Functional heterogeneity of nociceptin/orphanin FQ receptors revealed by (+)-5a Compound and Ro 64-6198 in rat periaqueductal grey slices

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Abstract

The nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor is a non-opioid branch of the opioid receptor family implicated in several neurological and psychological disorders, such as pain, anxiety, depression, involuntary movement, addiction, seizure and dementia. Heterogeneity of NOP receptors has been proposed based on the findings of splicing variants and from binding and functional studies. We have previously reported that Ro 64-6198, a NOP receptor agonist, activated a subset, but not all, of N/OFQ-sensitive NOP receptors in midbrain ventrolateral periaqueductal grey (vlPAG). In this study, we found that a new NOP receptor ligand, (+)-5a Compound ((3aS,6aR)-1-(cis-4-isopropylcyclohexyl)-5-methyl-2-phenylethahydropirro[piperidine-4,1'-pyrrolo[3, 4-c]pyrrole]), also activated a subset of NOP receptors in vlPAG neurons. (+)-5a Compound (0.1–30 μM) concentration-dependently activated G-protein-coupled inwardly-rectifying potassium (GIRK) channels mediated through the NOP receptors in about 35% of the recorded vlPAG neurons. (+)-5a Compound (EC50 : 605 nM) was less potent (1/12) and efficacious (47%) than N/OFQ. In (+)-5a Compound-insensitive neurons, Ro 64-6198 was also ineffective, and vice versa, but N/OFQ activated GIRK channels through NOP receptors. In (+)-5a Compound-sensitive neurons, (+)-5a Compound precluded the effect of Ro 64-6198. Immunofluorescent and morphometric studies showed that most of the (+)-5a Compound-sensitive neurons were multipolar with intensive dendritic arborization and immunoreactive to glutamic acid decarboxylase-67. It is suggested that (+)-5a Compound activates a subset of NOP receptors, similar to the Ro 64-6198-sensitive subset, in the vlPAG neurons which are mostly GABAergic. These results further support the presence of functional heterogeneity of NOP receptors in the midbrain PAG.

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Introduction

An orphan G-protein-coupled receptor (GPCR) was cloned and named initially as ORL1 (opioid receptor-like receptor), because, although having a structure highly homologous to classical opioid receptors, it displayed little affinity for opioids (Mollereau et al. 1994). This receptor was de-orphanized through identifying its endogenous ligand, a heptadecapeptide named nociceptin (Meunier et al. 1995) or orphanin FQ (Reinscheid et al. 1995), and renamed as nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor. It is classified as a non-opioid
branch of the opioid receptor family (NC-IUPHAR, 2004). N/OFQ and NOP receptors are widely distributed in the brain and involved in many neurological functions or disorders, including pain, anxiety, depression, addiction, feeding, learning and memory, motor control and central cardiovascular regulation (Chiou et al. 2007; Lambot, 2008). N/OFQ also regulates neurotransmitter release in the autonomic nervous system and plays a role peripherally in the pathogenesis of asthma, urine retention or cardiovascular function (Chiou et al. 2007; Lambot, 2008). Among these, the anxiolytic effect of N/OFQ (Jenck et al. 1997) or Ro 64-6198 (Wichmann et al. 2000), a non-peptide NOP receptor agonist, is noteworthy. Recently, several newly developed non-peptide NOP receptor agonists also showed anxiolytic effect in various animal models (Hirao et al. 2008; Varty et al. 2008). Unlike benzodiazepines, the NOP receptor agonist did not affect ethanol-induced hypnosis (Hirao et al. 2008). Recently, Ro 64-6198 was shown to have analgesic effects in monkeys at very low systemic doses similar to a reference opioid, alfentanil, but without any of the side-effects of opioids, such as respiratory depression or abuse liability (Ko et al. 2009). Given these potential clinical applications, it is important to characterize the pharmacological profiles of non-peptide NOP receptor ligands and to understand their mode of action.

Ro 64-6198 is the first non-peptide NOP receptor agonist with good brain permeability (Wichmann et al. 2000) and has been used in several studies to reveal the functional roles and nature of NOP receptors (Ko et al. 2009; Shoblock, 2007). Interestingly, Ro 64-6198, although having similar binding affinity and efficacy as N/OFQ at cloned NOP receptors, mimicked some, but not all, of the effects of N/OFQ in several functional studies (Chiou et al. 2007; Goeldner et al. 2008; Reiss et al. 2008; Shoblock, 2007). Moreover, the NOP receptor transcript was found to have splicing variants although no coding product was reported (Peluso et al. 1998). Mathis et al. (1999, 1997), also reported that there are two (high and low) specific binding sites of N/OFQ in the rodent brain. These findings suggest the possibility of NOP receptor heterogeneity.

