibition of control twitch in mouse and rat vas deferens and guinea pig ileum experiments, as % of 10 μM carbachol-induced contraction in mouse colon studies, as % of K^+-evoked 5-HT overflow in mouse cerebral cortex synaptosomes studies.

TABLE 2

pA_2 values of naloxone, J-113397 and UFP-101 vs N/OFQ and UFP-102

in the mouse and rat vas deferens and in the guinea pig ileum

	mouse vas deferens		**guinea pig ileum**		**rat vas deferens**	
	N/OFQ	***UFP-102***	***N/OFQ***	***UFP-102***	***N/OFQ***	***UFP-102***
Naloxone	< 6.0	< 6.0	< 6.0	< 6.0	< 6.0	< 6.0
J-113397	7.85 (7.65-8.05)	8.12 (7.91-8.33)	7.75 (7.57-7.93)	7.79 (7.53-8.05)	7.77 (7.57-7.97)	7.87 (7.63-8.11)
UFP-101	7.29 (7.10-7.48)	7.22 (7.06-7.38)	7.18 (6.98-7.38)	6.91 (6.62-7.20)	7.30 (7.05-7.55)	7.33 (7.05-7.61)

Data are the mean ± S.E.M. of at least 5 experiments. The confidence limits 95% are given in brackets

