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RESEARCH PAPER

In vitro and *in vivo* pharmacological characterization of nociceptin/orphanin FQ tetrabranched derivatives

A Rizzi[1], D Malfacini[1], M C Cerlesi[1], C Ruzza[1], E Marzola[2], M F Bird[3], D J Rowbotham[3], S Salvadori[2], R Guerrini[2], D G Lambert[3] and G Calo[1]

[1]*Department of Medical Sciences, Section of Pharmacology and National Institute of Neuroscience, and* [2]*Department of Chemical and Pharmaceutical Sciences and LTTA, University of Ferrara, Ferrara, Italy, and* [3]*Department of Cardiovascular Sciences, Division of Anaesthesia, Critical Care and Pain Management, Leicester Royal Infirmary, University of Leicester, Leicester, UK*

Correspondence
Girolamo Calo, Department of Medical Sciences, Section of Pharmacology, University of Ferrara, Via Fossato di Mortara 19, 44121 Ferrara, Italy. E-mail: g.calo@unife.it

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BACKGROUND AND PURPOSE

An innovative chemical approach, named peptide welding technology (PWT), allows the synthesis of multibranched peptides with extraordinary high yield, purity and reproducibility. With this approach, three different tetrabranched derivatives of nociceptin/orphanin FQ (N/OFQ) have been synthesized and named PWT1-N/OFQ, PWT2-N/OFQ and PWT3-N/OFQ. In the present study we investigated the *in vitro* and *in vivo* pharmacological profile of PWT N/OFQ derivatives and compared their actions with those of the naturally occurring peptide.

EXPERIMENTAL APPROACH

The following *in vitro* assays were used: receptor and [^{35}S]-GTPγS binding, calcium mobilization in cells expressing the human N/OFQ peptide (NOP) receptor, or classical opioid receptors and chimeric G proteins, electrically stimulated mouse vas deferens bioassay. *In vivo* experiments were performed; locomotor activity was measured in normal mice and in animals with the NOP receptor gene knocked out [NOP(–/–)].

KEY RESULTS

In vitro PWT derivatives of N/OFQ behaved as high affinity potent and rather selective full agonists at human recombinant and animal native NOP receptors. *In vivo* PWT derivatives mimicked the inhibitory effects exerted by the natural peptide on locomotor activity showing 40-fold higher potency and extremely longer lasting action. The effects of PWT2-N/OFQ were no longer evident in NOP(–/–) mice.

CONCLUSIONS AND IMPLICATIONS

The results showed that the PWT can be successfully applied to the peptide sequence of N/OFQ to generate tetrabranched derivatives characterized by a pharmacological profile similar to the native peptide and associated with a higher potency and marked prolongation of action *in vivo*.

Abbreviations

DOP, δ opioid peptide receptor; KOP, κ opioid peptide receptor; MOP, μ opioid peptide receptor; mVD, mouse vas deferens; N/OFQ, nociceptin/orphanin FQ; NOP, N/OFQ peptide receptor; NOP(–/–), mice knockout for the NOP receptor gene; NOP(+/+), wild-type mice; PWT, peptide welding technology; LA, locomotor activity

Table of Links

TARGETS	LIGANDS
δ opioid receptor	Dynorphin A
κ opioid receptor	[^{3}H]-diprenorphine
μ opioid receptor	DPDPE
NOP receptor	GTPγS
	Naloxone
	Norbinaltorphimine
	Probenecid
	SB-612111

This Table lists key protein targets and ligands in this document, which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Most of the currently marketed drugs are small molecules characterized by oral bioavailability and suitable pharmacokinetic properties. However, at least in general, relatively low selectivity for the target is often the cause of their side effects. In contrast, peptides are characterized by an extraordinary selectivity of action. However, the pharmacokinetic properties of peptides have been disappointing due to their low bioavailability, poor ability to cross barriers and high susceptibility to enzymatic degradation. To improve the pharmacokinetic properties of peptides, several different strategies have been used, including cyclization, substitutions with unnatural amino acids, modifications of the peptide bond, chemical protections at the N- and C-terminal, and use of multibranched compounds. The advantage of a multibranched peptide is its reduced susceptibility to enzymatic degradation, while the biological effects and the high selectivity of action of the peptide sequence are maintained. In the peptide receptor field, multibranched molecules have been synthesized and evaluated using different peptide sequences including neurotensin (Falciani *et al.*, 2007a), melanocyte-stimulating hormone (Brabez *et al.*, 2011), enkephalins (Falciani *et al.*, 2007b) and nociceptin/orphanin FQ (N/OFQ) (Bracci *et al.*, 2003). In these examples, the biological activity of the peptide sequences was maintained and their metabolic stability was prolonged by several fold. However, the synthesis of these molecules is rather challenging and is in general characterized by low yield and purity of the final product.

We have recently developed an innovative chemical approach, named peptide welding technology (PWT), that allows the synthesis of multibranched peptides with an extraordinary high yield, purity and reproducibility. Details of the PWT chemical strategy have been recently published (Guerrini *et al.*, 2014). With this approach, three different tetrabranched derivatives of N/OFQ have been synthesized and named PWT1-N/OFQ, PWT2-N/OFQ and PWT3-N/OFQ. These compounds differ exclusively in the core used: $(Lys)_2$-Lys-NH_2 for PWT1-N/OFQ, Cyclam for PWT2-N/OFQ and $(Lys)_2$-ethylendiamine for PWT3-N/OFQ. The chemical structures of the PWT derivatives of N/OFQ are shown in Figure 1.

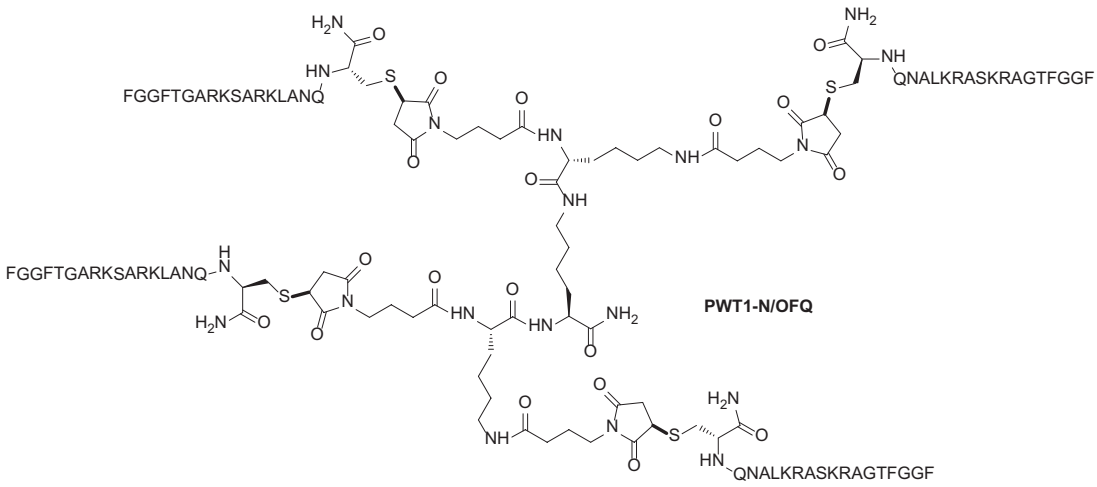
N/OFQ (FGGFTGARKSARKLANQ) was identified as the endogenous ligand of a previously described orphan receptor (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995), now referred to as the N/OFQ peptide (NOP) receptor. NOP is a GPCR whose activation produces inhibition of cAMP levels, stimulation of potassium currents and inhibition of calcium channels. The peptide and its receptor are widely distributed in the nervous system where, via inhibitory mechanisms, they modulate several different biological functions (Lambert, 2008).

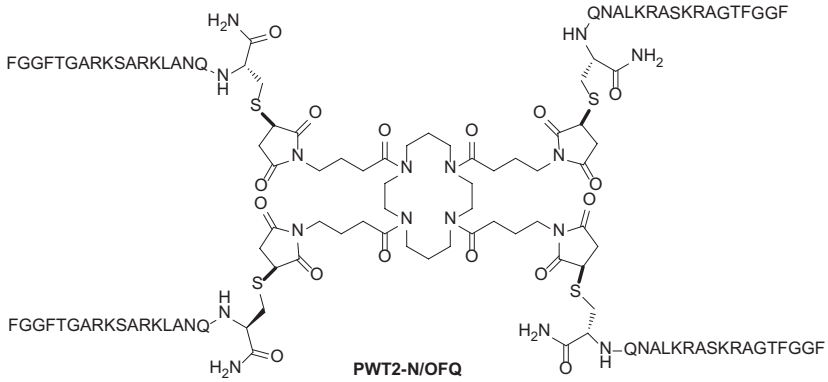
In the present study, we investigated the *in vitro* and *in vivo* pharmacological profile of PWT N/OFQ derivatives and compared their actions with those of the naturally occurring peptide.