We have previously found that Ro 64-6198 activated only a subset of the N/OFQ-sensitive NOP receptors in ventrolateral periaqueductal grey (vPAG) neurons in isolated rat midbrain slices (Chiou et al. 2004). (+)-5a Compound ((3a,5,6aR)-1-(cis-4-isopropylcyclohexyl)-5’-methyl-2’-phenylhexahydropipera
dine-4,1’-pyrrolo[3,4-c]pyrrolole) is a non-peptide ligand of NOP receptors with a structure backbone analogous to Ro 64-6198 (Kolczewski et al. 2003). It acts as a full agonist of NOP receptors and is 1.7- and 25-fold less potent than N/OFQ in expressed hNOP receptor-mediated GTP(Y)S binding and cAMP reduction, respectively (Kolczewski et al. 2003). Importantly, (+)-5a Compound is more selective for NOP receptors over other opioid receptors than Ro 64-6198, with overall >1000-fold binding selectivity (Kolczewski et al. 2003).

In this study, using the same midbrain slice preparations, we have quantitatively characterized the pharmacological profile of (+)-5a Compound at native NOP receptors of vPAG neurons, and compared its effect with N/OFQ and Ro 64-6198. The midbrain PAG was chosen because it is one of the brain regions with dense distribution of NOP receptors (Anton et al. 1996; Neal et al. 1999). Activation of NOP receptors has been reported to result in activation of G protein-coupled inwardly rectifying K+ (GIRK) channels, inhibition of Ca2+ channels and reduction of cAMP (Henderson & McKnight, 1997; Mogil & Pasternak, 2001). Through NOP receptor-mediated GIRK channel activation, N/OFQ can reduce neuronal excitability via membrane hyperpolarization in several brain regions, including PAG (Liao et al. 2009; Vaughan et al. 1997). We, therefore, used the magnitude of GIRK current as a functional assay for NOP receptor activity in vPAG neurons. The effect of (+)-5a Compound and its interactions with N/OFQ and Ro 64-6198 were examined and compared in Ro 64-6198-sensitive and -insensitive neurons.

Methods

Brain slice preparations

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Taiwan University. All efforts were made to minimize the number of animals used. The dissection of PAG slices, electrophysiological recordings and data analyses were similar to our previous study (Liao et al. 2009).

The midbrain blocks containing PAG were rapidly dissected from Wistar rats (postnatal days 9–18). Coronal slices (400 μm) were then sectioned with a vibrotome (Microslicer DTK-100, Japan) and equilibrated at room temperature in artificial cerebral spinal fluid (aCSF). The aCSF consisted of (mM): 117 NaCl, 4.5 KCl, 2.5 CaCl2, 1.2 MgCl2, 1.2 NaH2PO4, 25 NaHCO3 and 11.4 dextrose (pH 7.4), and was oxygenated with 95% O2/5% CO2. After equilibration for at least 1 h, one slice was mounted on a submerged recording chamber and continuously perfused with aCSF at 30 °C at a rate of 2–3 ml/min.
Blind patch-clamp whole-cell recording was performed with 4–8 MΩ glass microelectrodes filled with internal solution consisting of (m M): 125 K+ gluconate, 5 KCl, 0.5 CaCl₂, 5 BAPTA, 10 Hepes, 5 MgATP and 0.33 GTP Tris (pH 7.3). In order to elucidate if (+)-5a Compound, similarly to N/OFQ, also induced the NOP receptor-mediated GIRK current, a hyperpolarization voltage ramp protocol was applied. After whole-cell configuration was formed, the potential of the recorded neuron was held at -70 mV, stepped to -60 mV for 100 ms, ramped from -60 mV to -140 mV for 400 ms, and then stepped back to -70 mV (Fig. 1, inset). The membrane currents elicited by voltage ramps were acquired through an Axopatch 200B amplifier (Molecular Devices/Axon Instruments, USA) with pClamp 7 software (Molecular Devices/Axon Instruments) and simultaneously recorded with a chart recorder (Gould RS3200) to monitor the time-course of drug effects. The access resistance (10–15 MΩ) was monitored during the recording period. Only those neurons with unchanged access resistance before and after drug treatments were accepted to ensure that the clamp efficiency had not deteriorated during the recording period.

Functional heterogeneity of NOP receptors

The effect of the tested NOP receptor agonist was quantified by the percent increment of the membrane current at -140 mV (I_{-140}) taking its own I_{-140} before treatment as 100%. An I_{-140} increment >5% and the induced current having a reversal potential at around -90 mV (the equilibrium potential of K+ ions) was considered to be effective. For establishing the concentration–response curve of (+)-5a Compound, the percent increment of I_{-140} in each neuron was normalized to the maximal effect (E_{max}) produced by 1 μM N/OFQ, which was 39.4 ± 4% increment (n = 26) (Chiou et al. 2002). To quantitatively evaluate the antagonistic effect of various receptor antagonists, their interactions with (+)-5a Compound were examined in the same neuron. Given that not all neurons were sensitive to (+)-5a Compound (see Results section), it was not practical to pre-apply the intended antagonist. Therefore, the tested receptor antagonist was applied to (+)-5a Compound-sensitive neurons after the response of (+)-5a Compound had reached a steady state, which usually took 20–25 min. The response of the tested antagonist was continuously monitored thereafter. In the study verifying whether NOP receptor-mediated GIRK channels were functional in those (+)-5a Compound-unresponsive neurons, N/OFQ was examined after (+)-5a Compound had been
applied for at least 20 min. In this set of experiments, Ro 64-6198 was tested in some (+)-5a Compound-unresponsive neurons before N/OFOQ was added in order to verify if the neurons unresponsive to (+)-5a Compound were also insensitive to Ro 64-6198.

