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以藥理學方法在大鼠中腦環導水管灰質腦切片研究 Nociceptin/Orphanin FQ 受體之功能異質性

A Study on the Functional Heterogeneity of Nociceptin/Orphanin FQ Receptors in Rat Periaqueductal Gray Slices by Pharmacological Approach

廖彥昱

YAN-YU LIAO

指導教授：邱麗珠 博士

Advisor: LIH-CHU CHIOU, Ph.D.

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本論文係廖彥昱君（學號 D93443001）在國立臺灣大學藥理學系、所完成之碩（博）士學位論文，於民國 100 年 01 月 28 日承下列考試委員審查通過及口試及格，特此證明

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摘要

在 1994 年，一種新的鴉片受體被發現，因其結構相似於傳統鴉片受體，但卻對傳統鴉片受體致效劑缺乏親和力，同時也未發現其內生性致效劑，所以一開始被稱爲類鴉片孤兒受體(opioid receptor like 1, ORL1)。它的內生性致效劑在一年後被發現並命名爲 nociceptin 或 orphanin FQ，在 2002 年國際藥理學聯合會以其內生性致效劑之名正式將此受體命名爲 NOP 受體(nociceptin/orphanin FQ peptide receptor)。研究顯示此 N/OFQ-NOP 受體系統與許多神經及精神疾病有關，如疼痛、焦慮、憂鬱、非自主運動、成癮、發作及痴呆等。

從 NOP 受體基因剪接變異體(splicing variants)被發現及一些受體結合和功能性研究顯示有 NOP 受體異質性(heterogeneity)的存在。我們之前發現 Ro 64-6198 這個 NOP 受體致效劑在中腦環導水管灰質腹側區(ventrolateral PAG)只能活化一部份的 NOP 受體，而在本研究中，我們發現另一個新的非胜肽 NOP 受體結合劑(+)-5a Compound ((3a*S*,6a*R*)-1-(*cis*-4-Isopropylcyclohexyl)-5'-methyl-2'–phenylhexahydro spiro[piperidine-4,1'-pyrrolo[3,4-*c*]pyrrole])在相同的標本也只能活化一部份 NOP 受體，這些可被(+)-5a Compound 活化的 NOP 受體與 Ro 64-6198 所活化的 NOP 受體是屬於同一群，(+)-5a Compound (0.1-30 μM)能濃度相依的透過 NOP 受體活化 35% 所記錄神經細胞的 G 蛋白偶合向內整流鉀離子通道(GIRK channels)，其 IC_{50} 爲 605 nM，效價(potency)及所能引發反應的最大效力(efficacy)分別爲 N/OFQ 的 1/12 倍及 47%，對(+)-5a Compound-insensitive 的細胞，給予 Ro 64-6198 同樣也無法引發反應，但在這些細胞中 N/OFQ 則可有效的活化 G 蛋白偶合向內整流鉀離子通道，另外在(+)-5a Compound-sensitive 的細胞，給予 Ro 64-6198 則反應程度並無差異，從免疫螢光染色的實驗顯示(+)-5a Compound-sensitive 的細胞主要是 GABAergic，此外，(+)-5a Compound-sensitive 的細胞在型態上，其神經分支比(+)-5a Compound-insensitive 的細胞來得複雜。

N/OFQ(1-11)是具藥理活性的 N/OFQ 代謝產物，從放射線標定的研究中，

^{125}I-[Tyr^{10}]N/OFQ(1-11)及 ^{125}I-[Tyr^{14}]N/OFQ 這兩個 NOP 受體的放射線結合劑在鼠腦中有不同的分布，其中 ^{125}I-[Tyr^{10}]N/OFQ(1-11) 的結合位置可能是 ^{125}I-[Tyr^{14}]N/OFQ 所顯現出兩種不同親和力結合處中的高親和力結合處，基於這個發現，我們於是做了在環導水管灰質區對 Ro 64-6198/(+)-5a Compound 有反應的 NOP 受體就是 ^{125}I-[Tyr^{10}]N/OFQ(1-11)這個結合位置的假設，因此，我們藉由測試 [Tyr^{10}]N/OFQ(1-11)能否區別(+)-5a Compound-sensitive 及-insensitive 細胞來驗證這個假設。在紀錄的環導水管灰質腹側區細胞中，[Tyr^{10}]N/OFQ(1-11)在濃度 3-300 μM 時能引發向內整流性鉀離子電流，其 EC_{50} 爲 9.0 μM，比 N/OFQ 弱了 173 倍，而效力（efficacy）則與 N/OFQ 相似，[Tyr^{10}]N/OFQ(1-11)顯露了與(+)-5a Compound 不同的藥理特性，它不管在(+)-5a Compound-sensitive 或-insensitive 細胞中都有作用，這些結果顯示 [Tyr^{10}]N/OFQ(1-11)是一個效力比 N/OFQ 差的 NOP 受體全效致效劑，而且在環導水管灰質腹側區細胞中，[Tyr^{10}]N/OFQ(1-11)無法顯露出 NOP 受體功能異質性。

爲了闡明 NOP 受體多樣性的可能，發展及鑑定新的 NOP 受體拮抗劑是很重要的。因此在本研究中，我們也在中腦環導水管灰質腹側區細胞中測試 Compound 24 (1-Benzyl-N-[3-[spiroisobenzofuran-1(3H),4'-piperidin-1-yl]propyl]pyrrolidine-2-carboxamide)及 SB-612111 ((-)-*cis*-1- Methyl-7-[[4-(2,6- dichlorophenyl)piperidin-1-yl]methyl]-6,7,8,9-tetrahydro-5*H*-benzocyclohepten-5-ol)這兩個新發展的 NOP 受體拮抗劑是否具有能顯露出 NOP 受體功能異質性的特性，然而在我們所紀錄到可被 N/OFQ 所活化的全部細胞，N/OFQ 的作用都會被 Compound 24 及 SB-612111 所拮抗，因此這兩種 NOP 受體拮抗劑並無法顯露 NOP 受體功能異質性。Compound 24 (0.3-10 μM)能濃度相依的抑制 N/OFQ 所誘導出的向內整流鉀離子電流，其 IC_{50} 爲 2.6 μM，Compound 24 在環導水管灰質腹側區神經細胞的抑制效價比在小鼠輸精管標本及表現人類 NOP 受體的培養細胞所得到的效價還差，而且在相同的環導水管灰質區標本，Compound 24 的作用比另一個胜肽類 NOP 受體拮抗劑 UFP-101 還要

慢，當 Compound 24 濃度高達 10 μM 時對 NOP 受體並不具有內在致效劑的活性，不過只要 Compound 24 的濃度高於 3 μM 時也會抑制 mu 鴉片受體致效劑 DAMGO(D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin)所誘導出的向內整流鉀離子電流。而 SB-612111 也能濃度相依的拮抗 N/OFQ 所引發的向內整流鉀離子電流，從濃度反應曲線估出其 IC_{50} 值爲 87.7 nM，而且 SB-612111 就算濃度高到 1 μM 並不會產生內在致效劑活性，也不會影響 mu 鴉片受體致效劑 DAMGO 所引發的向內整流鉀離子電流。

總結，在環導水管灰質腹側區(+)-5a Compound 與 Ro 64-6198 一樣都只活化一部份的 NOP 受體，而這些對(+)-5a Compound 有反應的神經細胞大多屬於 GABAergic，而且對(+)-5a Compound 有反應及無反應的細胞，兩者在細胞型態上並不相同，這些結果進一步支持在中腦環導水管灰質區的 NOP 受體有功能異質性的存在。不過[Tyr^{10}]N/OFQ(1-11)在藥理特性上與(+)-5a Compound 並不相同，而且利用[Tyr^{10}]N/OFQ(1-11)以及 Compound 24 與 SB-612111 這兩個新的 NOP 受體拮抗劑也無法觀察到 NOP 受體功能異質性的現象。此外，在 Compound 24 與 SB-612111 的定量性研究上，證明 Compound 24 是一個效價普通且選擇性亦非理想的 NOP 受體拮抗劑，另一方面，SB-612111 則是一個有效且具選擇性，同時又具非胜肽類優勢的 NOP 受體拮抗劑，它可說是能用來探究內生性 N/OFQ 生理角色最好的 NOP 受體拮抗劑。

關鍵詞：NOP 受體、環導水管灰質區、G 蛋白偶合向內整流鉀離子通道、鴉片受體、異質性

Abstract

In 1994, a novel opioid receptor family, opioid receptor like 1 (ORL1) orphan receptors, was identified to be structurally similar to classical opioid receptors, but insensitive to traditional opioids. Its endogenous ligand was later identified to be a heptadecapeptide and termed nociceptin or orphanin FQ. This receptor was consensusly renamed after its endogenous ligand as nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor in IUPHAR2002. The N/OFQ-NOP receptor system was 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 gray (vlPAG). In this study, we found that a new non-peptide ligand of NOP receptors, (+)-5a Compound ((3a*S*,6a*R*)-1-(*cis*-4-Isopropylcyclohexyl)-5'-methyl-2'-phenylhexahydrospiro[piperidine -4,1'-pyrrolo[3,4-*c*]pyrrole]), also activated a subset of NOP receptors as the same subset affected by Ro 64-6198 in vlPAG neurons. (+)-5a Compound (0.1-30 μM) concentration-dependently activated G-protein-coupled inwardly rectifying potassium (GIRK) channels through the NOP receptors in about 35% of the recorded vlPAG neurons. (+)-5a Compound (EC_{50}: 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 was effective in activating GIRK channels through NOP receptors. In (+)-5a Compound-sensitive neurons, (+)-5a Compound precluded the effect of Ro 64-6198. Immunofluorecent 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.

N/OFQ(1-11) is a pharmacologically active metabolite of N/OFQ. The distribution of its radioligand, ^{125}I-[Tyr^{10}]N/OFQ(1-11), resembled the high affinity, but not all, binding sites of ^{125}I-[Tyr^{14}]N/OFQ in rodent brains. Based on this finding we hypothesize that the Ro 64-6198/(+)-5a Compound-sensitive NOP receptor in the PAG is the binding site of ^{125}I-[Tyr^{10}]N/OFQ(1-11). Here, we validated this hypothesis by examining if [Tyr^{10}]N/OFQ(1-11) can differentiate (+)-5a Compound-sensitive and -insensitive vlPAG neurons. [Tyr^{10}]N/OFQ(1-11), like N/OFQ, induced GIRK current through NOP receptors in the vlPAG neurons. It was 173 times less potent (EC_{50}: 9.0 μM) but equi-efficacious, as compared with N/OFQ. [Tyr^{10}]N/OFQ(1-11) displayed different pharmacological profiles as (+)-5a Compound. It was effective in both (+)-5a Compound-sensitive and -insensitive neurons. These results suggest that [Tyr^{10}]N/OFQ(1-11) is an NOP full agonist and less potent than N/OFQ. The functional heterogeneity of NOP receptors, therefore, can not be revealed by [Tyr^{10}]N/OFQ(1-11) in vlPAG neurons.