Methods

Cell culture and membrane preparation

CHO cells stably expressing human classical opioid receptors (MOP, KOP and DOP receptors) were maintained in Nutrient F12 containing 10% FBS; CHO cells expressing human NOP receptors were maintained in DMEM/Nutrient F12 (50/50)





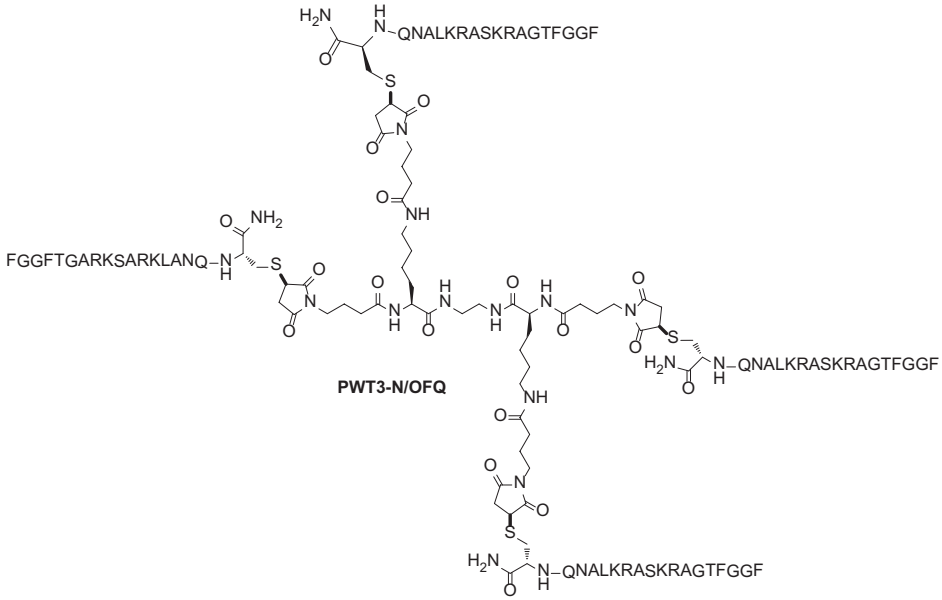


Figure 1

Chemical structures of PWT derivatives of N/OFQ.

with 5% FBS; all media were further supplemented with penicillin (100 IU·mL^{-1}), streptomycin (100 μg·mL^{-1}) and fungizone (2.5 μg·mL^{-1}). Stock cultures additionally contained geneticin (G418) (200 μg·mL^{-1}) for $CHO_{MOP/KOP/DOP}$ cells, or G418 (200 μg·mL^{-1}) and hygromycin B (200 μg·mL^{-1}) for CHO_{NOP} cells. Cultures were sustained at 37°C with 5% carbon dioxide humidified air and subcultured twice weekly. Membranes were prepared from freshly harvested cells. Cells were suspended in homogenizing buffer consisting of either Tris (50 mM) for [^{3}H]-diprenorphine displacement binding, Tris (50 mM), $MgSO_4$ (5 mM) for [^{3}H]-UFP-101 displacement binding or Tris (50 mM), EGTA (0.2 mM) for [^{35}S]-GTPγS assays. Cell suspensions were homogenized and membranes were collected via centrifugation at 20 374× *g* for 10 min at 4°C. This process was repeated three times and protein concentrations were determined (Lowry *et al.*, 1951).

Receptor binding

CHO_{NOP} membranes (40 μg) were incubated in 0.5 mL of buffer consisting of Tris (50 mM), BSA (0.5%), ~0.8 nM [^{3}H]UFP-101 and varying concentrations (10 μM–1 pM) of ligands. Non-specific binding was determined in the presence of 1 μM N/OFQ. In addition, 50 μg of membranes from CHO cells expressing classical opioid receptors was incubated in 0.5 mL buffer consisting of Tris (50 mM), BSA (0.5%), ~0.8 nM [^{3}H]-DPN and a range of concentrations of ligands. Non-specific binding was determined in the presence of 10 μM naloxone.

For both displacement assays, reactions were incubated at room temperature for 1 h and terminated by vacuum filtration through polyethylenimine (0.5%)-soaked Whatman GF/B filters (Fisher Scientific, Loughborough, UK), using a Brandel harvester. Radioactivity was determined following an 8 h extraction of filters in ScintiSafe Gel using liquid scintillation spectroscopy.

Stimulation of [^{35}S]-GTPγS binding

CHO_{NOP} membranes, 40 μg, were incubated in 0.5 mL buffer containing Tris (50 mM), EGTA (0.2 mM), $MgCl_2$ (1 mM), NaCl (100 mM), BSA (0.1%), bacitracin (0.15 mM), GDP (100 mM) and ~150 pM [^{35}S]-GTPγS. NOP ligands were included in varying concentrations, and non-specific binding was determined in the presence of 10 μM GTPγS. Reactions were incubated for 1 h at 30°C with gentle shaking and terminated by vacuum filtration through dry Whatman GF/B filters. Radioactivity was determined following an 8 h extraction of filters in ScintiSafe Gel using liquid scintillation spectroscopy.

Calcium mobilization

CHO cells stably co-expressing the human NOP, KOP or MOP receptor and the C-terminally modified $G\alpha_{qi5}$ and CHO cells expressing the DOP receptor and the $G\alpha_{qG66Di5}$ protein were generated as previously described (Camarda *et al.*, 2009; Camarda and Calo, 2013). Cells were maintained in culture medium consisting of DMEM/HAM'S F-12 (50/50) supplemented with 10% FCS, penicillin (100 IU·mL^{-1}), streptomycin (100 μg·mL^{-1}), fungizone (2.5 μg·mL^{-1}), geneticin (G418; 200 μg·mL^{-1}) and hygromycin B (200 mg·mL^{-1}). Cell cultures were kept at 37°C in 5% CO_2 humidified air. Experimental cultures were free from selection agents (hygromycin B, G418). When confluence was reached (3–4 days), cells were subcultured as required using trypsin/EDTA and used for experimentation. Cells were seeded at a density of 50 000 cells per well into 96-well black, clear-bottom plates. After 24 h of incubation, cells were loaded with medium supplemented with 2.5 mM probenecid, 3 μM of the calcium-sensitive fluorescent dye Fluo-4 AM and 0.01% pluronic acid for 30 min at 37°C. Afterwards, the loading solution was aspirated and 100 μL per well of assay buffer: HBSS supplemented with 20 mM HEPES, 2.5 mM probenecid and 500 μM Brilliant Black was added. Stock solutions (1 mM) of peptides and PWT compounds were made in distilled water and stored at –20°C. SB-612111 was solubilized (10 mM) in DMSO. Serial dilutions of ligands for experimental use were made in HBSS/HEPES (20 mM) buffer (containing 0.03% BSA fraction V). After the cell culture and compound plates had been placed in the FlexStation II (Molecular Device, Union City, CA USA), fluorescence changes were measured. Online additions were carried out in a volume of 50 μL per well. To facilitate drug diffusion into the wells in antagonist-type experiments, the present studies were performed at 37°C and three cycles of mixing (25 μL from each well) were performed immediately after antagonist injection.

Electrically stimulated mouse vas deferens (mVD)

Tissues were taken from NOP(+/+) or NOP(–/–) male mice. The mVD tissues were prepared as previously described (Calo *et al.*, 1996). Tissues were suspended in 5 mL of organ baths containing heated Krebs solution (composition in mM: NaCl 118.5, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10 and $CaCl_2$ 2.5) oxygenated with 95% O_2 and 5% CO_2. The bath temperature was set at 33°C. Tissues were continuously stimulated through two platinum ring electrodes with supramaximal rectangular pulses of 1 ms duration and 0.05 Hz frequency. A resting tension of 0.3× *g* was applied to the vas deferens. The electrically-evoked contractions (twitches) were measured isotonically with a strain gauge transducer (Basile 7006; Ugo Basile s.r.l., Varese, Italy) and recorded with the PC-based acquisition system Power Lab (ADInstrument, Colorado Springs, CO, USA). Following an equilibration period of 60 min, the contractions induced by electrical field stimulation were stable. At this time, cumulative concentration–response curves to N/OFQ, its PWT derivatives or DPDPE were performed.

Mouse locomotor activity

All experimental procedures adopted for *in vivo* studies were as humane as possible and complied with the ARRIVE guidelines (Kilkenny *et al.*, 2010) and the standards of the European Communities Council directives (86/609/EEC) and national regulations (D.L. 116/92). Male CD-1 mice (30–38 g, Harlan, San Pietro in Natisone, Udine, Italy) and male NOP(+/+) or NOP(–/–) mice were used. The total number of mice used for this study was 360. They were housed in Plexiglas® cages (Tecniplast, Buguggiate, Varese, Italy), under standard conditions (22°C, 55% humidity, 12 h light–dark cycle, lights on 07:00 h) with food and water available *ad libitum* for at least 5 days before experiments were begun. Experiments were performed according to the procedures

described previously (Guerrini *et al.*, 2009). For these experiments, the ANY-maze video tracking system was used (Ugo Basile, Varese, Italy, application version 4.52c Beta). Mice were positioned in a square plastic cage (40 × 40 cm), 1 mouse per cage. Four mice were monitored in parallel. Mouse horizontal activity was monitored by a camera, whereas vertical activity was measured by an infrared beam array. The parameters measured were cumulative distance travelled (total distance in metres that the animal travelled during the test), immobility time (the animal is considered immobile when 90% of it remains in the same place for a minimum of 2.5 s) and the number of rearing (the number of beam breaks due to vertical movements; this input is triggered when the beam is interrupted for a minimum of 200 ms). N/OFQ, PWT1-N/OFQ, PWT2-N/OFQ and PWT3-N/OFQ were given i.c.v. (2 μL per mouse). Free hand i.c.v. injections were given, under isoflurane anaesthesia (just sufficient to produce a loss of the righting reflex), in the left ventricle according to literature procedures (Laursen and Belknap, 1986).