**Immunofluorescence staining**

For immunofluorescence studies, 0.2% Lucifer Yellow was added to the internal solution. After recording, the slice containing the recorded neuron which had been filled with Lucifer Yellow was fixed, re-sectioned and subjected to an immunofluorescent staining of glutamic acid decarboxylase-67 (GAD67), a synthesizing enzyme of GABA (Erlander et al. 1991). Briefly, after recording, the slices were fixed with 4% paraformaldehyde at 4°C for 1 d, and then dehydrated in 30% sucrose. Dehydrated slices were embedded and re-sectioned in 50-μm sections with a cryostat microtome (Leica CM3050S, Leica Microsystems, Germany). Slice sections were rinsed and washed with phosphate-buffered saline (PBS) three times, followed by 0.3% Triton X-100 containing PBS (PBST) plus 0.5% bovine serum albumin (BSA) and then blocked in PBST containing 1% BSA and 10% normal goat serum (NGS) for 1 h. Then, slice sections were incubated with the mouse monoclonal antibody against GAD67 (diluted 1:1000) (Chemicon, USA) in PBST containing 1% BSA overnight at 4°C. Slice sections were then washed with PBST three times, followed by secondary antibody, Alexa 594 (diluted 1:100) (Invitrogen, USA) for 1 h. Fluorescent images were acquired with Zeiss LSM 510 Meta confocal microscope (Zeiss, USA) and edited with LSM 510 software (Zeiss).

**Morphometric analysis**

To compare the morphology of (+)-5a Compound-sensitive and -insensitive neurons, the recorded neurons were reconstructed and analysed. In brief, the recorded neuron was filled with biocytin (1%), which had been added in the internal solution during recording. After recording, slices were fixed with 4% paraformaldehyde at 4°C for 1 d. After several washes with PBS, sections were treated with 1% H2O2 diluted in PBS for 3 h, then incubated with 1% Triton X-100 containing PBS plus 10% NGS and 2% BSA overnight at room temperature. Biocytin-filled neurons were then revealed using a avidin-biotin-peroxidase complex (ABC) kit (PK-4000, Vector Laboratories, USA), followed by a 3,3’-diaminobenzidine (DAB) kit (SK-4100, Vector Laboratories). The DAB staining was visualized and processed by a 3,3’-diaminobenzidine (DAB) kit (SK-4100, Vector Laboratories). The image of the identified neuron was captured under an Olympus BX51 microscope (Olympus, Japan) equipped with a CIX9000 CCD camera (MicroBrightField, USA), and reconstructed three-dimensionally using Neurolucida (MicroBrightField). Morphometric analysis of the recorded neurons, including the soma area and dendritic arborization, was performed by Neurolucida Explorer. The dendritic complexity was analysed by Sholl concentric ring analysis.

**Statistics**

Data are expressed as the mean ± S.E.M with the number of neurons tested. Usually one neuron was recorded in each slice and 3–4 slices were taken from one rat. Student’s t test, one-way ANOVA and χ² test were used for statistical analysis and a p value <0.05 was considered to be significant.

**Results**

(+)-5a Compound and Ro 64-6198 were synthesized as reported previously (Jenck et al. 2000; Kolczewski et al. 2003). UFP-101 was a generous gift from Dr Calò and Dr Guerrini (University of Ferrara, Italy). N/OFOQ and AF-DX-116 were purchased from Tocris (UK) and naloxone was purchased from Sigma (USA). All drugs were dissolved in de-ionized water except (+)-5a Compound and Ro 64-9198, which were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was kept below 0.1%, which did not affect the membrane currents elicited by voltage ramps (Chiou et al. 2004).

(+)-5a Compound activates GIRK channels in vlPAG neurons

(+)-5a Compound (10 µM) shifted the holding current (ihold in Fig. 1a) outwardly and increased the membrane current elicited by a hyperpolarization ramp command from −60 to −140 mV in vlPAG neurons (Fig. 1a). Figure 1b shows the current-voltage (I-V) curves of the membrane currents in a vlPAG neuron before and after treatment with 10 µM (+)-5a Compound, and after washout. The current induced by (+)-5a Compound, obtained by subtracting the current in the control from that in the presence of (+)-5a Compound, displayed inward rectification (Fig. 1c). The mean reversal potential of (+)-5a Compound was −58 ± 2 mV. However, application of Ro 64-6198 was tested in some (+)-5a Compound-unresponsive neurons before N/OFOQ was added in order to verify if the neurons unresponsive to (+)-5a Compound were also insensitive to Ro 64-6198.
Compound-elicited current was $-90.5 \pm 1.3 \text{ mV (n = 49)}$, resembling the equilibrium potential of $K^+$ ions estimated by the Nernst equation. Therefore, the $K^+$ channel in vPAG neurons activated by $\text{(+-)5a Compound}$, as by N/OFQ (Chiou et al. 2002), is the GIRK channel, an inwardly rectifying $K^+$ channel that is coupled to G proteins (Ikeda et al. 1997).