To clarify the possible diversity of NOP receptors, it is important to develop and characterize novel NOP receptor antagonists. In this study, we also examined if two newly developed NOP receptor antagonists, 1-Benzyl-N-[3-[spiroisobenzofuran -1(3H),4'-piperidin-1-yl]propyl]pyrrolidine-2-carboxamide (Compound 24) and (-)-*cis*-1-Methyl-7-[[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl]-6,7,8,9-tetrahydro-5 *H*-benzocyclohepten-5-ol (SB-612111), can reveal the heterogeneity of NOP receptors in vlPAG slices. Both Compound 24 and SB-612111 antagonized the effect of N/OFQ in all the recorded neurons. Therefore, the heterogeneity of NOP receptors can not be revealed by these two antagonists. However, we further quantitatively characterized their interactions with N/OFQ in vlPAG slices. Compound 24, at 0.3-10 μM, attenuated N/OFQ-induced GIRK current concentration-dependently. The antagonistic potency of Compound 24 in vlPAG neurons (IC_{50}: 2.6 $\pm$ 0.6 μM) was, however, lower than that

obtained in mouse vas deferens preparations or expressed human NOP receptors. The action kinetic of Compound 24 was slower than UFP-101, a peptide antagonist, in the same preparations. Compound 24 had no intrinsic agonistic activity at NOP receptors at concentrations up to 10 μM. However, at concentrations higher than 3 μM, it also attenuated the GIRK current induced by DAMGO ([D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin), a mu-opioid receptor agonist. As for SB-612111, it also concentration-dependently antagonized N/OFQ-induced GIRK current in vlPAG neurons. The IC_{50} value of SB-612111 estimated from concentration-response curve is 87.7±1.2 nM. SB-612111 had no intrinsic agonistic activity and did not affect the GIRK current induced by DAMGO when tested at concentrations of up to 1 μM.

In conclusion, (+)-5a Compound activates a subset of NOP receptors, similar to the Ro 64-6198-sensitive subset, in vlPAG neurons which are mostly GABAergic. Moreover, (+)-5a Compound-sensitive or -insensitive neurons are morphologically distinct. These results further support the presence of functional heterogeneity of NOP receptors in the PAG. However, the pharmacological profiles of [Tyr^{10}]N/OFQ(1-11) are unlike that of (+)-5a Compound and the functional heterogeneity of NOP receptors can not be revealed by either [Tyr^{10}]N/OFQ(1-11) or new NOP receptor antagonists, Compound 24 and SB-612111. Quantitative studies of Compound 24 and SB-612111 in the vlPAG showed that Compound 24 acts as a competitive NOP receptor antagonist but its potency and selectivity are moderate. On the other hand, SB-612111 is a pure, potent and selective antagonist of NOP receptors with the merits of non-peptide nature, high potency, and selectivity. SB-612111 is the best NOP receptor antagonist available for exploring the functional roles of endogenous N/OFQ.

Key words：Nociceptin/orphanin FQ Receptor; Periaqueductal Gray; Potassium channel; (+)-5a Compound; [Tyr^{10}]N/OFQ(1-11); Compound 24; SB-612111.

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Abbreviation

ABC	avidin-biotin-peroxidase complex
ACC	anterior cingulated cortex
aCSF	artificial cerebral spinal fluid
BSA	bovine serum albumin
DAB	3,3'-diaminobenzidine
DAMGO	[D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin
DMSO	dimethyl sulfoxide
GAD67	glutamic acid decarboxylase-67
GABA	γ-amino-butyric acid
GIRK	G-protein coupled inwardly rectifying potassium channels
GPCR	G-protein coupled receptor
H_2O_2	hydrogen peroxide
I_{-140}	membrane current at -140 mV
I_{hold}	holding current
I-V	current-voltage
LY	lucifer yellow
NalBzOH	naloxone benzoylhydrazone
N/OFQ	nociceptin/orphanin FQ
NOP	nociceptin/orphanin FQ peptide
ORL1	opioid receptor-like 1
PAG	periaqueductal gray
PBS	phosphate buffered saline
RVM	rostral ventromedial medulla
vlPAG	ventrolateral periaqueductal gray

Introduction

1. N/OFQ and NOP receptor

An orphan G-protein coupled receptor (GPCR) was cloned in 1994 from a search to discover subtypes of opioid receptors and named initially as opioid receptor like 1 (ORL1), because, although having a structure highly homologous to classical opioid receptors, it displayed little affinity for opioids (Mollereau et al., 1994). One year later, this orphan receptor was de-orphanized through identifying its endogenous ligand, a heptadecapeptide named nociceptin (Meunier et al., 1995) or orphanin FQ (Reinscheid et al., 1995). The ORL1 receptor was classified as the fourth member of the opioid receptor family 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 is derived from a precursor protein, preproN/OFQ (ppN/OFQ) which consists of 181, 187 and 176 amino acids, respectively, in the rat, mouse and human (Nothacker et al., 1996). The intracellular events triggered by the binding of N/OFQ with the NOP receptor, and mediated by the activation of G-protein, include inhibition of adenylyl cyclase, activation of phospholipase C and K^+ channels and inhibition of Ca^{2+} channels.

The regional distribution of N/OFQ and the NOP receptor have been well

described. Regions with high levels of NOP receptor include the cerebral cortex, hypothalamus, hippocampus, amygdale, periaqueductal gray, substantia nigra, central gray, locus coeruleus and spinal cord (Anton et al., 1996; Berthele et al., 2003; Florin et al., 1997; Neal et al., 1999a). The localization of N/OFQ corresponds reasonably well with the NOP receptor. As with the receptor, N/OFQ immunoreactivity and mRNA levels detected using *in situ* hybridization are closely correlated. N/OFQ is found in ventral forebrain, hypothalamus, hippocampus, amygdala, ventral tegmentum, periaqueductal gray, substantia nigra, central gray, locus coeruleus, spinal cord (Boom et al., 1999; Neal et al., 1999b; Nothacker et al., 1996; Witta et al., 2004).

2. NOP receptor ligands

When the NOP receptor was cloned initially, several commonly used opioid drugs including etorphine and buprenorphine have been demonstrated to bind to NOP receptors (Wnendt et al., 1999). However, their binding affinities at the NOP receptors are relatively insignificant compared to their activity at classical opioid receptors. Thereafter, a wide range of selective agonist and antagonist ligands for NOP receptors have been developed, which show little or no affinity to classical opioid receptors and so allow NOP receptor mediated responses to be studied in isolation (Chiou et al., 2007).

2.1. NOP receptor agonist

2.1.1. Peptide NOP receptor agonist

Since N/OFQ was identified to be a NOP receptor agonist, several peptide-based NOP receptor agonists have been reported (Table 1). The shortest N/OFQ derivative possessing the same affinity for NOP receptor as N/OFQ is the truncated and amidated N/OFQ(1-13)-NH_2 (Guerrini et al., 1997). C-terminal amidation protects from degradation by carboxypeptidases and is now a standard feature of most N/OFQ-based peptide ligands. Substituting the 14^{th} and 15^{th} residues with Arg and Lys, respectively, gave an agonist, [Arg^{14}, Lys^{15}]N/OFQ. It was reported to be the first N/OFQ derivative with higher affinity and biological potency *in vitro* than N/OFQ (Okada et al., 2000; Rizzi et al., 2002b). Conformationally restricted peptide synthesis also generated an agonist, cyclo[Cys^{10}, Cys^{14}]N/OFQ, with comparable affinity to N/OFQ (Ambo et al., 2001). The cyclic peptide is the first conformationally restricted peptide with potent activity and may serve as a good template for studying the bioactive conformation of N/OFQ. [(pF)Phe^4, Arg^{14}, Lys^{15}]N/OFQ-NH_2 (UFP-102) appears to be a more promising one with higher potency and longer duration of action (Carra et al., 2005). Substituting the 7^{th} amino acid residue of UFP-102 with aminoisobutyric acid (Aib), a more potent agonist, UFP-112, of NOP receptors (Arduin et al., 2007) was developed. It behaves as a selective full agonist, producing long lasting effect *in vivo*. The

tetrapeptide OS-461 and its analogues, OS-462 and OS-500, developed by Nippon Shinyaku (Economidou et al., 2006b) were reported to have NOP agonistic activity and analgesic effects in mice. In 2007, a truncated cyclic analogue with cysteine residues at positions 7 and 10, c[Cys(7,10)]N/OFQ(1-13)-NH_2, was developed to improve the metabolic stability (Kitayama et al., 2007). Although it acts as a high-potency full agonist of NOP recetpors, the expected metabolic enzymatic protection was not achieved.

2.1.2. Non-peptide NOP receptor agonist

Buprenorphine and lofentanil are a few classic opioid drugs having agonistic activity at NOP receptors (Hawkinson et al., 2000). Ro 64-6198 is the first synthetic non-peptide agonist presented by F. Hoffmann-La Roche Ltd. in 2000 (Jenck et al., 2000). It was widely used to characterize the functional roles of the N/OFQ-NOP system. In 2003, F. Hoffmann-La Roche Ltd. developed another non-peptide agonist, (+)-5a Compound, with a structure backbone analogous to Ro 64-6198 (Kolczewski et al., 2003) (See the section 2.1.2.2). NNC 63-0532 is a spiroxatrine analogue with high affinity for NOP receptors but only 12-fold selectivity for NOP receptors as compared with MOP receptors (Thomsen and Hohlweg, 2000). In 2005, a potent non-peptide NOP receptor full agonist, W-212393, was development by Mitsubishi Pharma (Teshima et

al., 2005). This compound is fairly selective versus other opiate receptors, but also exhibits nanomolar affinity for the serotonin transporter. In 2006, Bignan et al. (2006) at Johnson & Johnson Company presented novel piperidin-indoles and pyrrolo-pyridines as NOP receptor agonists with low nanomolar affinity and more than 25-100 times selectivity versus other opioid receptors. In 2008, Hirao et al. (2008a) at Pfizer Company revealed two brain penetrating non-peptide agonists, PCPB and MCOPPB, which have subnanomolar affinity for NOP. In 2008, Varty et al. at Schering-Plough Company developed an NOP receptor agonist, SCH 221510 (Varty et al., 2008). It produced robust anxiolytic-like activity at doses that do not seem to produce nonspecific disruption of overt behaviors such as locomotor activity and did not tolerate after chronic dosing. In 2009, the same company developed another NOP receptor agonist, SCH 225288 (McLeod et al., 2009) with good oral absorption rate and effective in several cough models, including capsaicin-induced coughs in guinea pigs, and mechanically-evoked coughs in cats and infectious tracheobronchitis-induced coughs in canines. SCH 486757 (McLeod et al., 2010) is another orally active NOP receptor agonist from the same company. It has higher selectivity to NOP receptors than SCH 225288 and produces a level of antitussive efficacy equivalent to codeine in capsaicin-evoked cough model without the side effect liabilities associated with opioid antitussives. In addition, it does not show potential to induce abuse liabilities. This

compound has recently entered human clinical trials for cough treatment. In 2010, Pfizer Company also presented a new NOP receptor agonist, HPCOM (Hayashi et al., 2010). It showed potent affinity, high selectivity and long-lasting metabolic stability. When administered by *s.c.* injection, it had high plasma concentration with high plasma-to-brain selectivity (*s.c.*), and exhibited good antiallodynic activity on injured paw in neuropathic pain model rats derived from peripheral-selective antiallodynic activity. These non-peptide NOP receptor agonists are shown in Table 2. Among these, Ro 64-6198 is the first developed NOP receptors and has been widely used to elucidate the function of NOP receptors and determine the potential of NOP receptor as a therapeutic target, especially in anxiety. Our laboratory also had contribution in this field using this compound (Chiou et al., 2004). We used this compound as a reference compound to further explore the effects of (+)-5a Compound. These two lignads are specially introduced in the followings.