Data analysis and statistical procedures

In vitro data were expressed as mean ± SEM of at least three separate experiments. The concentration of drug producing 50% displacement of specific binding (IC_{50}) was corrected for the competing mass of radioligand according to Cheng and Prusoff (1973) to yield K_i using K_D. K_D values of [^{3}H]-UFP-101 at NOP and [^{3}H]-diprenorphine at classical opioid receptors were taken from previous studies (Kitayama *et al.*, 2003; Ibba *et al.*, 2008). [^{35}S]-GTPγS data are expressed as stimulation factor (agonist specific binding/basal specific binding). In calcium mobilization experiments, maximum change in fluorescence, expressed as percentage over the baseline fluorescence, was used to determine agonist response. Non-linear regression analysis using GraphPad Prism software (5.0; GraphPad Software, San Diego, CA, USA) allowed logistic iterative fitting of the resultant responses and the calculation of agonist potencies and maximal effects. Agonist potencies were given as pEC_{50} (the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect).

In vivo data are expressed as mean ± SEM of *n* animals. Data were analysed using one-way ANOVA followed by Dunnett's *post hoc* test or with Student's *t*-test. Differences were considered statistically significant when $P < 0.05$.

Results

Receptor binding

In CHO_{NOP} cell membranes, N/OFQ displaced [^{3}H]-UFP-101 binding in a concentration-dependent manner showing high affinity (pK_i 9.42). Similar results were obtained with PWT derivatives of N/OFQ; however, they displayed increased affinity by 5 (PWT3-N/OFQ) up to 15 (PWT1-N/OFQ) fold (Table 1). To investigate selectivity over classical opioid receptors, similar experiments were performed using membranes obtained from CHO cells expressing the MOP, KOP and DOP receptors and [^{3}H]-diprenorphine as radioligand. N/OFQ up to 1 μM did not bind to KOP and DOP receptors while it displayed micromolar affinity at MOP receptors (pK_i 6.06). All PWT derivatives showed higher affinity than N/OFQ at classical opioid receptors. However, their selectivity for the NOP receptor (ranging from 2570- to 14 125-fold) was never inferior to that displayed by the natural peptide (2291-fold). In parallel experiments, standard ligands for classical opioid receptors displayed the expected high affinity (morphine, pK_i 8.57 for MOP receptors; Dmt-Tic, pK_i 8.87 for DOP receptors; and norbinaltorphimine, pK_i 10.69 for KOP receptors).

Stimulation of [^{35}S]-GTPγS binding

In CHO_{NOP} cell membranes, N/OFQ stimulated [^{35}S]-GTPγS binding in a concentration-dependent manner with maximal effects of 3.5 ± 0.2 and pEC_{50} of 8.84 (Figure 2, top panel). PWT derivatives of N/OFQ mimicked the stimulatory effect of the natural peptide showing similar maximal effects but higher potency (pEC_{50} 9.71–10.12; Figure 2, top panel).

Calcium mobilization

In CHO_{NOP} cells stably expressing the $G\alpha_{qi5}$ chimeric protein, N/OFQ and its PWT derivatives evoked a concentration-dependent stimulation of calcium release (Figure 2, bottom panel). N/OFQ displayed high potency (pEC_{50} 9.39) and maximal effects (237 ± 15%). PWT derivatives of N/OFQ mimicked the peptide stimulatory effects showing similar maximal effects but slightly lower potency (pEC_{50} 8.75–9.16; Figure 2, bottom panel). Inhibition experiments were performed by testing increasing concentrations of the NOP receptor antagonist SB-612111 against a fixed concentration of agonist approximately corresponding to its EC_{80} (10 nM for

Table 1

Affinities of N/OFQ and its PWT derivatives in membranes of CHO cells expressing the human NOP or classical opioid receptors

	NOP	MOP	KOP	DOP
	pK_i ($CL_{95\%}$)	pK_i ($CL_{95\%}$)	pK_i ($CL_{95\%}$)	pK_i ($CL_{95\%}$)
N/OFQ	9.42 (9.23–9.57)	6.06 (5.80–6.32)	<6	<6
PWT1-N/OFQ	10.60 (10.10–11.10)	6.45 (6.21–6.69)	6.40 (6.01–6.79)	6.38 (6.07–6.69)
PWT2-N/OFQ	10.30 (9.99–10.61)	6.60 (6.31–6.89)	6.36 (5.80–6.92)	<6
PWT3-N/OFQ	10.10 (9.74–10.46)	6.69 (6.19–7.19)	6.33 (5.98–6.68)	<6

Data are the mean of three separate experiments performed in duplicate.

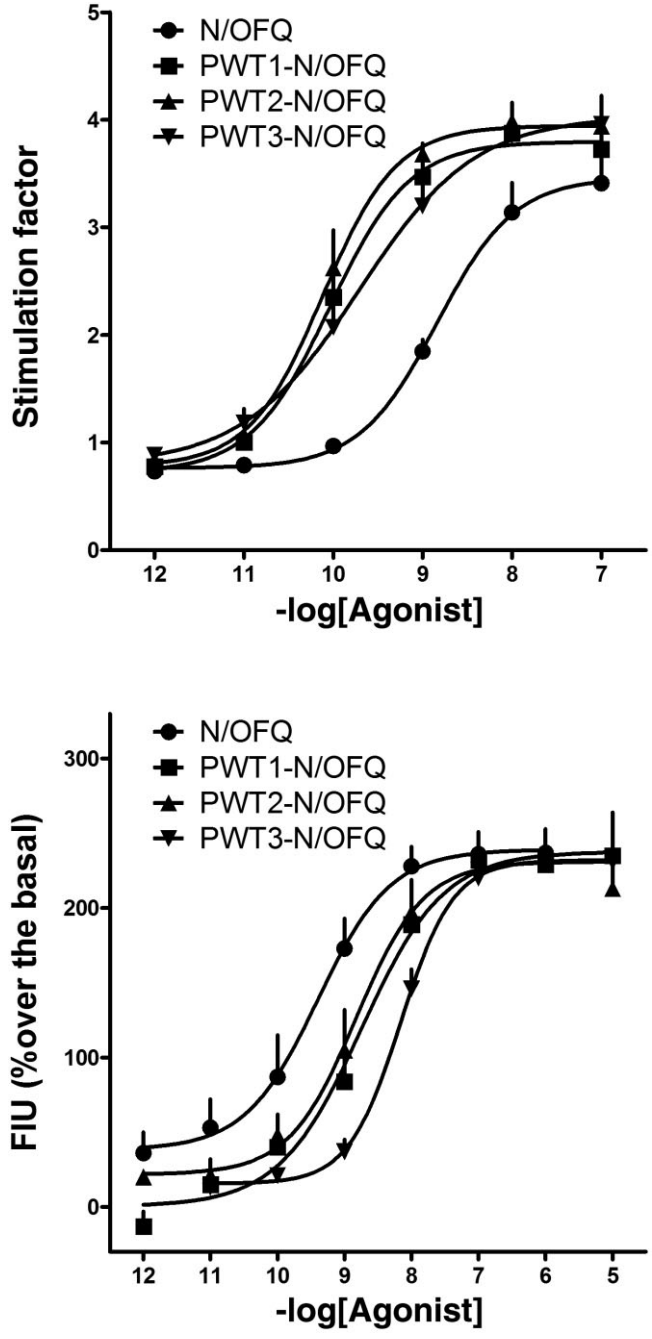


Figure 2

Concentration–response curves to N/OFQ and its PWT derivatives in [^{35}S]-GTPγS binding experiments performed in CHO_{NOP} cell membranes (top panel) and in calcium mobilization experiments performed on CHO cells co-expressing the NOP receptor and the $G\alpha_{qi5}$ chimeric protein (bottom panel). Data are mean ± SEM of four experiments performed in duplicate.

N/OFQ and 30 nM for all PWT derivatives). SB-612111 concentration-dependently inhibited the stimulatory effect of N/OFQ, displaying a pK_B value of 8.01. Similar results were obtained when challenging the antagonist against the stimulatory effect of PWT compounds (pK_B 7.82–8.23).