Figure 1

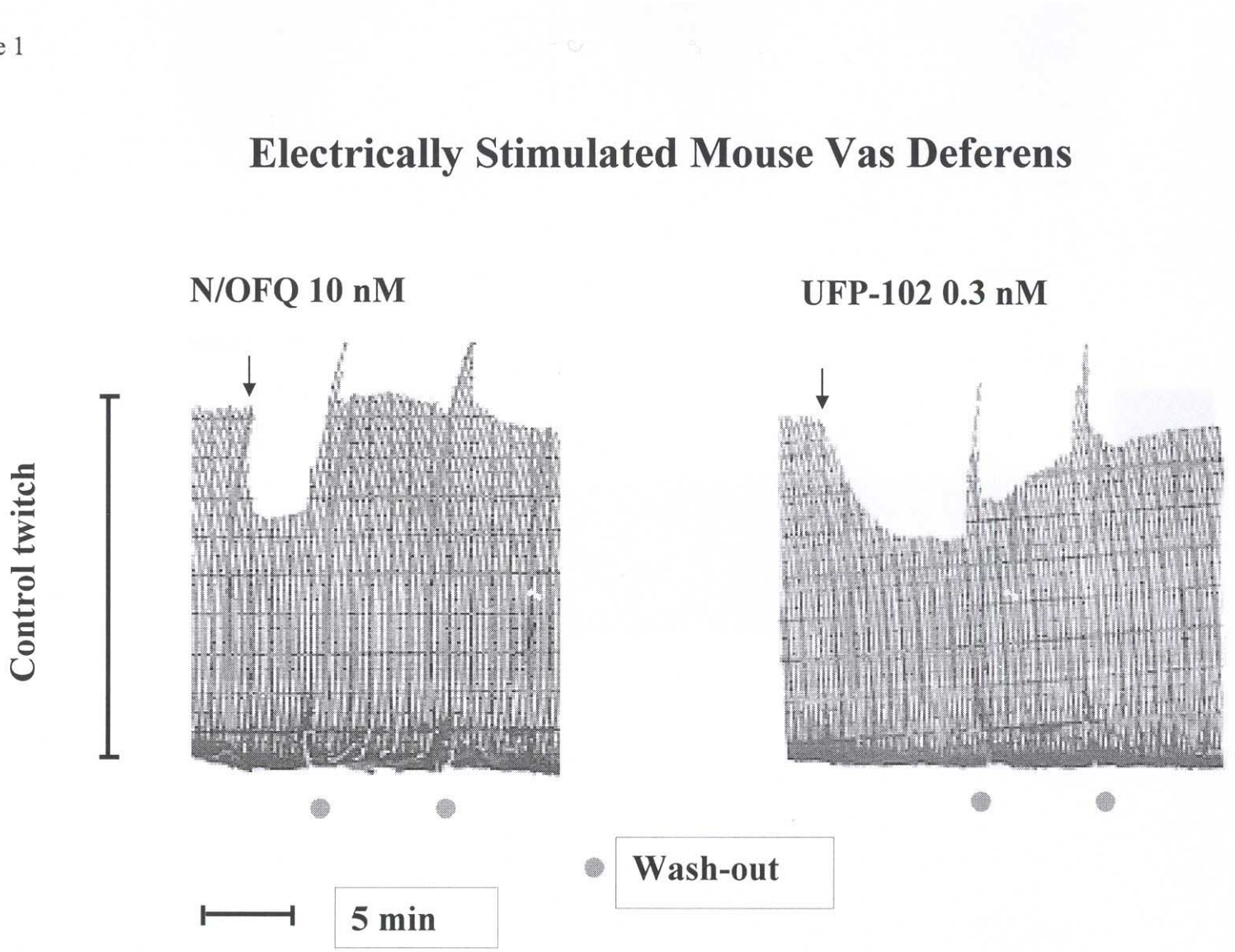

Electrically Stimulated Mouse Vas Deferens
N/OFQ 10 nM
UFP-102 0.3 nM
Control twitch
Wash-out
5 min