2.1.2.1. Ro 64-6198

Ro 64-6198 (Fig. 1) was shown initially to have subnanomolar affinity and high selectivity for NOP receptors (Jenck et al., 2000). Functional studies showed that Ro 64-6198 acted as a potent NOP receptor agonist in CHO_{hNOP} cells and peripheral and brain preparations (Chiou et al., 2004; Gehlert et al., 2006; Jenck et al., 2000; Rizzi et al., 2001; Wichmann et al., 2000). Ro 64-6198 has been used in several studies to reveal

the functional roles and nature of NOP receptor. In most studies, Ro 64-6198 mimicked the effects of N/OFQ, including memory impairment (Goeldner et al., 2008; Higgins et al., 2002; Kuzmin et al., 2009), high-dose N/OFQ-induced hypolocomotion (Kuzmin et al., 2004) and hyperphagia (Ciccocioppo et al., 2002), reduction of alcohol-induced conditioned place preference (Kuzmin et al., 2003), antitussion (McLeod et al., 2002) and orexigenic effect (Economidou et al., 2006a). However, Ro 64-6198 did not mimick all of the effects of N/OFQ in several functional studies (See the section 6.4). This raises the possibility that Ro 64-6198 and N/OFQ may activate a different functional subset of NOP receptors.

2.1.2.2. (+)-5a Compound

(+)-5a Compound ((3a*S*,6a*R*)-1-(*cis*-4-isopropylcyclohexyl)-5'-methyl-2' -phenylhexahydrospiro[piperidine-4,1'-pyrrolo[3,4-*c*]pyrrole]) (Fig. 1) 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 GTPγS 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).

2.2. NOP receptor antagonist

2.2.1. Peptide NOP receptor antagonist

Several peptide NOP receptor antagonists have been developed so far (Table 3). However, early developed peptide antagonists are flawed by their non-specificity, such as the conformational restricted peptide III-BTD or by their residual agonist activity, such as acetylated hexapeptides (Ac-RYYRIK-NH_2 and Ac-RYYRWK-NH_2) and the first proposed antagonist, [$Phe^1\psi(CH_2\text{-}NH)Gly^2$]N/OFQ(1-13)-$NH_2$ (Chiou et al., 2007). ZP-120 (Ac-RYYRWKKKKKK-NH_2) acted as a partial agonist at NOP receptor with higher potency and longer duration than N/OFQ. Its blood-brain barrier impermeable merit makes it useful in improving cardiovascular function without central effects (Kapusta et al., 2005). [$Nphe^1$]N/OFQ(1-13)-NH_2 is the first antagonist demonstrated to antagonize N/OFQ-effects but have no intrinsic activity at NOP receptors (Calo' et al., 2000b). Several physiological or pathological roles of N/OFQ have thereafter been revealed by using this pure and selective antagonist. [$Nphe^1$,Arg^{14},Lys^{15}]N/OFQ-NH_2 (UFP-101), derived by a combination of two N/OFQ-modified peptides, [Arg^{14},Lys^{15}]N/OFQ and [$Nphe^1$]N/OFQ(1-13)-NH_2, is a potent and competitive antagonist (Calo' et al., 2002). UFP-101 has been proven to be a more potent antagonist than [$Nphe^1$]N/OFQ(1-13)-NH_2 and have a longer duration *in vivo*. It has become a useful pharmacological tool available for the investigation of the central and peripheral

biological functions regulated by the N/OFQ-NOP receptor system and for defining the therapeutic potential of NOP receptor ligands. In 2006, Gunduz et al. synthesized a potent hexapeptide alcohol, Ac-RYYRIK-ol, by substituting the C-terianl amide of Ac-RYYRIK-NH_2 with an alcoholic function (Gunduz et al., 2006). However, tt displayed a complex pharmacological profile which is likely due to the low efficacy agonistic activity. Recently, a novel NOP receptor antagonist, Ac-RYYRIR-ol, representing the Lys^6 to Arg^6 replacement of its parent compound, Ac-RYYRIK-ol, had been synthesized (Bojnik et al., 2010). It also displays a complex pharmacological profile but do not show expected higher binding affinity than Ac-RYYRIK-ol.

2.2.2. Non-peptide NOP receptor antagonist

Putative non-peptide NOP receptor antagonists are summarized in Table 4. Naloxone benzoylhydrazone (NalBzOH) is a non-selective opioid receptor ligand, which was reported to competitively antagonize the effects of N/OFQ before any specific antagonist has been developed (Noda et al., 1998). A morphinan derivative, TRK-820, has been reported to interact with NOP receptor as an antagonist (Seki et al., 1999). This compound acts as a high affinity KOP agonist, whereas very high concentrations are required to reverse the actions of N/OFQ. In 1999, the first non-peptide antagonist selective for NOP receptor, J-113397, was developed by Banyu

Pharmaceutical in Japan. It is a very potent and selective antagonist of NOP receptors without intrinsic activity (Chiou and Fan, 2002; Ozaki et al., 2000b). However, it was later found to have psychomimetic central activity (Koizumi et al., 2004) which is likely attributed to its affinity at σ receptors (Chiou et al., 2007). Later, Calo's group at University of Ferrara (Italy) synthesized a high yield achiral analogue of J-113397, termed Trap-101 (Trapella et al., 2006), which is equipotent to enatiomer J-113397. In 2000, Japan Tobacco Inc. presented a non-peptide quinoline antagonist, JTC-801 (Shinkai et al., 2000), which binds to the NOP receptor with high affinity *in vitro*, yet only about 10-fold selectivity over the MOP receptor. In 2004, a potent and selective non-peptide antagonist, SB-612111, was reported by GlaxoSmithKline Pharmaceuticals (See section 2.2.2.2). In 2006, Goto et al at Banyu Pharmaceutical developed several structural diverse analogs of spiropiperidines as NOP receptor antagonists, among these, Compound 24 is a potent one (See the next paragraph). The same company kept developed several NOP antagonists with different chemical cores, including the benzimidazole derivatives (compound 28 is the most potent one in [^{35}S]GTPγS binding assay) (Okamoto et al., 2008), N-biarylmethyl spiropiperidines (compound 37 is a potent one) (Yoshizumi et al., 2008) and structureal diverse analogs of arylpyrazole (compound 31 is the most potent one) (Kobayashi et al., 2009). Recently, Volta et al in University of Ferrara (Italy) modified Trap-101 structure and introduced two methyl

groups in the hydroxymethyl function at the carbon 3 of the piperidine ring, thus obtaining a novel non-peptide antagonist, GF-4 (Volta et al., 2010). Such chemical modification did not alter the pharmacological activity of the compound (a pure and competitive NOP receptor antagonist) but slightly reduced its potency at recombinant NOP receptors and, more dramatically, *in vitro* selectivity over classical opioid receptors. In this study, we have the gifts of Compound 24 and SB-612111 from international collaboratory laboratories. Their detailed informations are introduced in the followings.

2.2.2.1. Compound 24

Compound 24 (1-Benzyl-N-[3-spiro[isobenzofuran-1(3H),4'-piperidine]-1-yl] propyl-D-proline amide) (Fig. 1) is a spiropiperidine analogue of NOP receptor antagonist developed by Goto et al. (2006). It was reported to have high affinity (K_d: 0.27 nM) at cloned human NOP receptors and be a potent antagonist (IC_{50}: 0.15 nM) in inhibiting GTPγS binding at cloned hNOP receptors expressed in CHO cells. In the *in vivo* study, Compound 24 significantly antagonized N/OFQ-induced reduction of locomotor activity (Goto et al., 2006). It also acted as a compeptitive antagonist in the assays of GTPγS and electrically stiumulated mouse vas deferens contraction (Fischetti et al., 2008).

2.2.2.2. SB-612111

SB-612111, (-)-*cis*-1-Methyl-7-[[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl] -6,7,8,9-tetrahydro-5*H*-benzocyclohepten-5-ol, (Fig. 1) is also an NOP receptor antagonist originally developed by GlaxoSmithKline Pharmaceuticals (Zaratin et al., 2004). It exhibits high affinity (Ki: 0.33-1.42 nM) at cloned human NOP receptors expressed in CHO cells and displayed 175-7594 times selectivity to NOP receptors among traditional opioid receptors (Khroyan et al., 2009; Spagnolo et al., 2007; Zaratin et al., 2004). Functional studies showed that SB-612111 acted as a potent antagonist (K_b: 5 nM) in reversing the inhibitory effect of N/OFQ on forskolin-induced luciferase expression (Zaratin et al., 2004). It also attenuated N/OFQ-induced inhibition of cAMP accumulation and GTPγS binding at CHO cell-expressed human NOP receptors, and inhibition of [^{3}H]5-HT overflow in mouse cortical synaptosomes (Spagnolo et al., 2007). Furthermore, SB-612111 antagonized the inhibitory effect of N/OFQ on electrically stimulated contractions in mouse vas deferens and guinea pig ileum preparations (Spagnolo et al., 2007). In GTPγS binding, cAMP accumulation and rat vas deferens contraction assays, SB-612111 did not display intrinsic activity at NOP receptors (Spagnolo et al., 2007). It also effectively antagonized immediate calcium transients evoked by N/OFQ in CHO_{hNOP} cells stably expressing the chimeric $G\alpha_{qi5}$ protein (Camarda et al., 2009b). SB-612111 also antagonized the effects of N/OFQ *in vivo*,

including the hyperalgesia and the reversal of morphine tolerance induced by N/OFQ in the mouse hot plate test (Zaratin et al., 2004), the antinociceptive and the pronociceptive actions of N/OFQ given by *i.t.* and *i.c.v.* injections, respectively, in the tail-flick test (Rizzi et al., 2007), and the orexigenic effect of N/OFQ (Rizzi et al., 2007). SB-612111 also potentiated the antinociception induced by buprenorphine and other mixed NOP/MOP receptor agonists, but not by selective MOP receptor agonists, in mouse tail-flick test (Khroyan et al., 2009). Several *in vivo* studies reported that SB-612111 alone did not affect the nociceptive response in the mouse hot-plate test when given by *i.c.v.* injection (Zaratin et al., 2004) or in the tail-flick test when given by *i.p.* (Rizzi et al., 2007) or *s.c.* (Khroyan et al., 2009) administration. However, SB-612111 (*i.v.*) effectively reversed the thermal hyperalgesia in a rat carrageenan inflammatory model (Zaratin et al., 2004). It (*i.p.*) also induced a depressant-like effect in the mouse forced swimming test in a manner reversed by N/OFQ, and this effect was inactive in NOP receptor knockout mice (Rizzi et al., 2007). The latter two studies might suggest that endogenous N/OFQ has pronociceptive and antidepressant functions.

3. Physiological or pathological roles of endogenous N/OFQ

Studies on the role of the endogenous N/OFQ in physiological or pathological functions began from the time of the discovery of the NOP receptor and first relied on

administration of N/OFQ. Several approaches have been conducted in revealing the physiological or pathological roles of endogenous N/OFQ. Before the selective NOP receptor antagonists or transgenic mice were developed, the antisense oligonucleotide against NOP receptor had been applied (Rossi et al., 1997). Knockout mice have been developed without the gene encoding NOP receptors (Nishi et al., 1997) or ppN/OFQ (Kest et al., 2001; Koster et al., 1999). Many physiological or pathological roles of N/OFQ have been reported from animal studies, including pain regulation, anxiety, depression, addiction, feeding, learning and memory, motor control and central cardiovascular regulation (Chiou et al., 2007; Lambert, 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; Lambert, 2008).