In order to assess the selectivity of action of PWT derivatives of N/OFQ, calcium mobilization experiments were also performed in cells expressing chimeric G protein and the classical opioid receptors. Dermorphin, DPDPE and dynorphin A were used as standard agonists for MOP, DOP and KOP receptors, respectively. Opioid ligands were all inactive up to 1 μM in CHO_{NOP} cells (Table 2). In CHO_{MOP} cells, dermorphin evoked concentration-dependent stimulatory effects with pEC_{50} of 9.29 and maximal effects of 135 ± 21%. The stimulatory effects of dermorphin were mimicked by dynorphin A, but dynorphin A was approximately 300-fold less potent. DPDPE, N/OFQ and its PWT derivatives were found to be inactive. In CHO_{DOP} cells, DPDPE evoked concentration-dependent stimulatory effects with pEC_{50} of 9.57 and maximal effects of 86 ± 14%. Dynorphin A was also able to elicit calcium mobilization, producing similar maximal effects but being approximately 100-fold less potent. All other agonists were inactive. Finally, in CHO_{KOP} cells, dynorphin A stimulated calcium release with very high potency (pEC_{50} of 10.04) and maximal effects of 225 ± 10%. All other agonists were inactive.

Electrically stimulated tissues

The effects of N/OFQ, its PWT derivatives and DPDPE were investigated in the electrically stimulated mVD taken from NOP(+/+) and NOP(–/–) mice. In tissues taken from NOP(+/+) animals, N/OFQ inhibited the electrically-induced contractions in a concentration-dependent manner with potency value (pEC_{50}) of 7.68 and maximal effect of 92 ± 2% (Figure 3). PWT1-N/OFQ mimicked the inhibitory effect of N/OFQ showing higher potency (pEC_{50} 8.02) and similar maximal effects. Similar results were obtained with PWT2-N/OFQ and PWT3-N/OFQ (Figure 3). Interestingly, the inhibitory effect induced by N/OFQ occurred immediately after adding the peptide to the bath and was immediately reversible after washing the tissue. In contrast, PWT1-N/OFQ induced a slow inhibitory effect, which reached a plateau only after 10 min. More importantly, the effects induce by PWT1-N/OFQ were rather resistant to washing. Superimposable results both in terms of slow kinetic and washing-resistant effects were obtained with PWT2-N/OFQ and PWT3-N/OFQ. In tissues taken from NOP(–/–), the inhibitory effects elicited by the natural peptide N/OFQ were no longer evident (Figure 3). In contrast, the three PWT derivatives of N/OFQ maintained the ability to inhibit the electrically induced contractions in NOP(–/–) tissues, but had a reduced potency by more than 10-fold for PWT1-N/OFQ and approximately 100-fold for PWT2-N/OFQ and PWT3-N/OFQ (Figure 3). The selective DOP receptor agonist DPDPE produced similar inhibitory effects in NOP(+/+) and NOP(–/–) tissues showing high potency ($pEC_{50} \approx 8.5$) and maximal effects (≈ 95%) (data not shown).

Mouse locomotor activity (LA)

The effects of N/OFQ and PWT derivatives on spontaneous LA were evaluated in CD-1 mice. In a first series of experiments, the dose–response curve to N/OFQ and PWT derivatives was assessed by recording animal behaviour for 120 min. As shown in Figure 4, N/OFQ produced a complex pattern of effects depending upon the dose and time (first and

Table 2

Calcium mobilization studies

	NOP	MOP	DOP	KOP
	pEC_{50} ($CL_{95\%}$)	pEC_{50} ($CL_{95\%}$)	pEC_{50} ($CL_{95\%}$)	pEC_{50} ($CL_{95\%}$)
N/OFQ	9.39 (9.23–9.57)	<6	<6	<6
PWT1-N/OFQ	8.75 (8.35–9.15)	<6	<6	<6
PWT2-N/OFQ	8.83 (8.47–9.18)	<6	<6	<6
PWT3-N/OFQ	9.16 (9.08–9.24)	<6	<6	<6
Dermorphin	<6	9.29 (9.19–9.38)	<6	<6
DPDPE	<6	<6	9.57 (9.03–10.11)	<6
Dynorphin A	<6	6.76 (6.50–7.02)	7.63 (7.38–7.88)	10.04 (9.93–10.16)

Potencies of N/OFQ, its PWT derivatives and standard opioid agonists in CHO cells expressing the human NOP or classical opioid receptors and chimeric G proteins. Data are the mean of three separate experiments performed in duplicate.

second hours after injection). Over the time course of the experiment saline-injected animals displayed a progressive decrease in horizontal and vertical activity associated with an increased immobility time. N/OFQ 0.1 nmol did not produce statistically significant effects, whereas 1 nmol N/OFQ produced stimulatory effects, increasing horizontal activity in the first hour and vertical activity in the second hour. The highest dose of peptide (i.e. 10 nmol) induced robust inhibitory effects during the first hour. However, this action was short lasting, and during the second hour, a statistically significant reduction in immobility time was recorded. Thus, N/OFQ produced short lasting biphasic effects with lower doses producing stimulatory effects and higher doses eliciting inhibitory effects. Under the same experimental conditions, the effects of PWT1-N/OFQ, PWT2-N/OFQ and PWT3-N/OFQ were assessed and compared with those of N/OFQ. For the sake of clarity, these results are summarized in Table 3, where statistically significant effects are coloured red, indicating inhibitory and green stimulatory actions. All the three N/OFQ PWT derivatives mimicked N/OFQ effects, but were more potent. PWT1-N/OFQ 2.5 pmol produced slight stimulatory effects, whereas the same dose of PWT2-N/OFQ or PWT3-N/OFQ was inactive. Also, 25 pmol of all PWT derivatives produced stimulatory effects during both the first and the second hours. At higher doses, that is, 250 pmol PWT1-N/OFQ did not modify animal locomotor behaviour, whereas PWT2-N/OFQ and PWT3-N/OFQ reduced vertical activity. Of note, these inhibitory effects were more pronounced during the second hour after the injection (Table 3). In order to investigate in detail *in vivo* duration of action, the effects of N/OFQ (10 nmol) and PWT derivatives (all at 250 pmol) were measured in an overnight experiment. Mice were injected i.c.v. at 11:00 h and their LA was measured from 15:00 to 09:00 h the following day. As shown in Figure 5, mice injected with saline displayed an increase in the horizontal and vertical activity associated with a decrease in immobility time at 19:00 h when the light was turned off, then their LA progressively returned to baseline levels. Mice injected with N/OFQ 10 nmol displayed the same locomotor behaviour as that of control mice. In contrast, mice treated with PWT derivatives displayed a profound and sustained depression of locomotor behaviour. In fact, statistically significant inhibitory effects were observed, in response to all PWT derivatives, on cumulative distance travelled, immobility time and number of rearing (Figure 5). In order to further investigate the duration of action of PWT derivatives, an additional experiment was performed in which the LA of mice was measured for 60 min, 24 h after the agonist injection. Saline-injected mice displayed a progressive reduction in horizontal and vertical activity associated with an increase in immobility time (Figure 6). Animals treated 24 h previously with 10 nmol N/OFQ or 250 pmol PWT1–N/OFQ displayed the same motor behaviour as that of control mice. Animals treated with 250 pmol PWT3-N/OFQ displayed lower horizontal and vertical activity and higher immobility time compared with controls; however, these differences did not reach statistical significance. In contrast, 250 pmol PWT2-N/OFQ produced statistically significant inhibitory effects on all the parameters measured (Figure 6).

Finally, the involvement of the NOP receptor in the inhibitory effects exerted by PWT2-N/OFQ on LA was assessed by comparing its action in NOP(+/+) and NOP(−/−) mice. As summarized in Table 4, 250 pmol PWT2-N/OFQ produced robust inhibitory effects in NOP(+/+) mice. In fact, horizontal and particularly vertical activities were significantly reduced and this was associated with a statistically significant increase in immobility time. NOP(−/−) mice injected with saline displayed a locomotor behaviour similar to that of NOP(+/+) animals; however, in these mice, PWT2-N/OFQ did not significantly modify locomotor behaviour (Table 4).