Figure 2

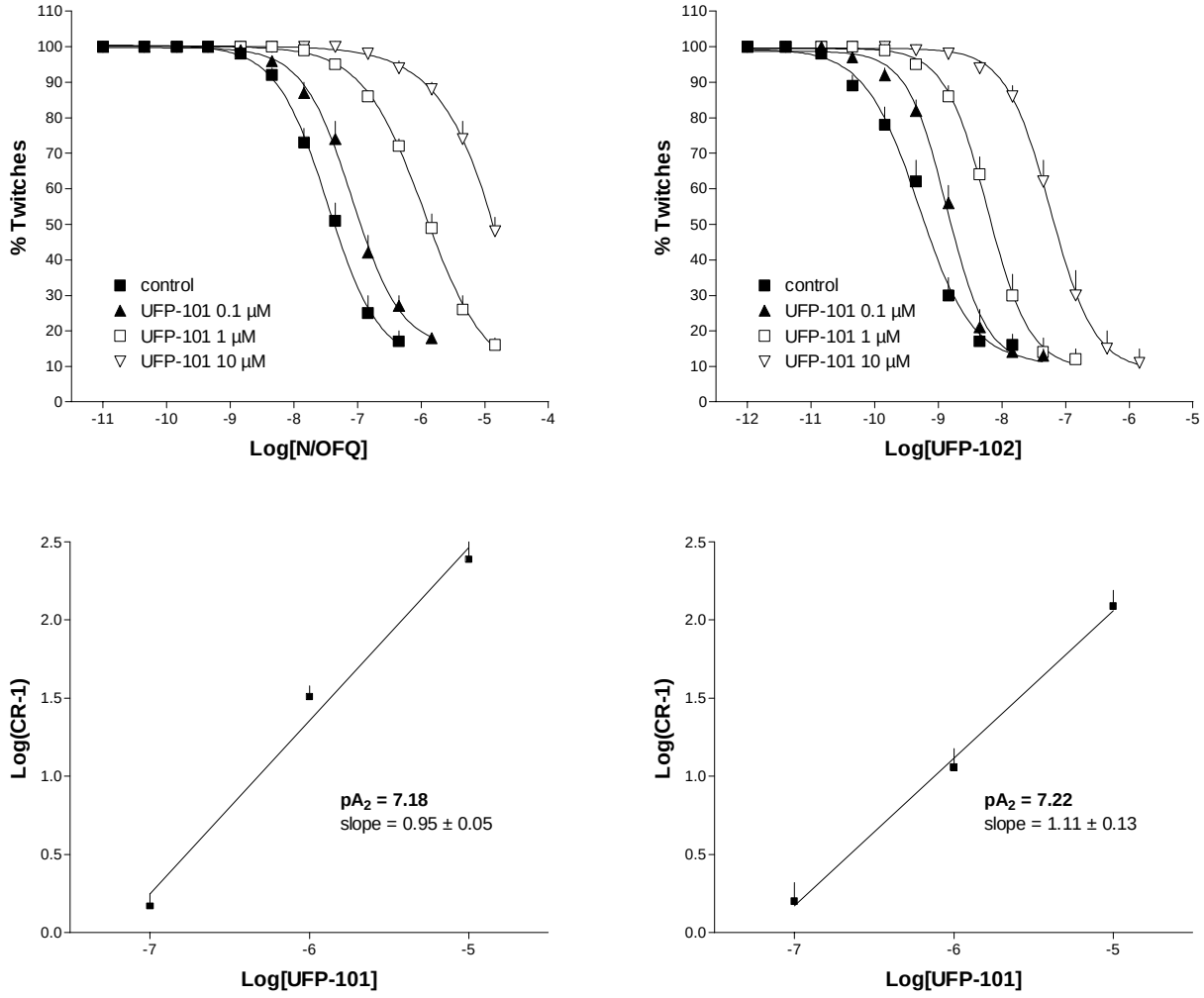

% Twitches
Log[N/OFQ]
control
UFP-101 0.1 µM
UFP-101 1 µM
UFP-101 10 µM
% Twitches
Log[UFP-102]
control
UFP-101 0.1 µM
UFP-101 1 µM
UFP-101 10 µM
Log(CR-1)
Log[UFP-101]
pA2 = 7.18
slope = 0.95 ± 0.05