4. Pain

Pain generally starts with a physical event, including a cut, burn, tear or bump. The sensation of pain usually depends on the activation of a set of neurons that includes primary afferent nociceptors, interneurons in the spinal cord, cells of the ascending tracts, thalamic neurons and neurons of the cerebral cortex. Hence, the pain system involves a set of ascending pathways that convey nociceptive information from

peripheral nociceptors to higher levels of the central nervous system, as well as descending pathway that modulate that information (Fig. 2).

4.1. Ascending pain pathway

Nociceptive information is transmitted mainly through the ascending pain pathway. Primary afferent nociceptors convey noxious information to the secondary sensory neurons within the dorsal horn of the spinal cord which sends projections upwardly. A subset of these projection neurons transmits information to the somatosensory cortex via the thalamus, providing information about the location and intensity of the painful stimulus. Other projection neurons engage the cingulated and insular cortices via connections in the brainstem and amygdale, cortributing to the affective component of the pain experience.

4.2. Descending inhibition pain pathway

The descending inhibition pain pathway is a well-characterized anatomical network that enables us to regulate nociceptive processing. The brain uses this pathway to send chemical substances and nerve impulses back down to the sensory neurons in the spinal cord to act against the pain message sent up by the pain receptors. The midbrain PAG, of pain-inhibiting circuitry is a crucial part, is best known and

contributes to opiate analgesia. In addition to PAG, a variety of brain regions are involved in this descending modulation, including the frontal lobe, anterior cingulated cortex (ACC), amygdale, hypothalamus and rostral ventromedial medulla (RVM). Figure 2 illustrates the key anatomical features of the descending inhibition pain pathway.

4.3. Effects of N/OFQ on pain

The sequence similarities between NOP and classical opioid receptors drive the studies that investigate the roles of endogenous N/OFQ focusing on pain regulation. N/OFQ shares similar cellular actions with opioids, such as inhibition of cAMP formation, activation of K^+ channels and inhibition of Ca^{2+} channels (Darland et al., 1998). However, different from conventional opioids that usually produce analgesia, N/OFQ produces controversial effects in pain regulation, such as supraspinal hyperalgesic but spinal analgesic effects (Mogil and Pasternak, 2001).

4.3.1 Supraspinal pain regulation of N/OFQ

Supraspinal hyperalgesia is the first reported action of N/OFQ *in vivo* using the hot-plate (Meunier et al., 1995) and tail-flick (Reinscheid et al., 1995) tests in the mouse. However, the hyperalgesic effect of *i.c.v*. N/OFQ in mice was found to be resulted from

an inhibition of stress-induced analgesia (Mogil et al., 1996). N/OFQ administered supraspinally reverses the effects of exogenous opioids (Pan 2000). Intracerebroventricular injection of N/OFQ was stressful, resulting in the release of central endogenous opioid peptide with their effects subsequently reversed by the delivered dose of N/OFQ. Indeed, when compared with non-injected animals rather than with *i.c.v.* vehicle-injected ones, *i.c.v.* N/OFQ-injected animals showed normal sensitivity to noxious stimulation, while *i.c.v*. vehicle-injected animals displayed a decreased nociceptive sensitivity (Mogil et al., 1996). In contrast, N/OFQ was ineffective in reversing analgesia induced by morphine given *i.t.* (Tian et al., 1997), indicating that the peptide acted as a supraspinal, but not a spinal anti-opioid peptide. In addition, all the studies, except the study of Yamamoto et al. (2001), show that *i.c.v.* blockade of NOP receptors produces antinociceptive responses by various approaches, including *i.c.v.* injection of J-113397 in rat carrageenan test (Okuda et al., 2000), or [Nphe^{1}]N/OFQ(1-13)-NH_2 (Calo' et al., 2000b), UFP-101 (Calo' et al., 2002) or NOP antisense oligonucleotide (Rossi et al., 1997) in rat tail-flick test. Morphine-induced supraspinal analgesia is also enhanced by N/OFQ blockade (Candeletti and Ferri, 2000; Rizzi et al., 2000). This may suggest the N/OFQ produces a basal nociceptive tone at the supraspinal level. The nociceptive tone of endogenous N/OFQ might be - at least in part - an effect secondary to anti-stress effect of N/OFQ. The tolerance induced by low

doses of morphine was reversed by NOP receptor antagonists and was absent in NOP receptor or N/OFQ knockout mice. However, the tolerance developed by escalating dose of morphine was not prevented in N/OFQ knockout mice (Chung et al., 2006; Kest et al., 2001). Therefore, the supraspinal nociceptive tone of endogenous N/OFQ may contribute to the tolerance development by low dose of morphine.

4.3.2 Spinal pain regulation of N/OFQ

At the spinal level, although N/OFQ injected intrathecally was initially reported to have no effect on thermal nociception (Reinscheid et al., 1995), a subsequent study reported a trend toward enhanced morphine analgesia by *i.t.* N/OFQ (Grisel et al., 1996) followed by additional support for spinal N/OFQ analgesia (King et al., 1997). In contrast to the supraspinal anti-opioid/hyperalgesic action, spinal administration produces a classical (opioid-like) antinociceptive response by inhibiting transmitter release at primary nocieptive afferent terminals (Inoue et al., 2003; Ko et al., 2006). Indeed N/OFQ has been shown to inhibit the release of transmitters involved in this pathway including glutamate (Faber et al., 1996). Some studies using different NOP antagonist injections intrathecally suggest that endogenous N/OFQ in the spinal cord palys a protective role in the inflammatory but not acute pain status (Rizzi et al., 2006; Yamamoto et al., 2001). However, negative results have also been reported by *i.t.*

injection with J-113397 in an inflammatory pain model (Fu et al., 2006) or the Nphe (Corradini et al., 2001) or JTC-801 (Muratani et al., 2002) in neuropathic pain models.

5. Periaqueductal gray (PAG)

The periaqueductal gray (PAG) is the gray matter located around the cerebral aqueduct within the tectum of the midbrain. It is a major component of a descending pain inhibitory system. The PAG projects to the RVM, which in turn sends its output to dorsal horn laminae important in nociceptive function. The PAG-RVM system is recognized as the central site of action of analgesic agents including opioids, cyclooxygenase inhibitors and cannabinoids (Hohmann et al., 2005; Leith et al., 2007; Yaksh et al., 1976). The major intrinsic circuit within the PAG is a tonically active GABAergic net work and inhibition of this network is an important mechanism for activation of outputs of the PAG. The PAG is subdivided from statistical cluster analysis and developmental studies into four subdivisions, dorsal, dorsolateral, ventrolateral and medial (Beitz and Shepard, 1985). The medial subdivision contains the smallest neurons and exhibits the lowest cell density. The highest cell density occurred in the dorsal subdivision. The lateral division of the PAG contained the largest neurons with cells in the dorsolateral portion being slightly larger than those located ventrolaterally. The study of the projections from the PAG to the nucleus raphe magnus (NRM) indicated

that the ventrolateral subdivision provides about 70% of the total PAG output to NRM, suggesting that the ventrolateral PAG is critically involved in descending inhibition pain pathway (Beitz, 1985).

5.1. Roles of N/OFQ in the PAG

Several brain regions that are involved in pain regulation and enriched with NOP receptors can be the sites of action for the analgesic effect of *i.c.v.* NOP receptor antagonists. The midbrain PAG is one of the very dense distribution areas in the brain of NOP receptors either by immunolabeling (Anton et al., 1996), *in situ* hybridization (Neal et al., 1999a) or autoradiography in rats (Neal et al., 1999a) or in mice (Florin et al., 1997). This area is enriched with ppN/OFQ mRNA in rats (Neal et al., 1999b; Nothacker et al., 1996) or in mice (Boom et al., 1999) or N/OFQ peptides in rats (Neal et al., 1999b) or in human (Witta et al., 2004). The midbrain PAG is the site initiating descending pain inhibitory pathway. The ventrolateral region of the PAG has been proven to be a crucial site for morphine-induced supraspinal analgesia (Yaksh et al., 1976). It is also the site of action that N/OFQ reverses morphine-induced analgesia (Morgan et al., 1997). J-113397 (*i.p.*) has been reported to have antinociceptive effect in rat carrageenan test, nerve injured rats and mouse formalin test but not in mouse hot-plate test (Okuda et al., 2000). It is interesting to note that Yamamoto et al. (2001)

found that *i.c.v.* injection of J-113397 increased the phase II, but not phase I, nociceptive responses in rat formalin test. This result is in contrast to the antinociceptive responses observed by other studies when NOP receptor antagonists were administered *i.c.v.* (Calo' et al., 2000b; Calo' et al., 2002; Okuda et al., 2000; Rizzi et al., 2006).

6. Heterogeneity of NOP receptors

The NOP receptor has been proposed to be heterogeneous from several aspects, including the presence of 1) splicing variants of NOP receptors, 2) two binding sites of ^{125}I-[Tyr^{14}]N/OFQ in the brain, 3) distict distriubiton in the bindings sites of ^{125}I-[Tyr^{10}]N/OFQ(1-11) and ^{125}I-[Tyr^{14}]N/OFQ, and 4) functional heterogeneity of NOP receptors.

6.1. Splicing variants of NOP receptors

The gene encoding murine NOP receptors, which is believed to be a single copy gene, termed *Oprl1* and localized on chromosome 2, was found to have alternative splicing sites (Nishi et al., 1997). Several splicing variant mRNAs have been found in the brain, although none of the encoded protein has yet been identified (Mogil and Pasternak, 2001).

6.2. Binding studies with ^{125}I-[Tyr^{14}]N/OFQ

Binding studies with ^{125}I-[Tyr^{14}]N/OFQ as a radioligand showed curvilinear scatchard plots in the mouse brain which are best fit by nonlinear regression analysis to two sites (Mathis et al., 1997). The high affinity site (K_D: 3.8 pM) is far less abundant in brain tissue (B_{max}: 31.6 fmol/mg protein) than the lower affinity site (K_D: 896 pM; B_{max}: 233 fmol/mg protein). The curvilinear scatchard plot in brain membranes contrasts sharply with linear scatchard plots seen in CHO cells transfected with NOP receptors (K_D: 36 pM) (Table 5). These results suggest there are high and low affinity binding sites of N/OFQ in rodent brains.