*(+-)5a Compound is less potent and less efficacious than N/OFQ*

The effect of $\text{(+-)5a Compound}$ on GIRK channels was concentration-dependent (0.1–30 $\mu M$). To establish its concentration–response curve (Fig. 2a), the magnitude of GIRK channel activation induced by $\text{(+-)5a Compound}$ was quantified from the percent increment of $I_{-140}$ obtained in each neuron as described in the Methods section and then, normalized to the maximal increment (39 ± 4% increment, $n = 26$) produced by 1 $\mu M$ N/OFQ (Chiou et al. 2002), expressed as the percentage of the maximal effect of N/OFQ. *(+-)5a Compound*, at 10 $\mu M$, produced a maximal effect of 47%, compared to that generated by N/OFQ (Fig. 2a).

For comparison, the concentration–response curves of N/OFQ and Ro 64-6198, taken from our previous studies (Chiou et al. 2002, 2004), are also shown in Fig. 2a. In the present study, 0.3 $\mu M$ N/OFQ was also tested and produced a similar increment (40.2 ± 5.4%, $n = 32$) of $I_{-140}$ (Fig. 2a) as it did previously (37.2 ± 5.3%, $n = 17$) (Chiou et al. 2002). The estimated EC$_{50}$ of *(+-)5a Compound* was 605 ± 2 nM, which is about 12 times higher than that of N/OFQ, i.e. 52 ± 6.8 nM (Chiou et al. 2002). The concentration–response current of *(+-)5a Compound* and Ro 64-6198 are similar (Fig. 2a) with $E_{\text{max}}$ values of 46.9 ± 4.6% and 61.5 ± 5.6% (Chiou et al. 2004), respectively.

*(+-)5a Compound activates GIRK channels in about half of the recorded neurons*

*(+-)5a Compound* (0.1–30 $\mu M$) activated GIRK channels in 49/92 (53%) recorded neurons and had no effect in the remaining 43 neurons. The responsiveness (>5% increment of $I_{-140}$) and the induced current having a reversal potential around $-90 \text{ mV}$ of a neuron was independent of its location; either at the superficial or deeper layer of the slice. The numbers of neurons sensitive to *(+-)5a Compound* or Ro 64-6198 over the total numbers of tested neurons under various concentrations are depicted in Fig. 2a. A scatter plot for the responses of all neurons tested with *(+-)5a Compound* showed that there was a sharp cutoff between *(+-)5a Compound*-sensitive and -insensitive neurons (Fig. 2b). Even at the highest tested concentration (30 $\mu M$), *(+-)5a Compound* was still ineffective in some neurons (Fig. 2), as observed previously with Ro 64-6198 (Chiou et al. 2004).
The effect of (+)-5a Compound was antagonized by NOP, but not opioid or M_2 muscarinic receptor antagonists

In (+)-5a Compound-sensitive neurons, the effect of (+)-5a Compound (0.1–30 μM) in 23/35 (66%) neurons recorded was antagonized by UFP-101 (Fig. 1a), which competitively antagonized the effect of N/OFQ in the same preparation (Chiou et al. 2005). However, in the remaining 12 neurons, the effects of (+)-5a Compound were not reduced by UFP-101. The effect of (+)-5a Compound was not affected by naloxone, a non-selective μ-opioid receptor antagonist. The I_{-140} increments after treatment with 10 μM (+)-5a Compound in the absence or presence of 1 μM naloxone were not significantly different (119.8 ± 2.5% vs. 120.3 ± 2.4%, n = 3).

Previous binding studies (Kolczewski et al. 2003) showed that (+)-5a Compound, at a higher concentration (10 μM), also had affinity at histamine H_3, muscarinic and α receptors, and sodium channels. It is unlikely that (+)-5a Compound activated GIRK channels through histamine H_3 receptors since there are few histamine H_3 receptors in PAG (Pillot et al. 2002), and activation of α receptors will block, not activate, K^+ channels (Zhang & Cuevas, 2005). Among the five subtypes (M1–M5) of muscarinic receptors, the M2 subtype exists in PAG and activation of M_2 muscarinic receptors also results in GIRK channel activation (Sanada et al. 2007). Therefore, we further examined if AF-DX-116, a selective antagonist of M_2 muscarinic receptors, would antagonize the effect of (+)-5a Compound. The result showed that AF-DX-116 did not alter the effect of (+)-5a Compound. After treatment with AF-DX-116 (3 μM), the increase in I_{-140} by 10 μM (+)-5a Compound was 99.9 ± 2.5% (n = 6) of that before treatment.