Log(CR-1)
Log[UFP-101]
pA2 = 7.22
slope = 1.11 ± 0.13

Figure 3

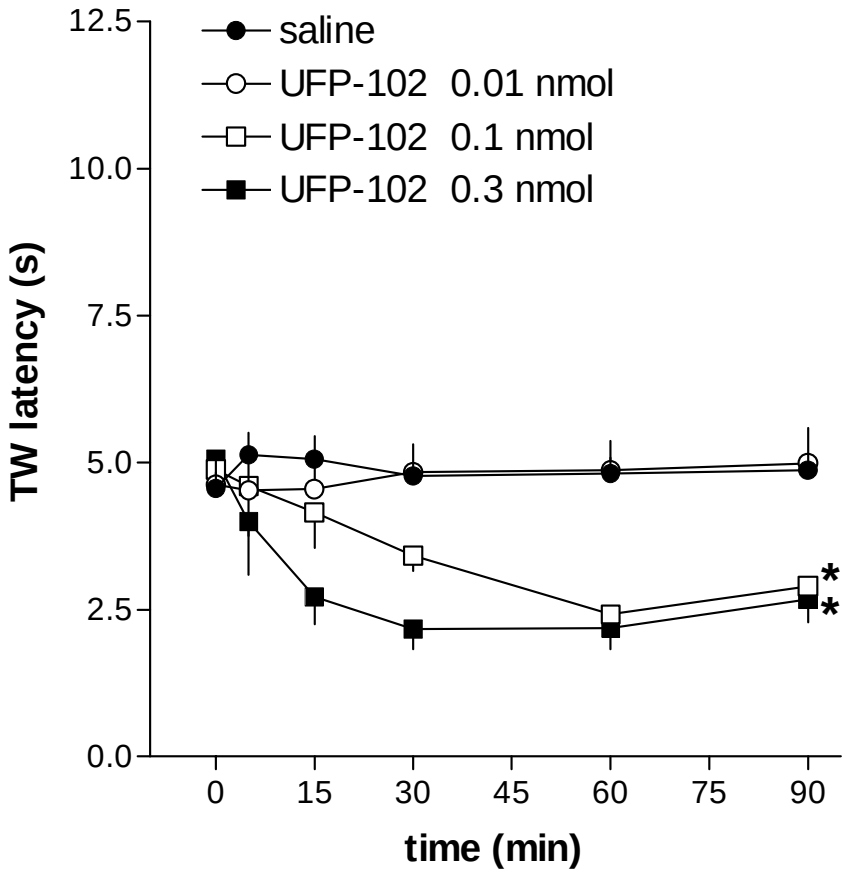

saline
UFP-102 0.01 nmol
UFP-102 0.1 nmol
UFP-102 0.3 nmol
TW latency (s)
12.5
10.0
7.5
5.0
2.5
0.0
0
15
30
45
60
75
90
time (min)
*
*