Discussion and conclusions

In this study, we investigated the pharmacological profile of tetrabranched derivatives of N/OFQ generated using the PWT. *In vitro* experiments demonstrated that PWT compounds bound the NOP receptor with high affinity and behaved as full agonists both at human recombinant and animal native receptors. While NOP receptor selectivity over classical opioid receptors was not affected, knockout studies

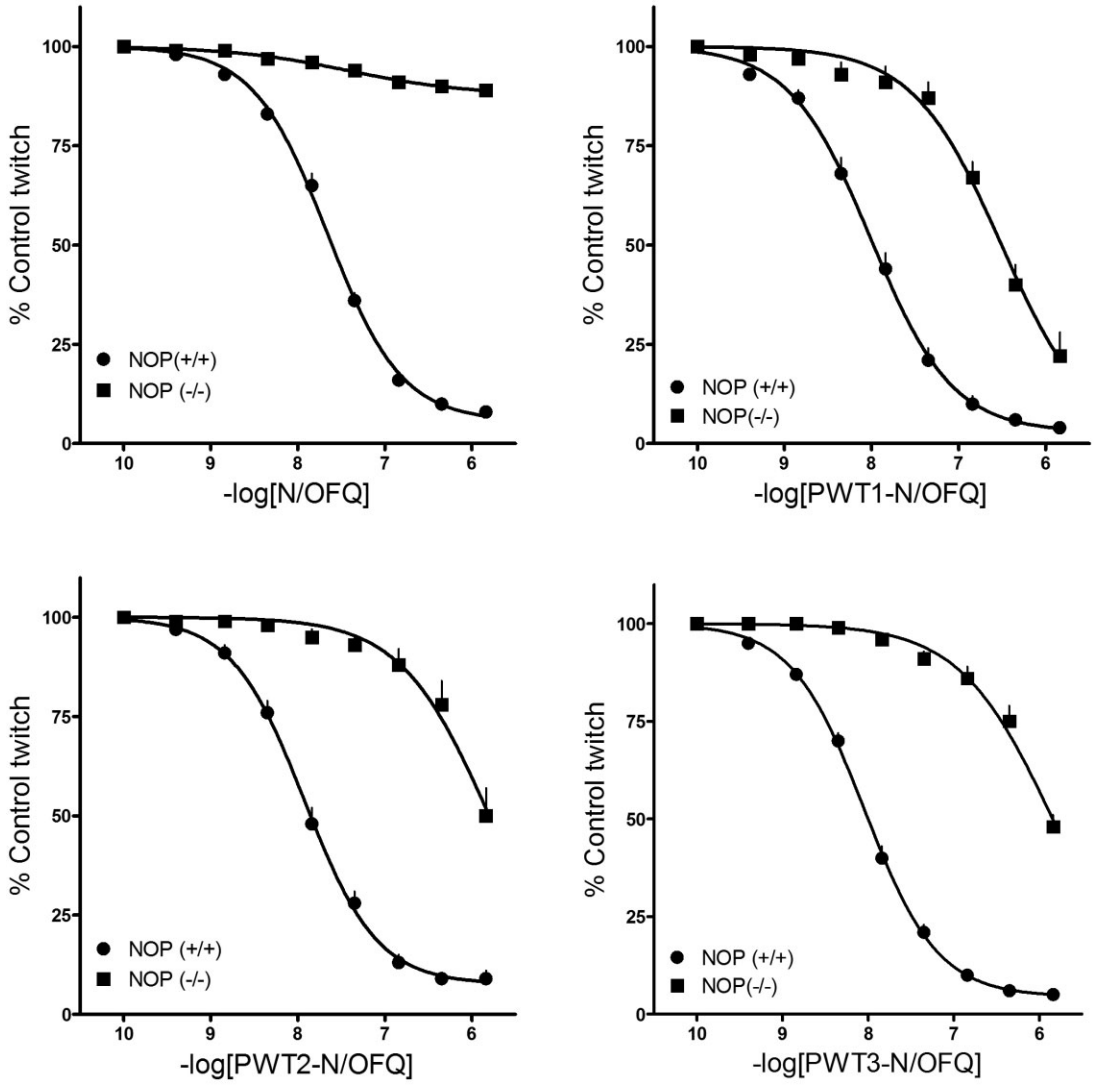


Figure 3

Concentration–response curves for N/OFQ and its PWT derivatives in electrically-stimulated mouse vas deferens taken from NOP(+/+) and NOP(–/–) animals. Data are mean ± SEM of four experiments.

in isolated tissues demonstrated a decline in selectivity of the PWT compounds, compared with N/OFQ, which was modest or moderate depending upon the type of core. *In vivo* studies have confirmed the ability of PWT compounds to mimic the effects of N/OFQ with high potency. In addition, the *in vivo* duration of action of PWT derivatives was far longer than that of N/OFQ. Overall, the results obtained in the present study demonstrate that the PWT can be successfully applied to N/OFQ to generate novel NOP receptor ligands that display similar biological activity to that of the natural peptide, but have an extremely prolonged duration of action.

In receptor binding studies, N/OFQ displayed subnanomolar affinity (pK_i 9.42) for the NOP receptor, in line with previous results (9.62, Fischetti *et al.*, 2009; 9.68, Spagnolo *et al.*, 2007). PWT derivatives of N/OFQ displaced [^{3}H]-UFP-101 binding and displayed 3- to 10-fold higher affinity than N/OFQ. This result is in line with previous findings that showed that N/OFQ dendrimers displaced [^{3}H]-N/OFQ binding from rat brain membranes with 4.5-fold higher affinity than N/OFQ (Bracci *et al.*, 2003). Similar results were also reported with multibranched derivatives of other peptide sequences including Leu-enkephalin (9-fold), Met-enkephalin (4-fold) and neurotensin(8–13) (12-fold) (Bracci *et al.*, 2003). Even larger increases in affinity of multibranched peptides, from 20- to 350-fold, depending upon the linker unit used, were reported in the case of melanocyte-stimulating hormone (Brabez *et al.*, 2011). However, no differences were found when the full sequence of neurotensin was used (Bracci *et al.*, 2003). Thus, an increase in the binding affinity of multibranched peptides compared with the natural sequences seems to be a general rule, even if there are significant differences, which depend on the specific peptide sequence and the linker used.

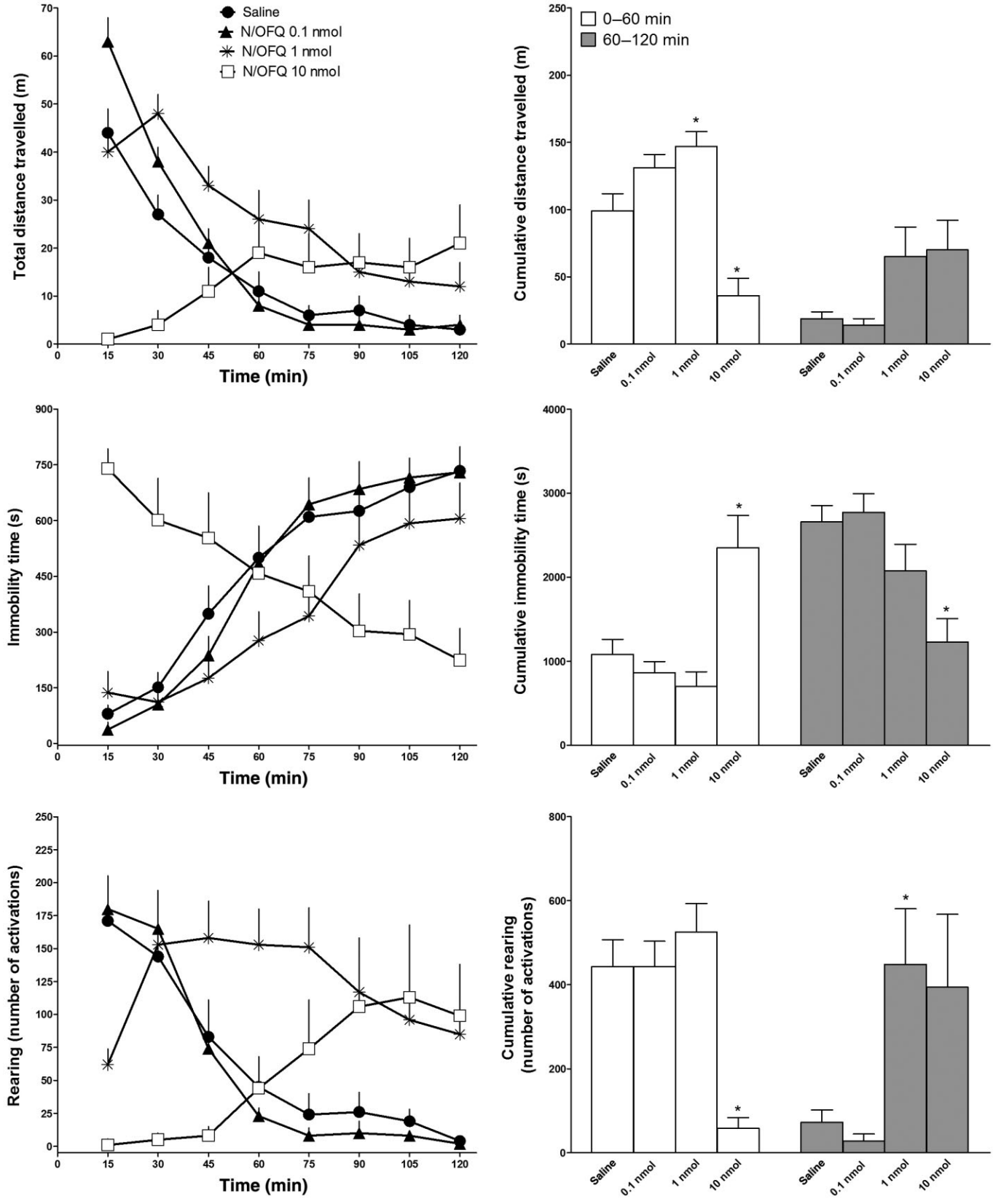


Figure 4

Dose–response curves for the effect of N/OFQ on mouse locomotor activity. Results are shown as time course in the left panels and as cumulative effects during the first and second hours of observation in the right panels. Data are mean ± SEM of 12 animals for each point. *$P < 0.05$ according to one-way ANOVA followed by the Dunnett's test for multiple comparisons.