6.3. Autoradiographic studies with ^{125}I-[Tyr^{10}]N/OFQ(1-11) and ^{125}I-[Tyr^{14}]N/OFQ

Autoradiographic studies, using ^{125}I-[Tyr^{10}]N/OFQ(1-11) and ^{125}I-[Tyr^{14}]N/OFQ as radioligands, showed that their distributions in the brain of either mice (Mathis et al., 1999) or rats (Letchworth et al., 2000) are different. Binding parameters of ^{125}I-[Tyr^{10}]N/OFQ(1-11) revealed an affinity (K_D) of 235 pM, which is over 100-fold lower than its K_i in inhibition of against ^{125}I-[Tyr^{14}]N/OFQ binding in mouse brain. The binding site of ^{125}I-[Tyr^{10}]N/OFQ(1-11) was, therefore, proposed to be the high affinity site for ^{125}I-[Tyr^{14}]N/OFQ, based from their similar maximal binding densities (B_{max}) (Table 5). The binding density of ^{125}I-[Tyr^{10}]N/OFQ(1-11) is one sixth of that of

^{125}I-[Tyr^{14}]N/OFQ (1.3±0.2 and 7.8±0.3 fmol/mg, respectively) in the rat midbrain PAG (Letchworth et al., 2000), suggesting that [Tyr^{10}]N/OFQ(1-11) might affect a portion of N/OFQ-sensitive NOP receptors. Recently, Dr. Pasternak's group showed that, in both wild type and NOP-knockout mice, N/OFQ displayed similar K_i values in competing the binding of ^{125}I-[Tyr^{10}]N/OFQ(1-11) at the high affinity site (Majumdar et al., 2009). This suggests that the high affinity binding site of N/OFQ still exists in NOP-knockout mice.

6.4. Functional heterogeneity of NOP receptors revealed from the results of Ro 64-6198

As reported previously, we have proven that Ro 64-6198 activates only a subset of the N/OFQ-sensitive NOP receptors in rat PAG (Chiou et al., 2004). Several studies also indicate that Ro 64-6198 mimicked some, but not all, of the effects of N/OFQ (Table 7). N/OFQ (*i.c.v.*) produced anxiolytic effect in both rats and mice but Ro 64-6198 (*i.p.*) was less effective in producing anxiolytic effect in mice at the doses unaffecting motor activity (Jenck et al., 2000). N/OFQ affects locomotor activity in a biphasic manner, increasing locomotion at lower doses but decreasing it at higher doses. Ro 64-6198 failed to fully reproduce the effect of N/OFQ, instead, it inhibited locomotor activity at all doses (Kuzmin et al., 2004). Ro 64-6198 mimicked the effects of N/OFQ to inhibit

contraction of mouse vas deferens, but the effects of Ro 64-6198 were not blocked by neither NOP nor MOP receptor antagonists (Rizzi et al., 2001). Ro 64-6198 regulated urination, but not hypotension or bradycardia, centrally as did N/OFQ (Dayan et al., 2001). In a neuropathic pain model, Ro 64-6198 was less effective in reducing allodynic responses than N/OFQ if given by *i.t.* administration, but was equopotent as N/OFQ if given by intraplantar injection.(Obara et al., 2005). In addition, Ro 64-6198 attenuated the expression of morphine sensitization at higher doses via a mechanism not blocked by a NOP receptor antagonist (Kotlinska et al., 2005). Although Ro 64-6198 increased food intake as N/OFQ, it did not decrease alcohol consumption as N/OFQ (Economidou et al., 2006a). However, it reduced alcohol self-administrtion and prevented relapse-like alcohol drinking (Kuzmin et al., 2007). Taken together, these effects of Ro 64-6198 suggest that there are functional heterogeneity of NOP receptors.

Aims of Study

Hypotheses:

1. **NOP receptors are functionally heterogeneous in vlPAG neurons. This was proven by our previous study using Ro 64-6198 and can also be revealed by (+)-5a Compound, [Tyr^{10}]N/OFQ(1-11) or new NOP receptor antagonists.**
2. **[Tyr^{10}]N/OFQ(1-11) is an agonist for the high, but not low, affinity binding site of N/OFQ in rodent brain. The Ro 64-6198-sensitive NOP receptors might be the NOP receptors exhibiting high binding affinity for N/OFQ.**

Aim 1: To investigate if (+)-5a Compound can also affect a portion of NOP receptors in vlPAG neurons.

(+)-5a Compound has a structure backbone analogous to Ro 64-6198 and Ro 64-6198 acts differently from N/OFQ at NOP receptors in rat PAG slices. If our hypothesis 1 is true, (+)-5a Compound should, like Ro 64-6198, also affect a portion of NOP receptors in vlPAG neurons.

Aim 2: To investigate if (+)-5a Compound affects the same subset of NOP receptors that Ro 64-6198 effects.

If both (+)-5a Compound and Ro 64-6198 affect a portion of NOP receptor in

vlPAG neurons, if they affect the same subset of NOP recpeotrs will be examined.

Aim 3: To characterize the neurochemical and morphological properties of the neurons sensitive to Ro 64-6198 and/or (+)-5a Compound.

If (+)-5a Compound affects the same subset of NOP receptors that Ro 64-6198 effects. The neurochemical and morphological properties of the neurons sensitive and insensitive to Ro 64-6198 and (+)-5a Compound will be examined.

Aim 4: To investigate if [Tyr^{10}]N/OFQ(1-11), an agonist for the high affinity binding site of N/OFQ in rodent brain, also affect a portion of NOP receptors in vlPAG neurons.

Binding studies showed that ^{125}I[Tyr^{14}]N/OFQ (low-affinity site) and ^{125}I[Tyr^{10}]N/OFQ(1-11) (high-affinity site) displayed distinct distribution sites in rodent brains. The binding densities of high-affinity site is one sixth of that of low-affinity sites in the rat PAG, suggesting that [Tyr^{10}]N/OFQ(1-11), which had higher selectivity for the high-affinity site than N/OFQ(1-11), might affect a portion of N/OFQ-sensitive NOP receptors. [Tyr^{10}]N/OFQ(1-11) had higher selectivity for the specific binding site of ^{125}I-[Tyr^{10}]N/OFQ(1-11) than its parent peptide, N/OFQ(1-11) (Table 6). Therefore, we used [Tyr^{10}]N/OFQ(1-11) as a tool to investigate if it can reveal the heterogeneity of

NOP receptors in vlPAG neurons.

Aim 5: To investigate if [Tyr^{10}]N/OFQ(1-11) affects the same subset of NOP receptors that (+)-5a Compound does.

If the aim 4 is true, then the pharmacological files of [Tyr^{10}]N/OFQ(1-11) will be compared with that of (+)-5a Compound to validate if they affect the same subset of NOP receptors.

Aim 6: To characterize the pharmacological properties of two novel NOP receptor antagonists, Compound 24 and SB-612111, and examine if they can differentiate the NOP receptors which are all sensitive to N/OFQ in vlPAG neurons.

We will further examine if the heterogeneity of NOP receptors can be revealed by NOP receptor antagonists, Compound 24 and SB-612111. In addition, their pharmacological properties in vlPAG neurons will be quantitatively characterized.

Materials and methods：

All experiments were conducted with Wistar rats of 9-18 days old and conformed to the guidelines of 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.

1. Brain slice preparations

The midbrain blocks containing the PAG were rapidly dissected from postnatal Wistar rats. Coronal slices (300 or 400 μm) were then sectioned with a vibrotome (microslicer DTK-100, Dosaka) and equilibrated at room temperature in the artificial cerebral spinal fluid (aCSF). The aCSF consisted of (in mM) 117 NaCl, 4.5 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$ and 11.4 dextrose (pH=7.4), and was oxygenated with 95% O_2/5% CO_2. After equilibration for at least one hour, one slices was mounted on a submerged recording chamber and continuously perfused with aCSF at a rate of 2-3 ml/min.

2. Electrophysiological recordings

Blind patch-clamp whole cell recording was performed with 4-8 MΩ glass microelectrodes filled with the internal solution consisting of (in mM): 125 K^+

gluconate, 5 KCl, 0.5 $CaCl_2$, 5 BAPTA, 10 HEPES, 5 MgATP and 0.33 GTPtris (pH=7.3). To elucidate if (+)-5a Compound, like N/OFQ, also induced the NOP receptor-mediated GIRK currents, 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. 3, inset). The membrane currents elicited by voltage ramps were acquired through an Axopatch 200B amplifier (Molecular Devices/Axon Instruments, Union City, CA) with a pClamp 7 software (Molecular Devices/Axon Instruments, Union City, CA) and simultaneously recorded with a chart recorder (Gould RS3200) at a low frequency response of 10 Hz 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 was not deteriorated during the recording period.

3. Quantitative analysis of NOP receptor ligands

3.1. NOP receptor agonists

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 increment of I_{-140} greater than 5% and the induced-current having a reversal potential at around -90 mV (the equilibrium potential of K^+ ions) was considered to be effective (defined as agonist-sensitive). For establishing the concentration-response curves of (+)-5a Compound or [Tyr^{10}]N/OFQ(1-11), 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%$\pm$4% increment (n=26) (Chiou et al., 2002a). The EC_{50} values of (+)-5a Compound and [Tyr^{10}]N/OFQ(1-11) were determined by the refection point of its concentration-response curve produced by logistic fitting: $E=E_{max}/[1+(D/EC_{50})^n]$, where E represents the percentage of increment, Emax the maximal increment, D the concentration of agonist and n the Hill coefficient. To quantitatively evaluate the antagonistic effect of various receptor antagonists, their interactions with (+)-5a Compound or [Tyr^{10}]N/OFQ(1-11) were examined in the same neuron. Given that not all neurons were sensitive to (+)-5a Compound or [Tyr^{10}]N/OFQ(1-11) (see Results), it was not practical to pre-apply the intended antagonist. Therefore, the tested receptor antagonist was applied to (+)-5a Compound or [Tyr^{10}]N/OFQ(1-11)-sensitive neurons after the response of (+)-5a Compound or [Tyr^{10}]N/OFQ(1-11) 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 or [Tyr^{10}]N/OFQ(1-11)-unresponsive neurons, N/OFQ was examined after (+)-5a Compound or [Tyr^{10}]N/OFQ(1-11) 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/OFQ was added in order to verify if the neurons unresponsive to (+)-5a Compound were also insensitive to Ro 64-6198. [Tyr^{10}]N/OFQ(1-11) was tested in the (+)-5a Compound-treated neurons in order to verify the correlation with (+)-5a Compound.

3.2. NOP receptor antagonists

To quantitatively estimate the antagonistic effect of Compound 24 or SB-612111 against N/OFQ- or DAMGO-induced GIRK current, Compound 24 or SB-612111 was applied after the response to N/OFQ or DAMGO had reached a steady state. The GIRK current induced by N/OFQ or DAMGO was quantified as the percent increment of the membrane current recorded at -140 mV (I_{-140}) in each neuron, taking its own I_{-140} before treatment as 100%. For establishing the concentration-response curve of Compound 24 or SB-612111 against N/OFQ- or DAMGO-induced GIRK current, the inhibitory effect of Compound 24 or SB-612111 in each neuron was calculated, taking the effect of N/OFQ or DAMGO in the same neuron as 100 %. The IC_{50} values of Compound 24 and SB-612111 were determined by the refection point of its concentration-response curve

in its inhibition of 0.1 μM N/OFQ-induced GIRK current produced by logistic fitting: $I=I_{max}/[1+(D/IC_{50})^n]$, where I represents the percentage of inhibition, Imax the maximal inhibition, D the concentration of antagonist and n the Hill coefficient.

4. Immunofluorescence staining

For immunofluorescence studies, 0.2% Lucifer yellow (LY) was added in 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 one day, and then dehydrated in 30% sucrose. Dehydrated slices were embedded and re-sectioned into 50 μm sections with a cryostat microtome (Leica CM3050S, Leica Microsystems, Nussloch, Germany). Slice sections were rinsed and washed with phosphate buffered saline (PBS) 3 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, Temecula, CA) in PBST containing 1% BSA overnight at 4 °C. Slice sections were then washed with PBST 3

times, followed by secondary antibody, Alexa 594 (diluted 1:100) (Invitrogen, Carlsbad, CA) for 1 hr. Fluorescent images were acquired with Zeiss LSM 510 Meta confocal microscope (Zeiss, Thornwood, NY) and edited with LSM 510 software (Zeiss, Thornwood, NY).