Figure 4

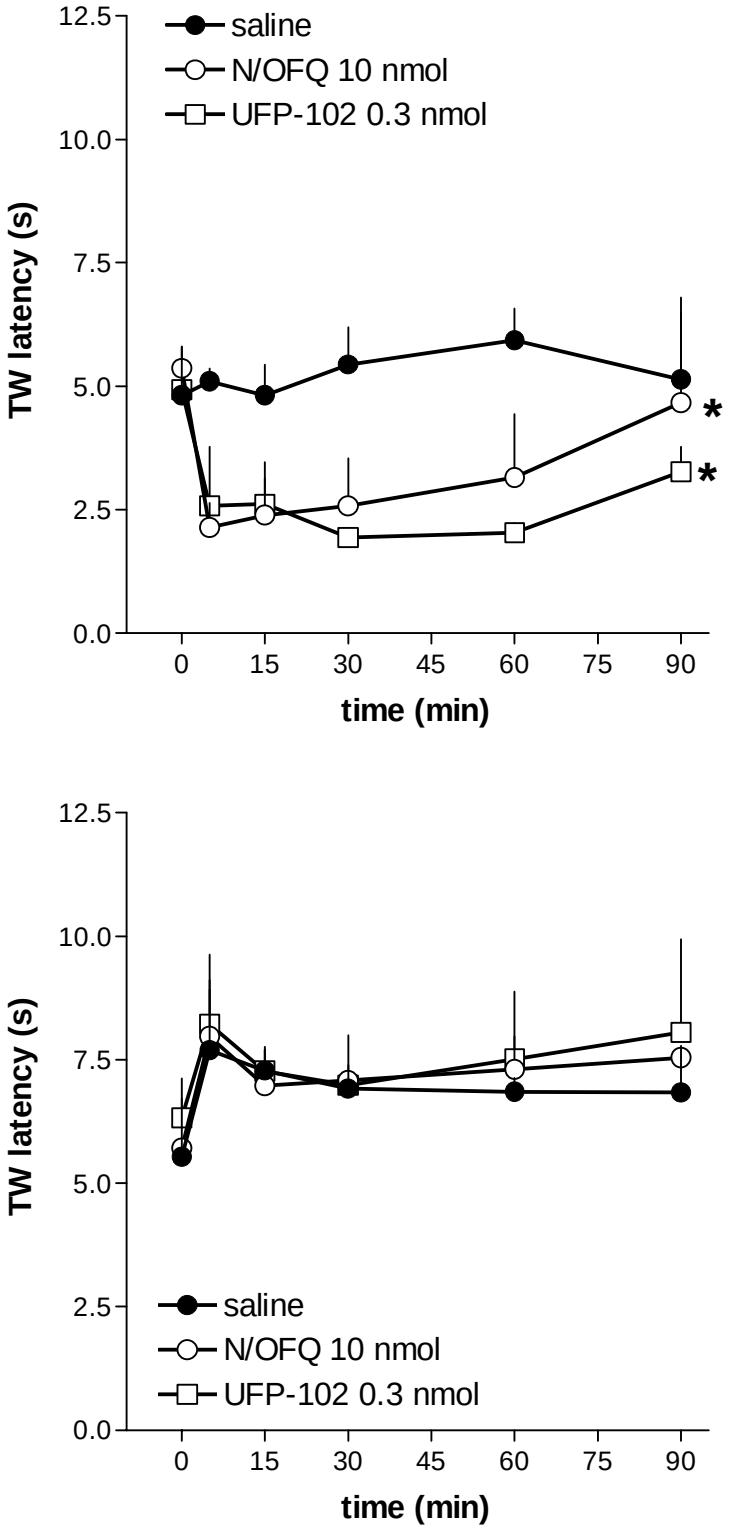
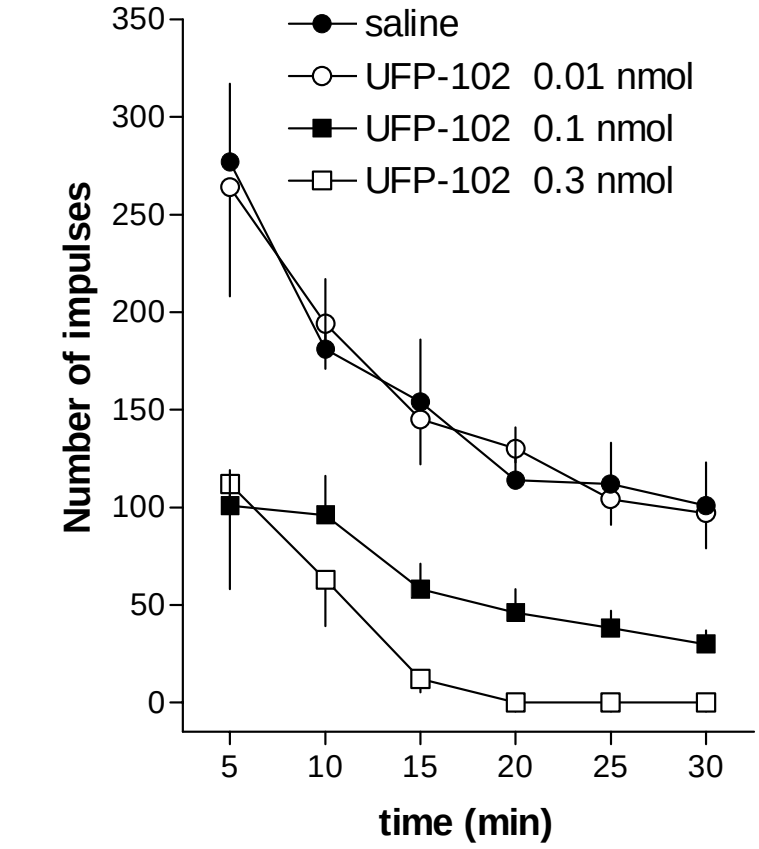