Table 3

Mouse locomotor activity

	0–60 min			60–120 min		
	Distance travelled (m)	Immobility time (s)	Number of rearing	Distance travelled (m)	Immobility time (s)	Number of rearing
Saline	99 ± 13	1080 ± 182	443 ± 64	19 ± 5	2660 ± 193	73 ± 29
N/OFQ 0.1 nmol	131 ± 10	863 ± 135	443 ± 61	14 ± 5	2774 ± 219	28 ± 17
N/OFQ 1 nmol	147 ± 11	702 ± 175	526 ± 67	65 ± 22	2077 ± 315	449 ± 132
N/OFQ 10 nmol	36 ± 13	2352 ± 386	58 ± 26	70 ± 22	1230 ± 279	394 ± 174
Saline	94 ± 12	1220 ± 182	348 ± 66	18 ± 5	2793 ± 187	60 ± 21
PWT1-N/OFQ 2.5 pmol	174 ± 19	597 ± 174	611 ± 76	79 ± 26	1962 ± 289	293 ± 86
PWT1-N/OFQ 25 pmol	191 ± 14	224 ± 47	538 ± 71	120 ± 18	956 ± 262	433 ± 93
PWT1-N/OFQ 250 pmol	109 ± 17	1113 ± 222	222 ± 60	24 ± 10	2775 ± 271	51 ± 28
Saline	130 ± 11	869 ± 156	364 ± 47	33 ± 10	2367 ± 274	133 ± 42
PWT2-N/OFQ 2.5 pmol	145 ± 15	755 ± 182	367 ± 74	65 ± 22	1794 ± 457	222 ± 86
PWT2-N/OFQ 25 pmol	237 ± 24	210 ± 30	455 ± 93	168 ± 33	635 ± 184	347 ± 116
PWT2-N/OFQ 250 pmol	81 ± 12	1131 ± 178	43 ± 8	1 ± 1	3414 ± 158	3 ± 3
Saline	119 ± 15	899 ± 189	509 ± 75	49 ± 14	1908 ± 382	296 ± 95
PWT3-N/OFQ 2.5 pmol	146 ± 22	913 ± 223	490 ± 91	41 ± 19	2461 ± 357	172 ± 88
PWT3-N/OFQ 25 pmol	147 ± 21	511 ± 196	310 ± 45	107 ± 18	817 ± 343	339 ± 73
PWT3-N/OFQ 250 pmol	98 ± 17	1083 ± 254	87 ± 20	24 ± 22	2997 ± 349	15 ± 15

Dose–response curves for N/OFQ and its PWT derivatives. Animal locomotor behaviour was measured for 2 h. Data are mean SEM of 12 animals for each point. Statistically significant results, $P < 0.05$ versus saline according to one-way ANOVA followed by the Dunnett's test for multiple comparisons, were coloured red indicating inhibitory and green, stimulatory effects.

In functional assays performed on the human recombinant NOP receptor ([^{35}S]-GTPγS binding and calcium mobilization) and at native NOP receptors expressed in the mVD, PWT compounds mimicked the actions of N/OFQ, producing similar maximal effects. Thus, PWT compounds always behaved as full agonists of NOP receptors. This applied to PWT1-N/OFQ, PWT2-N/OFQ and PWT3-N/OFQ and, therefore, we propose that the different cores used for generating these compounds have negligible, if any, effect on the ability of the peptide sequence to adopt the biologically active conformation responsible for full agonist pharmacological activity.

As far as potency is concerned, the following rank order of potency was obtained in the [^{35}S]-GTPγS binding assay and mVD assay PWT1-N/OFQ = PWT2-N/OFQ = PWT3-N/OFQ > N/OFQ. Similar findings were also obtained in previous studies performed using the vas deferens taken from CD-1 mice (Guerrini *et al.*, 2014). These results are in line with the increased affinity displayed by PWT compounds in binding studies. However, the opposite result was obtained in calcium mobilization studies, where N/OFQ was shown to be more potent than these PWT compounds. Since the rank order of agonist potency can be considered as a receptor fingerprint, this discrepant result deserves attention. The calcium mobilization assay is based on the aberrant signalling generated by the chimeric $G\alpha_{qi5}$ protein that forces the NOP receptor to signal via the calcium pathway. Therefore, it is possible that the aberrant signalling may induce modifications of the pharmacological profile of the receptor. However, the following considerations argue against this proposal. In a previous study, a large panel of NOP receptor ligands, including full and partial agonists as well as antagonists, were evaluated in the calcium assay, and the results obtained were the same as those from classical G_i-based assays and tissue studies (Camarda *et al.*, 2009). Moreover, it is reasonable to assume that N/OFQ and PWT compounds interact with the binding pocket of the NOP receptor [whose three-dimensional (3D) structure is now available (Thompson *et al.*, 2012)] with the same chemical structures, that is, the N/OFQ N terminal tetrapeptide FGGF (Calo and Guerrini, 2013). Thus, other reasons may account for the discrepant result. In the present study, the PWT compounds had a slow kinetic of action in the mouse vas deferens compared with N/OFQ. Camarda *et al.* (2009) showed that the calcium mobilization assay tends to underestimate the potency of agonists characterized by a slow interaction with the NOP receptor such as the peptides UFP-112 (Rizzi *et al.*, 2007), UFP-113 (Arduin *et al.*, 2007) and ZP120 (Rizzi *et al.*, 2002), and the non-peptide Ro 64-6198 (Rizzi *et al.*, 2001). This phenomenon may derive from the non-equilibrium conditions that characterize the calcium assay. In fact, the relatively long time needed to obtain full activation of the NOP receptor by slowly equilibrating agonists is not compatible with the rapid and transient nature of the calcium response. It is likely that this may account for the underestimation of the potency of N/OFQ PWT derivatives in this assay. Interestingly, Charlton and