N/OFQ activates GIRK channels via NOP receptors in (+)-5a Compound-insensitive vlPAG neurons

In those (+)-5a Compound-insensitive neurons, we further tested the effect of N/OFQ to verify if their NOP receptors and GIRK channels were functional. In 41/43 neurons which were insensitive to the pretreatment of (+)-5a Compound, N/OFQ (0.3 μM) activated GIRK channels (Fig. 3) in a comparable magnitude as in naive neurons (135.1 ± 3.2%, n = 41 vs. 137.2 ± 5.3%, n = 17). The effects of N/OFQ in these neurons were antagonized by UFP-101 (Fig. 3). This result suggests that (+)-5a Compound is ineffective in a subset of NOP receptors of vlPAG neurons, at which N/OFQ displays similar efficacy as in all neurons. This characteristic of (+)-5a Compound is similar to that of Ro 64-6198 (Chiou et al. 2004). Therefore, we further investigated the interactions between (+)-5a Compound and Ro 64-6198 in the same neurons.
(+)-5a Compound-insensitive neurons are also insensitive to Ro 64-6198, and vice versa

In a neuron that was unaffected by (+)-5a Compound, Ro 64-6198 also failed to induce any membrane current change (Fig. 4a). The same result was found in eight other neurons tested. The \( I_{-140} \) values after treatment with 10 \( \mu \text{M} \) (+)-5a Compound were 99.7 \( \pm \) 0.5% \( (n=9) \) of the controls and were 98.2 \( \pm \) 1.0% \( (n=9) \) after further treatment with 10 \( \mu \text{M} \) Ro 64-6198. In these neurons, N/OFQ activated GIRK channels in a manner antagonized by UFP-101 (Fig. 4a–c). Conversely, in five neurons which were unresponsive to Ro 64-6198, further addition of (+)-5a Compound also had no effect (Fig. 4d). The \( I_{-140} \) values after treatment with 10 \( \mu \text{M} \) Ro 64-6198 were 100.6 \( \pm \) 0.6% \( (n=5) \) of controls, and were 99.6 \( \pm \) 0.6% \( (n=5) \) after further treatment with 10 \( \mu \text{M} \) (+)-5a Compound. In these neurons, N/OFQ activated GIRK channels (Fig. 4d) in a manner blocked by UFP-101 (data not shown).

(+)-5a Compound precludes the effect of Ro 64-6198

In (+)-5a Compound-sensitive neurons, which had been treated with the maximal effective concentration of (+)-5a Compound \( (10 \mu \text{M}) \), further addition of Ro 64-6198 \( (10 \mu \text{M}) \) did not cause any additional change in membrane currents elicited by voltage ramps (Fig. 5a) in all of the 11 neurons tested. The \( I_{-140} \) values after treatment with 10 \( \mu \text{M} \) (+)-5a Compound were 118.8 \( \pm \) 3% of controls \( (n=11) \), and were 116.8 \( \pm \) 4% \( (n=11) \) after further treatment with 10 \( \mu \text{M} \) Ro 64-6198. This result suggests that (+)-5a Compound precluded the effect of Ro 64-6198.

Fig. 4. N/OFQ activated G-protein-coupled inwardly rectifying K\(^{+}\) channels through NOP receptors in the neurons insensitive to (+)-5a Compound, which were also insensitive to Ro 64-6198, and vice versa. (a) The chart recording of membrane currents in a ventrolateral periaqueductal grey (vlPAG) neuron which was insensitive to 10 \( \mu \text{M} \) (+)-5a Compound and also insensitive to 10 \( \mu \text{M} \) Ro 64-6198, but responsive to 0.3 \( \mu \text{M} \) N/OFQ. The effect of N/OFQ was reversed by 1 \( \mu \text{M} \) UFP-101. (b) I–V curves of the neuron shown in panel (a) before (i) and after treatment with (+)-5a Compound (ii), Ro 64-6198 (iii), or N/OFQ (iv). (c) The I–V curve of N/OFQ-induced current \( [(iv) \rightarrow (i)] \). (d) The chart recording of membrane currents in a vlPAG neuron, which was insensitive to 10 \( \mu \text{M} \) Ro 64-6198 and also insensitive to 10 \( \mu \text{M} \) (+)-5a Compound, but responsive to 0.3 \( \mu \text{M} \) N/OFQ. The results were reproduced in another eight and four neurons, respectively, under the conditions in panels (a) and (d).
N/OFQ further enhances GIRK current in (+)-5a Compound-sensitive neurons

In contrast to Ro 64-6198, N/OFQ (0.3 μM) further enhanced GIRK current in the neurons which were responsive to (+)-5a Compound (Fig. 5b). After further treatment with 0.3 μM N/OFQ, the \( I_{\text{GIRK}} \) values in the presence of the maximal effective concentration (10 μM) of (+)-5a Compound increased from 119.6 ± 3.6% to 132.6 ± 5.9% of controls (n = 8). The latter response is comparable to that produced by 0.3 μM N/OFQ alone (137.2 ± 5.3%, n = 17). When N/OFQ was reduced to 50 nM, which is equi-effective to 10 μM of (+)-5a Compound, the increase in \( I_{\text{GIRK}} \) in 10 μM (+)-5a Compound-pretreated neurons was the same as in non-pretreated neurons (129.2 ± 2.8%, n = 4 vs. 129.6 ± 3.1%, n = 4). This suggests that even at the maximal effective concentration, (+)-5a Compound did not interact with the equi-effective concentration of N/OFQ.