saline
N/OFQ 10 nmol
UFP-102 0.3 nmol
TW latency (s)
12.5
10.0
7.5
5.0
2.5
0.0
0
15
30
45
60
75
90
time (min)
*
*
saline
N/OFQ 10 nmol
UFP-102 0.3 nmol
TW latency (s)
time (min)

Figure 5


saline
UFP-102 0.01 nmol
UFP-102 0.1 nmol
UFP-102 0.3 nmol
Number of impulses
350
300
250
200
150
100
50
0
5
10
15
20
25
30
time (min)

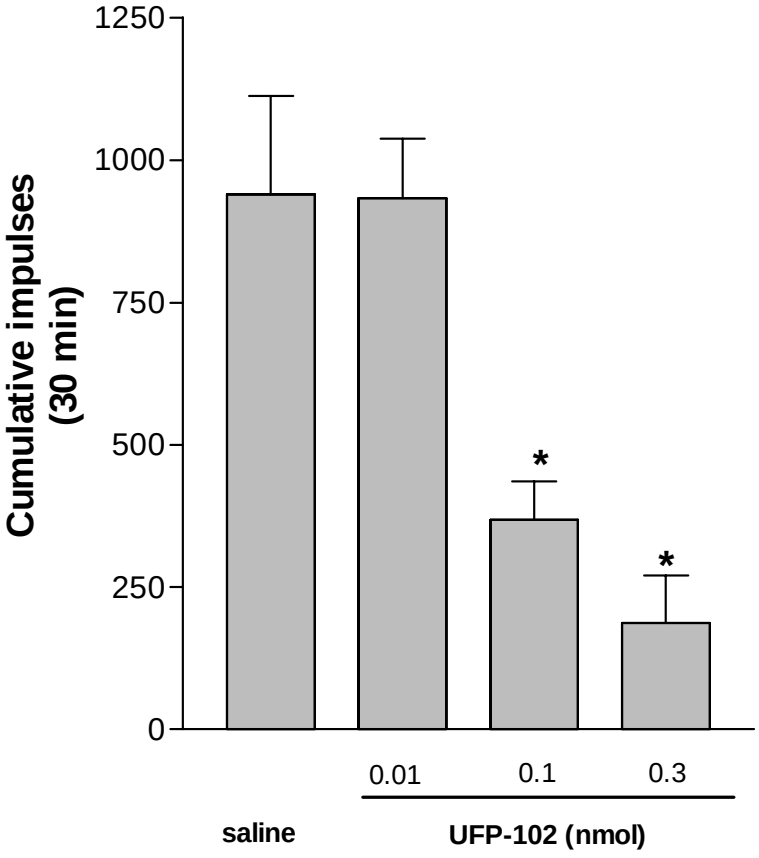
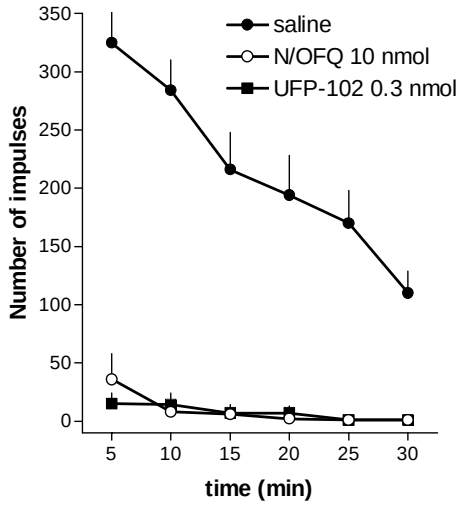
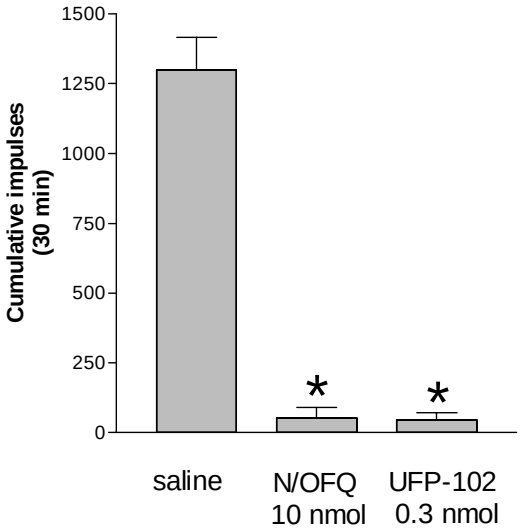
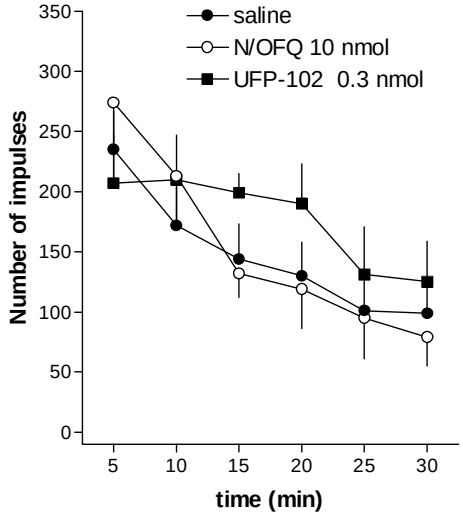

Cumulative impulses (30 min)
1250
1000
750
500
250
0
*
*
0.01
0.1
0.3
saline
UFP-102 (nmol)

Figure 6


saline
N/OFQ 10 nmol
UFP-102 0.3 nmol
Number of impulses
time (min)


Cumulative impulses (30 min)
saline
N/OFQ 10 nmol
UFP-102 0.3 nmol


saline
N/OFQ 10 nmol
UFP-102 0.3 nmol
Number of impulses
time (min)