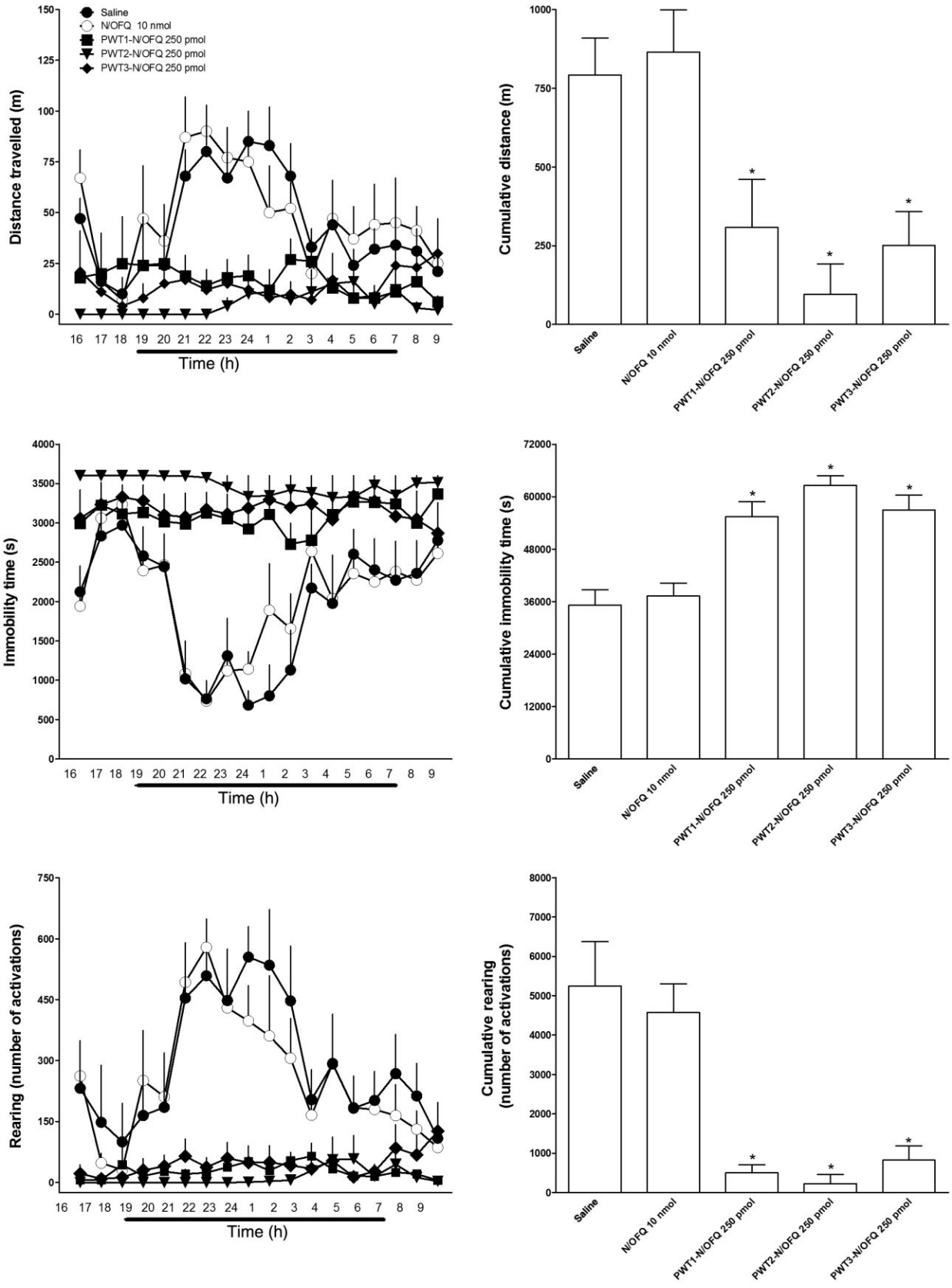


Figure 5

Effects of 10 nmol N/OFQ and 250 pmol of its PWT derivatives on mouse locomotor activity in overnight experiments. Results are shown as time course in the left panels and as cumulative effects in the right panels. Data are mean ± SEM of 12 animals for each point. *$P < 0.05$ according to one-way ANOVA followed by the Dunnett's test for multiple comparisons. The horizontal bar indicates the period in which the light was turned off.

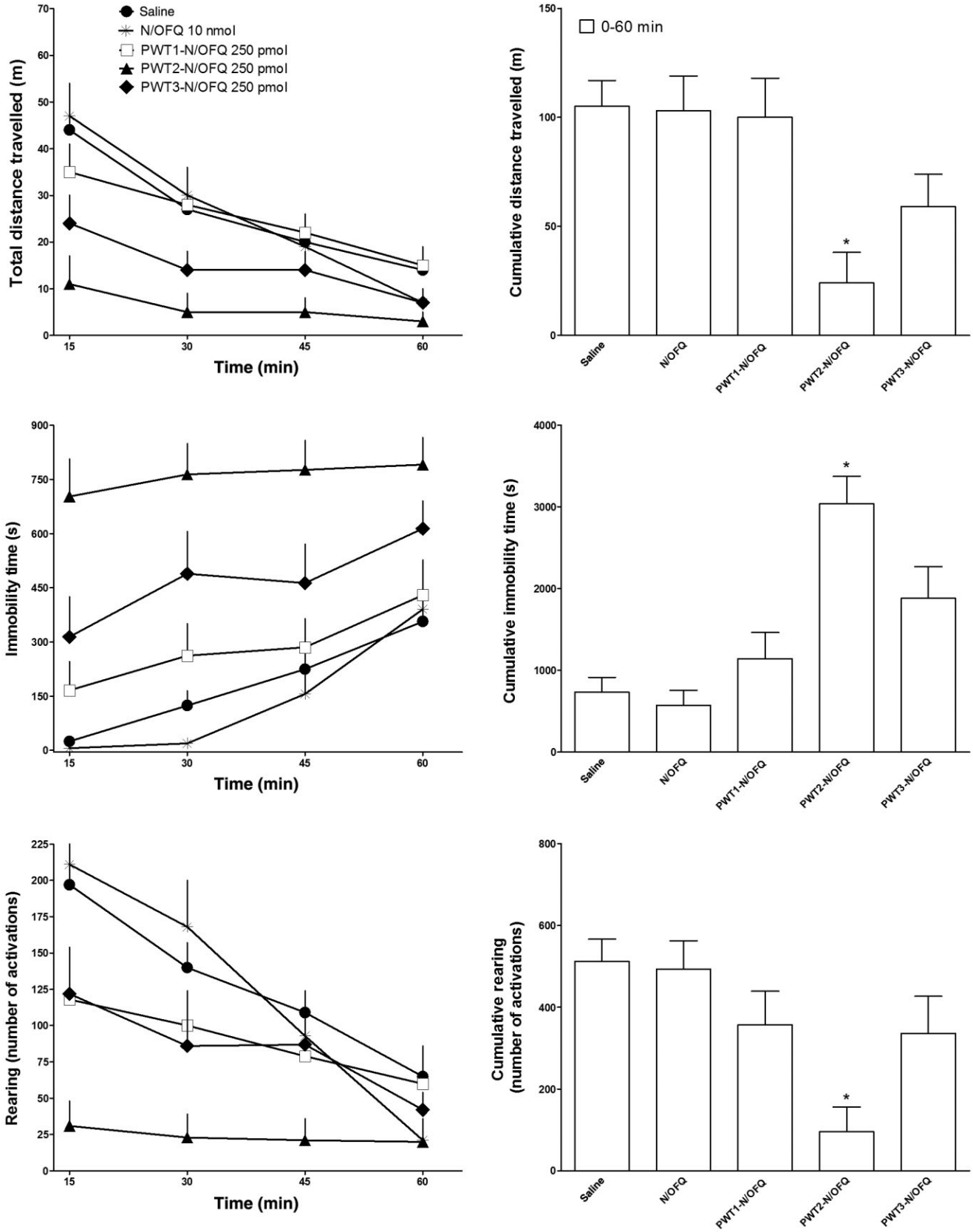


Figure 6

Effects of 10 nmol N/OFQ and 250 pmol of its PWT derivatives on mouse locomotor activity 24 h after their injection. Results are shown as time course in the left panels and as cumulative effects in the right panels. Data are mean ± SEM of 12 animals for each point. *$P < 0.05$ according to one-way ANOVA followed by the Dunnett's test for multiple comparisons.

Table 4

Effects of 250 pmol PWT2-N/OFQ on locomotor activity of NOP(+/+) and NOP(–/–) mice

	NOP(+/+)		NOP(–/–)	
	Saline	PWT2-N/OFQ	Saline	PWT2-N/OFQ
Distance travelled (m)	230 ± 26	104 ± 19*	203 ± 26	170 ± 10
Immobility time (s)	1805 ± 378	3305 ± 390*	2372 ± 525	2558 ± 454
Rearing (*n*)	911 ± 175	96 ± 19*	625 ± 181	663 ± 135

*$P < 0.05$ versus saline, according to the Student's *t*-test for unpaired data. Animal locomotor behaviour was measured for 2 h. Data are mean SEM of 12 animals for each point.

Vauquelin (2010) investigated this hypothesis in two different experimental systems modelled to mimic the [^{35}S]-GTPγS binding and the calcium assay with two different agonists: a high affinity slowly associating ligand (L1) and a lower affinity fast onset ligand (L2). The simulation displayed an opposite rank order of potency of agonists in the two assays with L1 > L2 in the system mimicking the [^{35}S]-GTPγS assay and L2 > L1 in the system mimicking the calcium assay (for details see figure 4 in Charlton and Vauquelin, 2010). Thus, the results of this simulation perfectly match our experimental data obtained with N/OFQ and its PWT derivatives; this suggests that the reversal of the rank order of agonist potency measured in the calcium assay is likely to derive from kinetic artefacts.

As far as receptor selectivity is concerned, receptor binding and calcium mobilization studies demonstrated that the high selectivity of N/OFQ over classical opioid receptors is maintained by PWT derivatives. The molecular basis for the selectivity of action of NOP receptor ligands over classical opioid receptors has been recently documented by comparing the 3D crystal structure of these receptors (Filizola and Devi, 2013). In the NOP receptor binding pocket, the N-terminal tetrapeptide of the N/OFQ-related peptide UFP-101 (Nphe-Gly-Gly-Phe) make the same hydrophobic interactions as the aromatic rings of C-24 (the ligand used to generate the NOP receptor crystal), and the N-terminal amino group forms a salt bridge with Asp^{130}, thus supporting a close similarity in the binding poses between small molecules and peptides (Thompson *et al.*, 2012). Although this information is relative to the inactive form of these receptors (i.e. in complex with antagonists), most likely similar mechanisms regulate the interaction of N/OFQ with the NOP receptor and its selectivity over classical opioid receptor as discussed in detail in Calo and Guerrini (2013). These same molecular mechanisms are not affected by applying the PWT to the N/OFQ sequence, as tetrabranched derivatives of N/OFQ displayed similar selectivity over classical opioid receptor as N/OFQ itself.