5. Morphometric analysis

To compare the morphology of (+)-5a Compound-sensitive and -insensitive neurons, the recorded neurons were reconstructed and analyzed. 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 one day. After several washes with PBS, sections were treated with 1% H_2O_2 diluted in PBS for 3 hr, 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, Burlingham, CA), followed by a 3,3'-diaminobenzidine (DAB) kit (SK-4100, Vector Laboratories, Burlingham, CA). The DAB staining was monitored under a microscope (ECLIPSE TE 2000-U, Nikon, Japan) and stopped when appropriate by rinsing the sections with PBS. The sections were then mounted with Histokitt mounting medium (Assistent, Germany). The image of the identified neuron was captured under the

Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a CX9000 CCD camera (MicroBrightField, Williston, VT), and reconstructed 3-dimensionally using Neurolucida (MicroBrightField, Williston, VT). Morphometric analysis of the recorded neurons, including the soma area and dendritic arborization, was performed by Neurolucida Explorer. The dendritic complexity was analyzed by the Sholl concentric ring analysis.

6. Chemicals

(+)-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 Drs. Calo' and Guerrini (University of Ferrara, Ferrara, Italy). Compound 24 was synthesized at University of Ferrara, Italy. We appreciate the generous gift of SB-612111 from Dr Toll (SRI international, Menlo Park, California, USA) (Khroyan et al., 2009). N/OFQ and AF-DX-116 were purchased from Tocris (Bristol, UK) and naloxone was from Sigma (St. Louis, MO). All drugs were dissolved in de-ionized water except (+)-5a Compound ,Ro 64-9198, Compound 24 and SB-612111 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).

7. 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 *t* test, Student pair *t* test, one sample *t* test, one way ANOVA and chi-square test were used for statistical analysis and a p value < 0.05 was considered to be significant.

Results

1. Functional heterogeneity of NOP receptors reveal by (+)-5a Compound

1.1. (+)-5a Compound activated GIRK channels in vlPAG neurons.

(+)-5a Compound (10 μM) shifted the holding current (I_{hold} in Fig. 3A) outwardly and increased the membrane current elicited by a hyperpolarization ramp command from -60 to -140 mV in vlPAG neurons (Fig. 3A). Fig. 3B 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. 3C). The mean reversal potential of (+)-5a Compound-elicited current was -90.5±1.3 mV (n=49), resembling the equilibrium potential of K^+ ions estimated by the Nernst equation. Therefore, the K^+ channel in vlPAG neurons activated by (+)-5a Compound, as by N/OFQ (Chiou et al., 2002a), is the GIRK channel, an inwardly rectifying K^+ channel that is coupled to G proteins (Ikeda et al., 1997).

1.2. (+)-5a Compound was less potent and less efficacious than N/OFQ.

The effect of (+)-5a Compound on GIRK channels was concentration-dependent (0.1-30 μM). To establish its concentration-response curve (Fig. 4A), the magnitude of

GIRK channel activation induced by (+)-5a Compound was quantified from the percent increment of I_{-140} obtained in each neuron as described in the Methods and then, after normalized to the maximal increment (39%±4% increment, n=26) produced by 1 μM N/OFQ (Chiou et al., 2002a), expressed as the percentage of the maximal effect of N/OFQ. (+)-5a Compound, at 10 μM, produced a maximal effect of 47%, as compared to that generated by N/OFQ (Fig. 4A). For a comparison, the concentration-response curves of N/OFQ and Ro 64-6198, taken from our previous studies (Chiou et al., 2004; Chiou et al., 2002a), are also shown in Fig. 4A. In this study, 0.3 μM N/OFQ was also tested and reproduced a similar increment (40.2%±5.4%, n=32) of I_{-140} (filled square in Fig. 4A) as it did before (37.2%±5.3%, n=17) (Chiou et al., 2002a). The estimated EC_{50} of (+)-5a Compound was 605±2 nM, which is about 12 times higher than that of N/OFQ, 52±6.8 nM (Chiou et al., 2002a). The concentration-response curves of (+)-5a Compound and Ro 64-6198 are similar (Fig. 4A) with E_{max}s of 46.9%±4.6% and 61.5%±5.6% (Chiou et al., 2004), respectively.

1.3. (+)-5a Compound activated GIRK channels in about half of the recorded neurons.

(+)-5a Compound (0.1-30 μM) activated GIRK channels in 49 out of 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 mV) of a neuron was independent of its location; either at the superficial or deeper layer of the slice. The numbers of the neurons sensitive to (+)-5a Compound or Ro 64-6198 over the total numbers of tested neurons under various tested concentrations are depicted in Fig. 4A. 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. 4B). Even at the highest tested concentration (30 μM), (+)-5a Compound was still ineffective in some neurons (Fig. 4), as observed before with Ro 64-6198 (Chiou et al., 2004).

1.4. The effect of (+)-5a Compound was antagonized by NOP, but not opioid or M2 muscarinic, receptor antagonists.

In (+)-5a Compound-sensitive neurons, the effect of (+)-5a Compound (0.1-30 μM) in 23 out of 35 (66%) neurons recorded was antagonized by UFP-101 (Fig. 3A), 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 H3, muscarinic and σ receptors, and sodium channels. It is unlikely that (+)-5a Compound activated GIRK channels through histamine H3 receptors since there are few histamine H3 receptors in the PAG (Pillot et al., 2002), and activation of σ receptors will block, not activate, K^+ channels (Zhang and Cuevas, 2005). Among the five subtypes (M1-M5) of muscarinic receptors, the M2 subtype exists in the PAG and activation of M2 muscarinic receptors also results in GIRK channel activation (Sanada et al., 2007). Therefore, we further examined if AF-DX-116, a selective antagonist of M2 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 I_{-140} increased by 10 μM of (+)-5a Compound was 99.9%±2.5% (n=6) of that before treatment.

1.5. N/OFQ activated 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 out

of 43 neurons which were insensitive to the pretreatment of (+)-5a Compound, N/OFQ (0.3 μM) activated GIRK channels (Fig. 5).in a comparable magnitude as in naïve 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. 5). 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.

1.6. (+)-5a Compound-insensitive neurons were 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. 6A). The same result was found in other 8 tested neurons. The I_{140} values after treatment with 10 μM (+)-5a Compound were 99.7%±0.5% (n=9) of the controls and were 98.2%±1.0% (n=9) after further treatment with 10 μM Ro 64-6198. In these neurons, N/OFQ activated GIRK channels in a manner antagonized by UFP-101 (Fig. 6A-C). Conversely, in 5 neurons which were unresponsive to Ro 64-6198, further addition of (+)-5a Compound also had no effect

(Fig. 6D). The I_{-140} values after treatment with 10 μM Ro 64-6198 were 100.6%±0.6% (n=5) of the controls, and were 99.6%±0.6% (n=5) after further treatment with 10 μM (+)-5a Compound. In these neurons, N/OFQ did activate GIRK channels (Fig. 6D) in a manner blocked by UFP-101 (data not shown).

1.7. (+)-5a Compound precluded the effect of Ro 64-6198.

In (+)-5a Compound-sensitive neurons, which have been treated with the maximal effective concentration of (+)-5a Compound (10 μM), further addition of Ro 64-6198 (10 μM) did not cause any additional change in membrane currents elicited by voltage ramps (Fig. 7A) in all of the 11 neurons tested. The I_{-140} values after treatment with 10 μM (+)-5a Compound were 118.8%±3% of the controls (n=11), and were 116.8%±4% (n=11) after further treatment with 10 μM of Ro 64-6198. This result suggests that (+)-5a Compound precluded the effect of Ro 64-6198.

1.8. N/OFQ further enhanced 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. 7B). After further treatment with 0.3 μM N/OFQ, the I_{-140} values in the presence of the maximal effective

concentration (10 μM) of (+)-5a Compound were increased from 119.6%±3.6% to 132.6%±5.9% of the 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, its increment on I_{140} in 10 μM (+)-5a Compound-pretreated neurons was also 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 equi-effective concentration of N/OFQ.

1.9. Most of the (+)-5a Compound-sensitive neurons were 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 on Table 8 and Figure 8.

As shown on Table 8, the mean resting membrane potential, input resistance and capacitance of (+)-5a Compound-sensitive (n=78) and -insensitive (n=70) neurons were not significantly different. Morphometric analysis data showed that (+)-5a Compound-sensitive (n=8 from 6 rats) and (+)-5a Compound-insensitive (n=6 from 5 rats) neurons had comparable soma size and total dendritic length. (+)-5a

Compound-sensitive neurons had more primary dendrites (Fig. 8A), resembling the triangular and multipolar cells described in the Golgi study (Beitz and Shepard, 1985). Besides, compared with (+)-5a Compound-insensitive neurons (Fig. 8B), 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 8C). Neurons sensitive to (+)-5a Compound had more intersections in the Sholl concentric rings particularly in the proximal regions (< 100 μm) (Fig. 8C), indicating that these neurons have more complicated dendritic arbors.

GABAergic neurons account for 50% of all the neurons in the PAG (Mugnaini and Oertel, 1985) and most of them are interneurons (Reichling and Basbaum, 1990). We, therefore, examined whether the recorded neurons were GABAergic through a colocalization of GAD67 immmunofluorescent 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. 9A), and the remaining 9 (22%) neurons were non-GABAergic, suggesting a higher proportion (31/40 vs. 9/40, $p<0.01$, chi-square 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. 9B) and the remaining 16 (53%) neurons were non-GABAergic (Fig. 9C).

2. Quantitative study of [Tyr^{10}]N/OFQ(1-11) in vlPAG

2.1. [Tyr^{10}]N/OFQ(1-11) activated inwardly rectifying potassium channels.

[Tyr^{10}]N/OFQ(1-11), at 3-300 μM, shifted the holding current (I_{hold} in Fig. 10A) outwardly and increased the membrane current elicited by hyperpolarization ramps from -60 to -140 mV voltage-dependently in vlPAG neurons (Fig. 10B). The currents increased at more negative potentials were greater than those at less negative potentials. Thus, the current-voltage (I-V) relationship of [Tyr^{10}]N/OFQ(1-11)-induced current, which was obtained by subtracting the currents in the control from that in the presence of [Tyr^{10}]N/OFQ(1-11), is characterized with inward rectification (Fig. 10C). The reversal potential of [Tyr^{10}]N/OFQ(1-11)-induced current was -92.4$\pm$1.8 mV (n=40), which corresponds to the equilibrium potential of potassium ions (-91 mV) according to the Nernst equation. Therefore, in vlPAG neurons, [Tyr^{10}]N/OFQ(1-11), like N/OFQ (Liao et al., 2010), activated IRK channels which are coupled to G-protein (Ikeda et al., 1997).

2.2. [Tyr^{10}]N/OFQ(1-11) was similarly efficacious, but less potent than, N/OFQ.