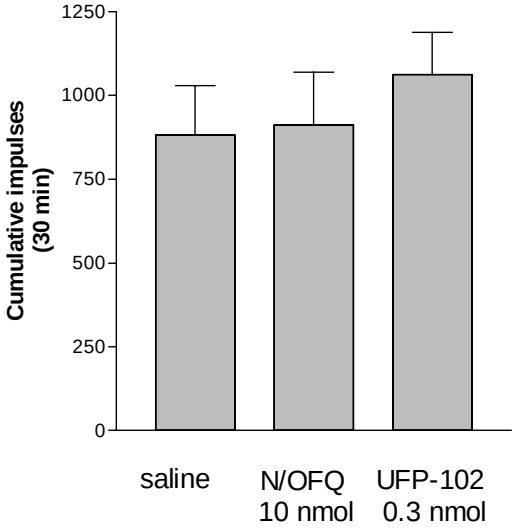
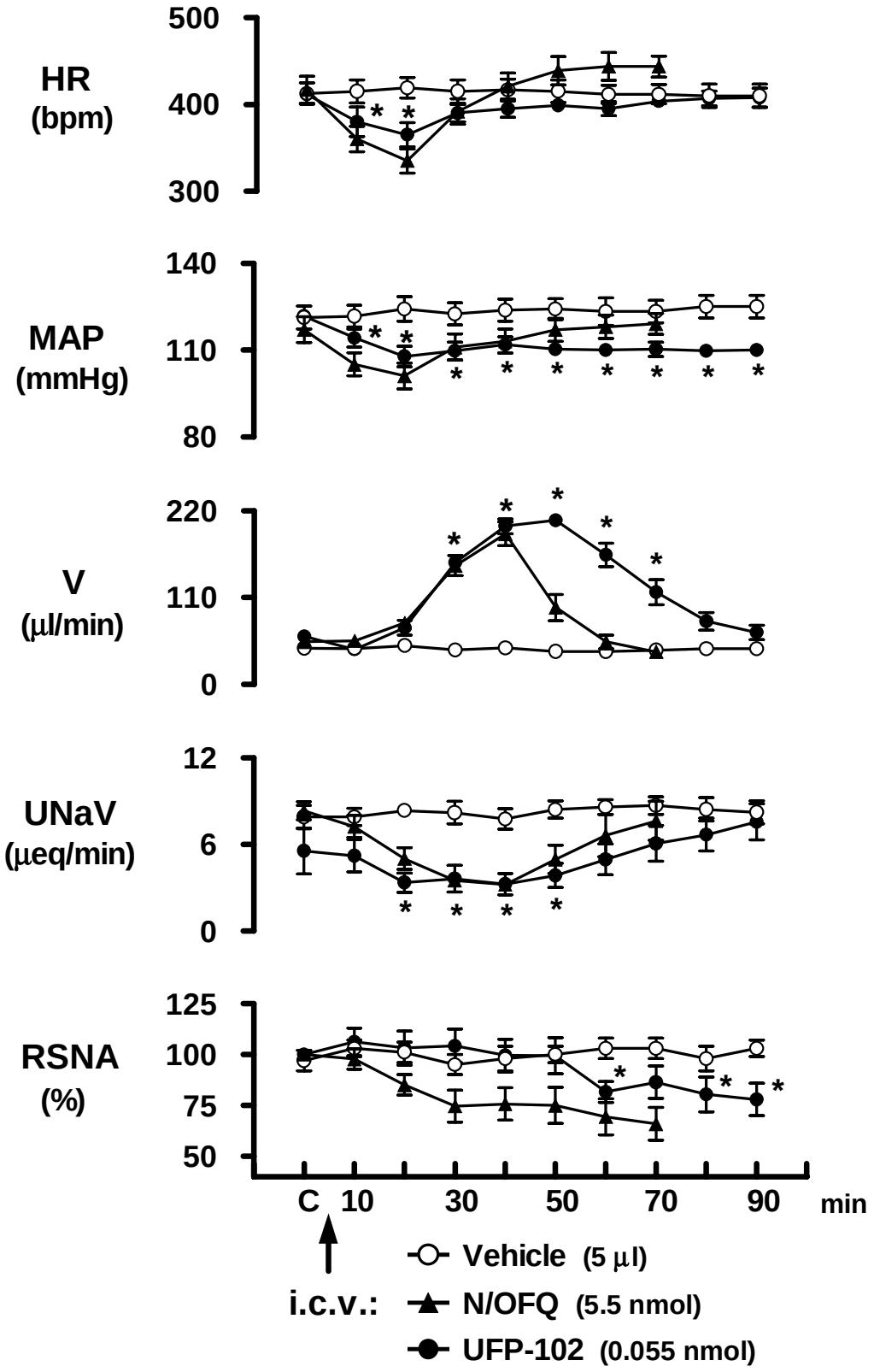

Cumulative impulses (30 min)
saline
N/OFQ 10 nmol
UFP-102 0.3 nmol

Figure 7


HR
(bpm)
500
400
300
MAP
(mmHg)
140
110
80
V
(µl/min)
220
110
0
UNaV
(µeq/min)
12
6
0
RSNA
(%)
125
100
75
50
C
10
30
50
70
90
min
i.c.v.:
Vehicle (5 µl)
N/OFQ (5.5 nmol)
UFP-102 (0.055 nmol)