Quite different results were obtained by comparing the action of N/OFQ and of its PWT derivatives in tissues taken from NOP(+/+) and NOP(–/–) mice. The inhibitory effect exerted by N/OFQ in the electrically-stimulated vas deferens of NOP(+/+) mice was no longer evident in tissues taken from NOP(–/–) animals. This is in line with previous findings obtained in NOP(–/–) mice (Carra *et al.*, 2005; Rizzi *et al.*, 2007) and recently confirmed in NOP(–/–) rats (Rizzi *et al.*, 2011). In parallel experiments, PWT derivatives of N/OFQ were still active in NOP(–/–) tissues, although their potency was reduced from 20 (PWT1-N/OFQ) to more than 100-fold (PWT2-N/OFQ and PWT3-N/OFQ). Thus, the absolute selectivity of N/OFQ seems to be affected by the application of the PWT, and tetrabranched peptides, at relatively high concentrations, are able to interact with an undefined receptor in this preparation to inhibit the twitch response. Interestingly, the loss of selectivity of tetrabrached peptides is moderate for PWT1-N/OFQ and modest for PWT2-N/OFQ and PWT3-N/OFQ; thus, it seems that the PWT2 and PWT3 cores are superior to PWT1 in maintaining the selectivity of action of the natural sequence. The partial loss of selectivity displayed by PWT derivatives in the mVD should not be overemphasized. In fact, in this preparation, the selective NOP receptor antagonist SB-612111 (100 nM) produced a similar rightward shift of the concentration–response curves to N/OFQ and to PWT compounds indicating that, at least for relatively low concentrations, the effects of tetrabranched peptides were exclusively due to activation of NOP receptors (Guerrini *et al.*, 2014). Moreover, in the mVD, off-target effects were reported for different non-peptide NOP receptor agonists. For instance, Ro 64-6198 (Jenck *et al.*, 2000), the most extensively published non-peptide NOP receptor agonist (Shoblock, 2007), mimicked N/OFQ inhibitory effect in the mVD. However, the effects of Ro 64-6198 could not be antagonized by selective NOP receptor antagonists even in the presence of naloxone (Rizzi *et al.*, 2001). Other molecules such as Ro 65-6570 (Wichmann *et al.*, 2000) or SCH 221510 (Varty *et al.*, 2008) were also able to inhibit the twitch response of the mVD; however, their effects were not sensitive to the NOP receptor antagonist J-113397 and their concentration–response curves in NOP(+/+) and in NOP(–/–) tissues were the same (Molinari *et al.*, 2012).

Collectively, the results from the *in vitro* studies demonstrated that PWT derivatives of N/OFQ behave as potent full agonists at human recombinant and animal native NOP receptors and display a selectivity profile for the NOP receptor, which is inferior to that of N/OFQ but clearly superior to that of the available non-peptide NOP receptor agonists. Thus, we further characterized the effects of PWT derivatives of N/OFQ *in vivo* in the mouse LA assay.

In the original article reporting its identification, N/OFQ was shown to inhibit LA after supraspinal administration (Reinscheid *et al.*, 1995). This was later confirmed in mice and rats in different laboratories (Lambert, 2008). However, some studies have reported biphasic effects of N/OFQ on LA with

low doses producing stimulation and high doses producing inhibition (Florin *et al.*, 1996). The exclusive involvement of the NOP receptor in this action of N/OFQ has been firmly demonstrated in both receptor antagonism and knockout studies (Calo and Guerrini, 2013). In the present study, in the first series of experiments we performed dose–response curves to N/OFQ and to its PWT derivatives by measuring the animal behaviour for 120 min. N/OFQ produced biphasic effects depending upon the dose and time. In particular, relatively low doses of peptide produced stimulatory effects, whereas high doses produced robust inhibitory effects during the first hour after administration and stimulatory effects during the second hour. A similar pattern of effects was measured in response to the supraspinal administration of PWT derivatives of N/OFQ, with the following major differences: agonist potency and onset and duration of action. The biphasic effect of N/OFQ on LA associated with the very different onset and duration of action of the natural peptide versus PWT derivatives makes the comparison of the dose–response curves and the calculation of agonist potency quite difficult. However, the profound inhibition of locomotor behaviour obtained immediately after the injection of 10 nmol of N/OFQ and after 2 h (PWT2-N/OFQ) or more (PWT1-N/OFQ and PWT3-N/OFQ) from the injection of PWT compounds suggests an approximate dose-ratio of 40. This increase in agonist potency is larger than that observed in *in vitro* studies. This may possibly derive from the lower susceptibility to peptidase action reported for multibranched peptides compared with the free peptide sequence. In fact, Bracci *et al.* (2003) demonstrated that N/OFQ was degraded within 2 h of incubation with serum or rat brain membranes, whereas a tetrabranched derivative of N/OFQ was still detectable after 24 h. Interestingly, a structure-based hypothesis of branched peptide resistance to proteolysis has been proposed previously (Falciani *et al.*, 2007b). Since peptide metabolism is likely to be more relevant *in vivo* than *in vitro*, it is reasonable to assume that the lower susceptibility to peptidase action contributes to the increased agonist potency displayed by these compounds *in vivo*. Another major difference between N/OFQ and PWT-N/OFQ action is the kinetic of action. The onset of PWT-N/OFQ action is delayed compared with that of N/OFQ and the duration of action seems to be longer. In fact, the inhibitory effects exerted by PWT compounds at the higher dose tested were more pronounced during the second than the first hour after injection. Interestingly, these *in vivo* differences parallel the kinetic of action of these compounds *in vitro* in the mVD where they mimicked N/OFQ actions but showed slower kinetic and wash-resistant effects. To further investigate the duration of action of PWT derivatives of N/OFQ, an overnight experiment was performed where the effects of equieffective doses of N/OFQ and PWT derivatives were compared. The results of this experiment clearly demonstrate that N/OFQ produces short lasting effects, whereas those elicited by PWT derivatives lasted for the whole period of observation. To further investigate possible differences in the duration of action of PWT derivatives, animals were injected with the same doses and their LA was measured 24 h after injection. Mice treated with N/OFQ and PWT1-N/OFQ displayed a locomotor behaviour similar to that of saline-treated animal, whereas mice treated with PWT3-N/OFQ displayed reduced LA, although the differences did not reach a statistical level of significance. In contrast, even after 24 h from the injection, mice treated with PWT2-N/OFQ displayed a statistically significant reduction in the horizontal and vertical activities and increase in immobility time.

Collectively, these *in vivo* studies demonstrated that PWT compounds mimic the effects of N/OFQ on LA, but with higher potency and much longer duration of action. In particular, PWT2-N/OFQ was found to be the PWT derivative showing the longest duration of action. Thus, the PWT2 seems to be the best core in terms both of retaining the selectivity of action of the native peptide sequence and of increasing, by several fold, the *in vivo* duration of action.

To investigate the involvement of the NOP receptor in the *in vivo* action of PWT2-N/OFQ, the effect of this compound on LA was assessed in NOP(+/+) and NOP(−/−) mice. PWT2-N/OFQ 250 pmol induced a robust inhibitory effect on LA of NOP(+/+) mice while being completely inactive in NOP(−/−) animals. This result clearly demonstrates that the action of PWT2-N/OFQ on LA, similar to what has previously been reported for N/OFQ (Nishi *et al.*, 1997; Carra *et al.*, 2005), is exclusively due to stimulation of NOP receptors.

Collectively, the results from the *in vitro* and *in vivo* studies demonstrate that PWT2-N/OFQ is a full agonist of the NOP receptor characterized by high potency, good selectivity and remarkable *in vivo* duration of action. Among available ligands, the most potent and selective agonist for the NOP receptor is the peptide UFP-112 ([(pF)Phe^{4}Aib^{7}Arg^{14}Lys^{15}]N/OFQ-NH_2), which was designed using a combination of several chemical modifications that increase NOP receptor affinity/potency and/or reduce susceptibility to enzymatic degradation (Rizzi *et al.*, 2007; Calo *et al.*, 2011). Compared with UFP-112, PWT2-N/OFQ displays slightly lower potency and selectivity of action. However, the duration of action of PWT2-N/OFQ is longer than that of UFP-112. In fact, the inhibitory effects of UFP-112 on LA lasted for about 6 h (Rizzi *et al.*, 2007), whereas those elicited by PWT2-N/OFQ were still evident after 24 h from injection.

In conclusion, the present study showed that the PWT can be successfully applied to the peptide sequence of N/OFQ to generate tetrabranched derivatives characterized by a pharmacological profile similar to the native peptide, but associated with a higher potency and a marked prolongation of action *in vivo*. The compound PWT2-N/OFQ displayed good selectivity and the longest duration of action *in vivo*. This NOP receptor ligand is therefore proposed as a novel research tool particularly to investigate those conditions in which a prolonged activation of the NOP receptor may evoke beneficial effects. More generally, the PWT strategy may be easily used to generate innovative and interesting ligands for peptide GPCRs. Using the PWT2 core, we are planning to generate and study several PWT derivatives obtained with different peptide sequences characterized by diverse pharmacological activity (antagonist, partial and full agonists). The information coming from these studies will allow us to firmly establish the value of the PWT strategy for the generation of innovative ligands for peptide receptors.

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Author contributions

A. R., D. J. R., D. G. L. and G. C. conceived and designed the experiments. A. R., D. M., M. C. C., C. R. and M. F. B. performed the experiments. A. R., D. M. and M. F. B. analysed the data. E. M., S. S. and R. G. contributed reagents/materials/analysis tools. A. R., R. G., D. G. L. and G. C. wrote the manuscript.

Conflict of interest

G. C., S. S. and R. G. are inventors of the patent application (EP13162532.9) focused on PWT and are founders of the University of Ferrara spin off company UFPeptides s.r.l., the assignee of such patent application.

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