The effect of [Tyr^{10}]N/OFQ(1-11) on GIRK channels was

concentration-dependent. To establish its concentration-response curve (Fig. 11), the magnitude of GIRK channel activation induced by [Tyr^{10}]N/OFQ(1-11) was quantified from the increment of I_{140} as described in Methods. The maximal increment was induced by 100 μM [Tyr^{10}]N/OFQ(1-11), which was 34.9±5.5% (n=22) and similar to the maximal effect induced by N/OFQ (1 μM), being 39.4±4% (n=26), in the same preparations (Chiou et al., 2002).

In order to compare potency of [Tyr^{10}]N/OFQ(1-11) with that of N/OFQ, Ro 64-6198 and (+)-5a Compound, the increment of [Tyr^{10}]N/OFQ(1-11) was normalized to the maximal increment (39.4±4%) produced by 1 μM N/OFQ (Chiou et al., 2002), expressed as the percentage of the maximal effect of N/OFQ, as shown in Fig. 11. The estimated EC_{50} value of [Tyr^{10}]N/OFQ(1-11) is 9.0±0.9 μM, which is about 173 times lower than that of N/OFQ, 52.0±6.8 nM (Chiou et al., 2002) obtained in the same preparations. [Tyr^{10}]N/OFQ(1-11) is also less potent than Ro 64-6198 or (+)-5a Compound (Fig. 11)

2.3. The effect of [Tyr^{10}]N/OFQ(1-11) was antagonized by UFP-101 but not naloxone.

To verify if the effect of [Tyr^{10}]N/OFQ(1-11) is mediated through NOP receptors, UFP-101 was applied after the effect of [Tyr^{10}]N/OFQ(1-11) had reached the steady

state. UFP-101 decreased the current induced by [Tyr^{10}]N/OFQ(1-11) but did not change its reversal potential (Fig. 10B). The I_{-140} induced by [Tyr^{10}]N/OFQ(1-11) (100 μM) was reduced by UFP-101 (1 μM) from 132.1±5.9% to 118.6%±4.7% (n=7). The reversal potentials of [Tyr^{10}]N/OFQ(1-11)-induced current in the absence and presence of UFP-101 were -92.4±1.8 mV (n=40) and -90.1±2.7 mV (n=20), respectively. Furthermore, the effect of [Tyr^{10}]N/OFQ(1-11) was unaffected by naloxone, a non-selective opioid receptor antagonist. The I_{-140} increments after treatment with 100 μM [Tyr^{10}]N/OFQ(1-11) in the absence or presence of 1 μM naloxone were not significantly different (134.4%±5.1% *vs*. 134.5%±5.4%, n=6, p=0.96, one sample *t*-test).

2.4. [Tyr^{10}]N/OFQ(1-11) further enhanced GIRK current in (+)-5a Compound-sensitive neurons.

[Tyr^{10}]N/OFQ(1-11) (3-10 μM) activated GIRK channels in 40/60 of the recorded neurons. This phenomenon appears to be similar to the results obtained with (+)-5a Compound (Liao et al., 2010) and Ro 64-6198 (Chiou et al., 2004), which had effect on the NOP receptors in a portion of vlPAG neurons. However, the higher the concentration of [Tyr^{10}]N/OFQ(1-11) tested, the fewer the insensitive neurons (Fig. 11A). This might due to the low potency of [Tyr^{10}]N/OFQ(1-11). When its

concentration was too low, the GIRK current was too small to be distinguished from the baseline. This unlikes the case of (+)-5a Compound or Ro 64-6198. They were ineffective in a portion of tested neurons even at the highest tested concentrations (Chiou et al., 2004; Liao et al., 2010). A distinguished cut-off in the response histogram was observed in the neurons treated with (+)-5a Compound (Fig. 2B in Liao et al., 2010) but not that with [Tyr^{10}]N/OFQ(1-11) (Fig. 11B).

To examine if the population of [Tyr^{10}]N/OFQ(1-11)-sensitive vlPAG neurons are the same subset as those affected by (+)-5a Compound, we applied (+)-5a Compound first and then [Tyr^{10}]N/OFQ(1-11) in the same vlPAG neurons. (+)-5a Compound (10 μM) activated GIRK channels in 12 out of 22 recorded neurons and had no effect in the remaining 10 neurons. In (+)-5a Compound-sensitive neurons, (+)-5a Compound reproduced an increment of I_{-140} (123.6%±4.2%, n=12), as did it before (118.5%±1.9%, n=26) (Liao 2010). In these (+)-5a Compound-sensitive neurons, [Tyr^{10}]N/OFQ(1-11) (100 μM) further enhanced GIRK currents (Fig 12A), increasing I_{-140} to 135.6%±6.3% of the controls (n=12), which is not different from that produced by 100 μM [Tyr^{10}]N/OFQ(1-11) alone (134.9%±5.5%, n=22).

2.5. [Tyr^{10}]N/OFQ(1-11) induced GIRK currents in (+)-5a Compound-insensitive neurons.

In those (+)-5a Compound-insensitve neurons, [Tyr^{10}]N/OFQ(1-11) was effective in activating GIRK currents in 8 out of 10 tested neurons. Fig. 12B demonstrates one of these neurons, in which (+)-5a Compound was ineffective, but [Tyr^{10}]N/OFQ(1-11) activated GIRK channels. The increment of I_{-140} were 131.3%$\pm$5.9% (n=8) (Fig. 12B), which is not different from that produced by 100 μM [Tyr^{10}]N/OFQ(1-11) alone (134.9%$\pm$5.5%, n=22).

2.6. [Tyr^{10}]N/OFQ(1-11) precludes the effect of N/OFQ.

The interaction of [Tyr^{10}]N/OFQ(1-11) with N/OFQ was further investigated. In neurons effectively affected by [Tyr^{10}]N/OFQ(1-11) at the maximal effective concentration (100 μM), further addition of N/OFQ (0.3 μM) did not cause any additional change in membrane currents (Fig. 13) in all of the 7 neurons tested. The I_{-140} values after treatment with 100 μM [Tyr^{10}]N/OFQ(1-11) were 137.7%$\pm$3.7% of controls (n=7), and were 138.1%$\pm$4.1% (n=7) after further treatment with 0.3 μM N/OFQ. The result suggests that [Tyr^{10}]N/OFQ(1-11) precludes the effect (GIRK channel activation) of N/OFQ in vlPAG neurons.

3. Quantitative study of Compound 24 in vlPAG

3.1. N/OFQ activated GIRK channels in vlPAG neurons.

As reported previously, N/OFQ shifted the holding current (I_{hold} in Fig. 14A) of vlPAG neurons outwardly and increased the membrane current elicited by hyperpolarization ramps from -60 to -140 mV voltage-dependently (Fig. 14). The outward current induced by N/OFQ at more negative potentials was greater than that at positive potentials (Fig. 14B). Thus, the current-voltage (I-V) curve of N/OFQ-induced current, which was obtained by subtracting the control current from that in the presence of N/OFQ, was characterized with inward rectification (Fig. 14C). The reversal potential of N/OFQ-induced current was -85±8 mV (n=23), resembling the equilibrium potential of K^+ ions calculated followed the Nernst equation. These results indicate that N/OFQ activated an inwardly rectifying K^+ current in vlPAG neurons which is mediated by G-protein (Ikeda et al., 1997).

3.2. Compound 24 antagonized the effect of N/OFQ concentration-dependently.

Compound 24 reduced the current induced by N/OFQ but did not change its reversal potential (Fig. 14), being -85±8 mV, n=23 and -84±7 mV, n=18, respectively, before and after treatment with Compound 24. To quantitatively compare the interaction of compound 24 with N/OFQ in each neuron, N/OFQ was applied first and, then, followed by Compound 24 plus N/OFQ. Compound 24 (0.3-10 μM) concentration-dependently reduced the GIRK current induced by 0.1 μM N/OFQ (Fig.

15, filled circles). The IC_{50} of Compound 24 estimated from the concentration-inhibition curve was 2.6 ± 0.6 μM (Fig. 15). The times to onset and steady state for the effect of Compound 24 were 10-15 and 25-30 min, respectively (Fig. 14). They are longer than those, being 6-8 and 15-20 min, respectively, needed in the same preparations for UFP-101 (Chiou et al., 2005).

3.3. Compound 24 reduced DAMGO-induced GIRK current at higher concentrations.

Activation of MOP receptors, but not κ-opioid (KOP) or δ-opioid (DOP) receptors (Chieng and Christie, 1994), also induces GIRK current in 30-60% of vlPAG neurons recorded (Behbehani et al., 1990; Chieng and Christie, 1994; Chiou and How, 2001; Chiou and Huang, 1999). Compound 24 was, therefore, challenged against the effect of DAMGO, a selective MOP receptor agonist, to examine the selectivity of Compound 24. As reported previously (Chiou and How, 2001), DAMGO shifted the holding current outwardly and increased the membrane current elicited by hyperpolarization ramps (Fig. 16). The current induced by DAMGO also displayed inward rectification and its reversal potential resembles the equilibrium potential of K^+ ions (Fig. 16B), indicating that DAMGO also activates GIRK channels. The effect of DAMGO was concentration-dependent but not observed in every recorded neuron (Chiou and How,

2001). Among 7 tested neurons, DAMGO (0.3 μM) activated GIRK channels in 5 neurons. In these DAMGO-sensitive neurons, further addition of 1 μM Compound 24, did not affect the GIRK current increased by DAMGO (Fig. 16A). The current after addition of 1 μM Compound 24 was 99.4±1.2 % of that with DAMGO alone. However, when the concentrations of Compound 24 were increased to 3-10 μM, it reduced the GIRK current induced by DAMGO concentration-dependently (Fig. 15, filled square). Compound 24, apparently, was 3 folds more potent in inhibiting the effect of N/OFQ than that of DAMGO (Fig. 15).

3.4. Compound 24 had no effect on membrane current *per se.*

Compound 24 was tested alone to investigate whether this compound has intrinsic agonistic activity at NOP receptors of vlPAG neurons. At the concentration up to 10 μM, Compound 24 had no effect on holding current or the membrane currents elicited by hyperpolarization ramps (Fig. 17). The I_{-140} after treatment with Compound 24 was 99.4±1.5 (n=4) of the control. In neurons unaffected by Compound 24, baclofen, which also activates GIRK channels in vlPAG neurons (Chiou and How, 2001), did activate a K^+ current characterized by inward rectification. This suggests that the negative result of Compound 24 alone is not due to a deterioration or absence of these K^+ channels in the recorded neurons.

4. Quantitative study of SB-612111 in vlPAG

4.1. SB-612111 antagonized the effect of N/OFQ in a concentration-dependently manner.

N/OFQ activated GIRK channels in vlPAG neurons (Fig. 18). The mean reversal potential of N/OFQ-induced current was -90.1±3.6 mV (n=44). To quantitatively analyze the antagonistic effect of SB-612111 against N/OFQ-induced GIRK current, 100 nM N/OFQ was applied first and, then, followed by SB-612111 plus N/OFQ. SB-612111 (0.01-3 μM) concentration-dependently reduced the GIRK current evoked by 100 nM N/OFQ (Fig. 19, filled circles) but did not change its reversal potential (Fig. 18). The IC_{50} of SB-612111 estimated from the concentration-inhibition curve was 87.7±1.2 nM (Fig. 19).