Most of the (+)-5a Compound-sensitive neurons are GABAergic

To characterize the differences between (+)-5a Compound-sensitive and -insensitive neurons, their electrophysiological properties, morphometric features and neurotransmitter identity were compared. These results are presented in Table 1 and Fig. 6.

As shown on Table 1, the mean resting membrane potential, input resistance and capacitance of (+)-5a Compound-sensitive (n = 78) and -insensitive (n = 70) neurons were not significantly different.

![Fig. 5. N/OFQ, but not Ro 64-6198, further increased G-protein-coupled inwardly rectifying K⁺ (GIRK) currents in (+)-5a Compound-sensitive neurons. Ro 64-6198, 10 μM (a) failed to, but N/OFQ, 0.3 μM (b) did further increase GIRK currents in neurons which were responsive to a pretreatment with (+)-5a Compound at the maximal effective concentration (10 μM). The results were reproduced in another 10 and seven neurons, respectively, under the conditions in panels (a) and (d).](image-url)

### Table 1. Comparison in the electrophysiological and morphometric properties between (+)-5a-insensitive and -sensitive neurons

<table>
<thead>
<tr>
<th>Parameters</th>
<th>(+)-5a-insensitive</th>
<th>(+)-5a-sensitive</th>
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<tbody>
<tr>
<td>Membrane potential (mV)</td>
<td>−66.7 ± 0.4</td>
<td>−67.2 ± 0.5</td>
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<td>Input resistance (MΩ)</td>
<td>329 ± 27</td>
<td>346 ± 33</td>
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<td>Capacitance (pF)</td>
<td>59.7 ± 2.5</td>
<td>61.7 ± 2.9</td>
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<tr>
<td>Soma area (μm²)</td>
<td>235.4 ± 56.7</td>
<td>364.3 ± 61.6</td>
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<tr>
<td>Number of primary dendrites</td>
<td>3.0 ± 0.3</td>
<td>4.4 ± 0.5*</td>
</tr>
<tr>
<td>Number of branching nodes</td>
<td>2.8 ± 0.3</td>
<td>7.0 ± 1.1**</td>
</tr>
<tr>
<td>Number of terminal tips</td>
<td>5.8 ± 0.4</td>
<td>11.5 ± 1.1***</td>
</tr>
<tr>
<td>Highest order of dendrites</td>
<td>2.8 ± 0.3</td>
<td>3.9 ± 0.3*</td>
</tr>
<tr>
<td>Total dendritic length (μm)</td>
<td>1476.7 ± 147.2</td>
<td>1732.8 ± 211.2</td>
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</table>

Data are mean ± S.E.M.
The electrophysiological properties were taken from 70 (+)-5a-insensitive and 78 (+)-5a-sensitive neurons. The recorded neurons either sensitive (n = 8 from six rats) or insensitive (n = 6 from five rats) to (+)-5a Compound were traced by biocytin-conjugated DAB staining and their 3D morphology was reconstructed by Neurolucida and morphometrically analysed by Neurolucida Explorer.

* \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.005 \) vs. the (+)-5a-insensitive group (Student’s t test).

Morphometric analysis data showed that (+)-5a Compound-sensitive (n = 8 from six rats) and (+)-5a Compound-insensitive (n = 6 from five rats) neurons had comparable soma size and total dendritic length. (+)-5a Compound-sensitive neurons had more primary dendrites (Fig. 6a), resembling the triangular...
and multipolar cells described in the Golgi study (Beitz & Shepard, 1985). Moreover, compared to (+)-5a Compound-insensitive neurons, the dendrites of (+)-5a Compound-sensitive neurons had more branching nodes leading to greater dendritic orders and more terminal tips. Using Sholl analysis, these two types of neurons could also be differentiated from their dendritic complexity [Fig. 6a(iii)]. Neurons sensitive to (+)-5a Compound had more intersections in the Sholl concentric rings particularly in the proximal regions (<100 µm) [Fig. 6a(iii)], indicating that these neurons have more complicated dendritic arbors.

GABAergic neurons account for 50% of all the neurons in PAG (Mugnaini & Oertel, 1985) and most of them are interneurons (Reichling & Basbaum, 1990). We, therefore, examined whether the recorded neurons were GABAergic through a co-localization of GAD67 immunofluorescent reactivity with Lucifer Yellow, which had been filled in the recorded neuron through the recording electrode after the recording was completed. For those neurons which were sensitive to (+)-5a Compound, 31/40 (78%) were GABAergic [Fig. 6b(i)], and the remaining nine (22%) neurons were non-GABAergic, suggesting a higher proportion (31/40 vs. 9/40, p < 0.01, χ² test) of (+)-5a Compound-sensitive neurons are GABAergic. On the other hand, in 30 (+)-5a Compound-insensitive neurons examined, only 14 (47%) neurons were GABAergic [Fig. 6b(ii)] and the remaining 16 (53%) neurons were non-GABAergic [Fig. 6b(iii)].

**Discussion**

In the present study, we demonstrated that (+)-5a Compound, similarly to N/OFQ (Chiou et al. 2002, 2004), activated GIRK channels in vPAG neurons.
through NOP receptors. However, (+)-5a Compound was less potent and efficacious than N/OFQ. (+)-5a Compound had a similar concentration–response curve as that of Ro 64-6198, and like Ro 64-6198, affected the NOP receptors in only one-third of the recorded neurons (Chiou et al. 2004). Importantly, (+)-5a Compound-insensitive neurons were also unresponsive to Ro 64-6198, and vice versa, while N/OFQ activated GIRK channels in these neurons via NOP receptors. Furthermore, (+)-5a Compound precluded the effect of Ro 64-6198, but not that of N/OFQ. Interestingly, (+)-5a Compound-sensitive or -insensitive neurons are morphologically distinct.

(+)-5a Compound, like Ro 64-6198, is less potent and efficacious than N/OFQ in the vlPAG

In vlPAG slices, (+)-5a Compound was 12 times less potent than N/OFQ and displayed only 47% efficacy of N/OFQ. Interestingly, in the same preparation, Ro 64-6198 was also 15 times less potent than N/OFQ and had 61% efficacy of N/OFQ (Chiou et al. 2004). The lower potencies of (+)-5a Compound and Ro 64-6198 compared to N/OFQ might be due to the larger amount of penetration barriers in the brain tissue, compared to cultured cells. Similar findings that NOP receptor ligands are less potent in native NOP receptors in rodent tissues than in expressed human NOP receptors have been reported (Calò et al. 2000).

The lower efficacy of (+)-5a Compound, compared to N/OFQ, could be because (+)-5a Compound acted as a partial agonist at the same NOP receptors where N/OFQ is a full agonist. However, there was a sharp cut-off in the responses to (+)-5a Compound between (+)-5a Compound-sensitive and -insensitive neurons (Fig. 2b). Furthermore, in (+)-5a Compound-insensitive neurons, N/OFQ was as effective as in all other neurons. Hence, it is unlikely that the ineffectiveness of (+)-5a Compound in those insensitive neurons is due to its partial agonist properties in those neurons which had lower density of NOP receptors. Therefore, the lower efficacy of (+)-5a Compound might be explained by the fact that it affected only one subset, whereas N/OFQ affected all of the NOP receptors in vlPAG neurons (see below).

(+)-5a Compound activated GIRK channels through NOP, but not opioid, σ, H₃ histamine or M₂ muscarinic receptors

Similar to N/OFQ (Chiou et al. 2002; Vaughan et al. 1997) and Ro 64-6198 (Chiou et al. 2004), (+)-5a Compound activated GIRK channels in vlPAG neurons. The effects of (+)-5a Compound in most of the tested neurons were mediated through NOP receptors since they were antagonized by UFP-101, which effectively antagonized the effect of N/OFQ in vlPAG neurons (Chiou et al. 2005). The ineffectiveness of naloxone excludes the involvement of opioid receptors. (+)-5a Compound displayed micromolar affinity at histamine H₃, muscarinic and σ receptors (Kolczewski et al. 2003). However, these receptors do not appear to be involved in (+)-5a Compound-induced GIRK activation in vlPAG neurons. First, there are few H₃ receptors in PAG (Pillot et al. 2002). Second, activation of σ receptors does not open GIRK channels (Zhang & Cuevas, 2005) although σ receptors do exist in PAG (Mei & Pasternak, 2007). Third, although M₂ muscarinic receptor activation in PAG induced GIRK currents (Sanada et al. 2007), AF-DX-116, a selective M₂ muscarinic receptor antagonist, did not antagonize the effect of (+)-5a Compound. Taken together, it is suggested that the effect of (+)-5a Compound in vlPAG neurons is mediated through NOP, but not opioid, σ, H₃ histamine or M₂ muscarinic receptors.

The effect of (+)-5a Compound in a small portion, 18% (34% of 53%), of the recorded neurons was also not antagonized by UFP-101. We did not further investigate these neurons for two reasons. First, it occurred in a limited number (n=12) of recorded neurons. Second, the intracellular dialysis nature of the whole-cell recording technique precluded us from further investigating if Ro 64-6198 also produced a UFP-101-insensitive effect in these neurons since these neurons have been treated with two drugs, (+)-5a Compound and UFP-101.

(+)-5a Compound activates a subset of NOP receptors in vlPAG neurons

Unlike N/OFQ which activated GIRK channels through NOP receptors in almost all (96%) of the recorded neurons (Chiou et al. 2002), (+)-5a Compound activated GIRK channels in only 53% of the recorded neurons. Among these neurons, 66% were sensitive to UFP-101, suggesting that the effects of (+)-5a Compound in 35% (66% of 53%) of vlPAG neurons are mediated through NOP receptors. However, in those (+)-5a Compound-insensitive neurons, NOP receptors are functional since N/OFQ still activated GIRK channels through NOP receptors. It is, therefore, suggested that (+)-5a Compound, similarly to Ro 64-6198 (Chiou et al. 2004), affected just one subset, not all, of the NOP receptors in vlPAG neurons.
Several findings from our study suggest that (+)-5α Compound and Ro 64-6198 activate the same subset of NOP receptors in vIPAG neurons. First, a similar ratio (35%) of the recorded neurons were sensitive to (+)-5α Compound and Ro 64-6198 (Chiou et al. 2004). Second, (+)-5α Compound-insensitive neurons were also unresponsive to Ro 64-6198, and vice versa, whereas N/OFQ activated GIRK channels in these neurons. Third, (+)-5α Compound, at the maximal effective concentration precluded the effect of Ro 64-6198.

A majority of the (+)-5α Compound-sensitive neurons are GABAergic

Morphological studies demonstrated that bipolar, triangular and multipolar neurons are present in PAG (Beitz & Shepard, 1985). (+)-5α Compound-sensitive neurons have 3–6 primary dendrites resembling the triangular and multipolar neurons. Interestingly, the ratio (53%) of (+)-5α Compound-sensitive neurons in PAG, in the present electrophysiological study is very close to the ratio (52%) of triangular plus multipolar neurons in the previous Golgi study (Beitz & Shepard, 1985). The triangular and multipolar neurons have more primary dendrites and complicated dendritic arbors implying that (+)-5α Compound-sensitive neurons may receive and integrate various excitatory/ inhibitory/regulatory inputs. Furthermore, our post-hoc GAD67 immunostaining verified that 78% of the (+)-5α Compound-sensitive neurons are GABAergic. Since GABAergic neurons account for 50% of all the neurons in PAG (Mugnaini & Oertel, 1985) and most of them are interneurons (Reichling & Basbaum, 1990), our findings suggest that (+)-5α Compound-sensitive neurons play an important role in GABA-mediated pain regulation. Activation of GIRK channels on GABAergic interneurons results in excitation of the vIPAG, leading to anti-nociception through activation of the descending pain inhibitory pathway. It remains to be elucidated if Ro 64-6198 or (+)-5α Compound, when injected into the vIPAG, will produce anti-nociception through this disinhibition mechanism.

Heterogeneity of NOP receptors

Although a single binding site of N/OFQ in the brain was originally reported (Dooley & Houghten, 1996) and most of the effects of N/OFQ were absent in NOP knockout mice (Burmeister et al. 2008; Kuzmin et al. 2009), heterogeneity of NOP receptors is suggested by the binding studies. Pasternak’s group (Mathis et al. 1997, 1999) has demonstrated two specific binding sites of N/OFQ with high and low affinity and distinct distributions in rodent brains. Recently, the same group’s meeting report (Majumdar et al. 2009) showed that, in both wild-type and NOP receptor knockout mice, N/OFQ displayed similar binding affinity at high-affinity binding sites of N/OFQ (Mathis et al. 1999). The function of N/OFQ at these high-affinity sites in NOP knockout mice remains to be elucidated. It also remains to be further elucidated if the NOP receptor subset sensitive to Ro 64-6198 and (+)-5α Compound resembles those high-affinity binding sites of N/OFQ.

Several lines of evidence showed that Ro 64-6198 mimicked some but not other functions of N/OFQ in vivo and in vitro, such as high-dose N/OFQ-induced hypolocomotion but not low-dose induced hyperlocomotion (Kuzmin et al. 2004), the centrally regulated urination but not hypotension or bradycardia (Dayan et al. 2001), the inhibition of vas deferens contraction in rats but not in mice (Rizzi et al. 2001), the anxiolytic action in rats being less effective in mice (Jenck et al. 2000), the increase in food intake but not the decrease in alcohol consumption (Economidou et al. 2006), and the anti-allodynic effect intraplantarly but less effective intrathecally in neuropathic rats (Obara et al. 2005). Compared with N/OFQ, Ro 64-6198 is more hydrophobic and has slower kinetics (Chiou et al. 2004; Jenck et al. 2000). Although these properties of Ro 64-6198 might make it display different potency and pharmacokinetics from N/OFQ, it is difficult to explain why Ro 64-6198 mimicked some, but not all, actions of N/OFQ. Taken together with the current findings of (+)-5α Compound and the finding of splicing variants of NOP receptors (Peluso et al. 1998), it is likely that there is functional heterogeneity of NOP receptors in the brain. It remains to be further elucidated if different subsets of NOP receptors mediate different functions of N/OFQ physiologically or pathologically when its CSF levels are altered (Barnes & Lambert, 2004; Chiou et al. 2007), and resemble different binding sites of N/OFQ in the brain.

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**Statement of Interest**

None.

**References**


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