4.2. SB-612111 did not affect the membrane current *per se*.

SB-612111 was tested alone for the possible intrinsic agonistic activity at NOP receptors of vlPAG neurons. At concentrations up to 1 μM, SB-612111 did not affect the holding current or the membrane currents elicited by voltage ramps (Fig. 20). The I_{-140} after treatment with SB-612111 was 101.5%±1.0% (n=7) of the control value. In these SB-612111 (1 μM) unaffected neurons, we further tested the effect of baclofen, a

$GABA_B$ receptor agonist that also activates GIRK channels in vlPAG neurons (Chiou, 2001), to verify if their GIRK channels were functional. Addition of 1 μM baclofen did activate a K^+ current characterized by inward rectification (Fig. 20). This demonstrates that the negative result of SB-612111 alone is not due to an absence of GIRK channels in the recorded neurons or a deterioration of the recording condition.

4.3. SB-612111 did not affect DAMGO-induced GIRK current.

Although SB-612111 was 174-fold more selective at expressed NOP receptors than expressed μ-opioid receptors, it still has nanomolar binding affinity at μ-opioid receptors (Zaratin et al., 2004). SB-612111 was, therefore, challenged against the effect of DAMGO, a selective μ-opioid receptor agonist, to examine the selectivity of SB-612111 in PAG slices. As reported previously (Liao et al., 2009), DAMGO (0.3 μM) outwardly shifted the holding current and increased the membrane currents elicited by hyperpolarization ramps (Fig. 21). The current elicited by DAMGO was also characterized with inward rectification and had a reversal potential at -88.9±2.9 mV, resembling the equilibrium potential of K^+ ions (Fig. 21C). This suggests that DAMGO also activates GIRK channels in vlPAG neurons. As reported previously (Chiou and How, 2001), DAMGO affected only a portion of vlPAG neurons. Among 13 tested neurons, DAMGO (0.3 μM) induced GIRK currents in 8 neurons. In these

DAMGO-sensitive neurons, further addition of SB-612111 at the concentration up to 1 μM, which markedly blocked the GIRK current induced by 100 nM N/OFQ (Fig. 19), did not affect the GIRK current increased by DAMGO (Fig. 21A). The current in the presence of DAMGO plus SB-612111 was 100.4±2.3 % (n=8) of that in the presence of DAMGO alone.

Discussion

1. Functional heterogeneity of NOP receptors

1.1. Functional heterogeneity of NOP receptors revealed by (+)-5a Compound and Ro 64-6198

1.1.1. (+)-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 as compared to N/OFQ might be due to the larger amount of penetration barriers in the brain tissue, compared with 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 (Calo' et al., 2000a).

The lower efficacy of (+)-5a Compound, as compared to N/OFQ, could be due to that (+)-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. 4B). 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).

1.1.2. (+)-5a Compound activates 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. 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 appear to be not involved in (+)-5a Compound-induced GIRK activation in vlPAG neurons. First, there are few H3 receptors in the PAG (Pillot et al., 2002). Second, activation of σ-receptors does not open GIRK channels (Zhang and Cuevas, 2005) although σ-receptors do exist in the PAG (Mei and Pasternak, 2007). Third, although M2-muscarinic receptor activation in the 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. 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 the recorded neurons. Second, the intracellular dialysis nature of the whole cell recording technique precluded us from further investigating if Ro 64-6198 also produced an UFP-101-insensitive effect in these neurons since these neurons have been treated with two drugs, (+)-5a Compound and UFP-101.

1.1.3. (+)-5a Compound activates a subset but not all 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 the GIRK channels in only 53% of the recorded neurons. Among these neurons, 66% was 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, similar to Ro 64-6198 (Chiou et al., 2004), affected only one subset, but not all, of the NOP receptors in vlPAG neurons.

1.1.4. (+)-5a Compound and Ro 64-6198 activate the same subset of NOP receptors in vlPAG neurons.

Several findings from this study suggest that (+)-5a Compound and Ro 64-6198 activate the same subset of NOP receptors in vlPAG neurons. First, a similar ratio (35%) of the recorded neurons were sensitive to (+)-5a Compound and Ro 64-6198 (Chiou et al., 2004). Second, (+)-5a Compound-insensitive neurons were also unresponsive to Ro 64-6198, and *vice versa*, whereas N/OFQ activated GIRK channels in these neurons. Third, (+)-5a Compound, at the maximal effective concentration precluded the effect of Ro 64-6198.

1.1.5. A majority of the (+)-5a Compound-sensitive neurons are GABAergic.

Morphological studies demonstrated that bipolar, triangular and multipolar neurons are present in the PAG (Beitz and Shepard, 1985). (+)-5a Compound-sensitive neurons have three to six primary dendrites resembling the triangular and multipolar neurons. Interestingly, the ratio (53%) of (+)-5a Compound-sensitive neurons in the

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

1.2. Functional heterogeneity of NOP receptors can not be revealed by [Tyr^{10}]N/OFQ(1-11).

1.2.1. [Tyr^{10}]N/OFQ(1-11) activates GIRK mediated by NOP but not MOP receptors.

The current induced by [Tyr^{10}]N/OFQ(1-11) had a reversal potential resembling the equilibrium potential of K^+ ions and was characterized with inward rectification. Therefore, this peptide derivative of N/OFQ mimics the action of N/OFQ, the endogenous peptide agonist of NOP receptors, to activate inwardly rectifying K^+ channels in vlPAG neurons (Liao et al., 2010; Vaughan et al., 1997), which are coupled to G-protein (Ikeda et al., 1997). The effect of [Tyr^{10}]N/OFQ(1-11) was antagonized by UFP-101, suggesting that the effect of [Tyr^{10}]N/OFQ(1-11) is mediated through NOP receptors. The ineffectiveness of naloxone excludes the involvement of opioid receptors in the effect of [Tyr^{10}]N/OFQ(1-11).

1.2.2. [Tyr^{10}]N/OFQ(1-11) is a full agonist of NOP receptor and less potent than N/OFQ.

The maximal increment of I_{140} induced by [Tyr^{10}]N/OFQ(1-11) is comparable to that produced by N/OFQ, suggesting [Tyr^{10}]N/OFQ(1-11) is a full agonist of NOP receptor in vlPAG neurons. However, [Tyr^{10}]N/OFQ(1-11) is 173 folds less potent than N/OFQ in the same preparation (Chiou et al., 2002). This is in agreement with the finding that N/OFQ(1-11) is 10-3950 folds less potent than N/OFQ in culture cell (Reinscheid et al., 1996; Rossi et al., 1997) and 1310 folds less potent in mouse brain homogenates (Mathis et al., 1997) (Table 9). N/OFQ(1-11) (*i.t.*) was also less effective

than N/OFQ in increasing the mouse tail-flick latency (King et al., 1997). Conversely, N/OFQ(1-11), when given by *i.c.v.* injection was more potent than N/OFQ in the mouse tail-flick latency (Rossi et al., 1997). It was approximately 2-fold more potent than N/OFQ in attenuating capsaicin-induced nociception, if given by intraplantar injection (Sakurada et al., 2005) (Table 10). The explanation for the finding that N/OFQ(1-11) was more potent than N/OFQ *in vivo* is that the antinociceptive effects of N/OFQ observed in these studies were mediated by its metabolite, N/OFQ(1-11), but not by N/OFQ itself (Rossi et al., 1997; Sakurada et al., 2005).

1.2.3. [Tyr^{10}]N/OFQ(1-11) affects both (+)-5a Compound-sensitive and -insensitive vlPAG neurons.

Autoradiographic binding studies showed that ^{125}I[Tyr^{14}]N/OFQ and ^{125}I[Tyr^{10}]N/OFQ(1-11), two radioligands of NOP receptors, displayed distinct distribution sites in rodent brains (Letchworth et al., 2000; Mathis et al., 1999). The binding density of ^{125}I[Tyr^{10}]N/OFQ(1-11) resembled the density of the high affinity site for ^{125}I[Tyr^{14}]N/OFQ in the brains of rats (Letchworth et al., 2000) and mice (Mathis et al., 1999). Therefore, the binding site of ^{125}I[Tyr^{10}]N/OFQ(1-11) was suggested to be the high affinity site for N/OFQ (Mathis et al., 1999). The binding densities of ^{125}I[Tyr^{10}]N/OFQ(1-11) is one sixth of that of ^{125}I[Tyr^{14}]N/OFQ (1.3$\pm$0.2 and 7.8$\pm$0.3

fmol/mg, respectively) in the rat midbrain PAG (Letchworth et al., 2000), suggesting that [Tyr^{10}]N/OFQ(1-11) might affect a portion of N/OFQ-sensitive NOP receptors.

Our previous studies demonstrated that, (+)-5a Compound and Ro 64-6198, two non-peptide NOP receptor agonists, affected the NOP receptors in a portion of NOP receptors in vlPAG neurons (Chiou et al., 2004; Liao et al., 2010), but N/OFQ was effective in almost all recorded neurons (Chiou et al., 2004). This raises the possibility that [Tyr^{10}]N/OFQ(1-11) might be, similar to (+)-5a Compound or Ro 64-6198, able to differentiate NOP receptor subsets in vlPAG neurons. However, the present results nullify this hypothesis. First, [Tyr^{10}]N/OFQ(1-11) displayed a different pharmacological profiles as (+)-5a Compound in activating GIRK currents in vlPAG neurons. (+)-5a Compound displayed only 47% efficacy of N/OFQ, but [Tyr^{10}]N/OFQ(1-11) was as efficacious as N/OFQ. Second, [Tyr^{10}]N/OFQ(1-11) affected both (+)-5a Compound-sensitive and -insensitive neurons. Third, [Tyr^{10}]N/OFQ(1-11) precluded the effect of N/OFQ in the same neuron, suggesting [Tyr^{10}]N/OFQ(1-11) affects all the N/OFQ-sensitive NOP receptors.

1.3. NOP receptor heterogeneity can not be differentiated by NOP receptor antagonists.

Our finding that functional heterogeneity of NOP receptors revealed by Ro

64-6198 and (+)-5a Compound, both are NOP receptor agonists, in vlPAG neurons. In the same preparation, N/OFQ activated GIRK channels through NOP receptors in almost all (96%) of the recorded neurons (Chiou et al., 2002). Compound 24 and SB-612111, both are NOP receptor antagonists, antagonized the effect of N/OFQ in all vlPAG neurons which sensitive to N/OFQ. In addition, both antagonists have no effect on membrane current when given alone. These results suggest that NOP receptor antagonists can not differentiate heterogeneity of NOP receptors.

1.4. Heterogeneity of NOP receptors

Although single binding site of N/OFQ in the brain was originally reported (Dooley and 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 results from binding studies. A saturation binding study with ^{125}I-[Tyr^{14}]N/OFQ in mouse brain revealed curvilinear Scatchard plots (Mathis et al., 1997), suggesting that there are high and low binding affinity sites of N/OFQ in the mouse brain. Autoradiographic studies also showed that the distribution sites of these two ligands were significantly different in the brain of mice (Mathis et al., 1999) and rats (Letchworth et al., 2000). However, our study using [Tyr^{10}]N/OFQ(1-11) can neither distinguish the subset of NOP receptors sensitive or insensitive to (+)-5a

Compound/Ro 64-6198 (Chiou et al., 2004; Liao et al., 2010) nor differentiate different affinity binding sites of NOP receptors (Mathis et al., 1999). Recently, the binding affinity of N/OFQ at the binding site of ^{125}I-[Tyr^{10}]N/OFQ(1-11) was found to be unchanged in the brains of NOP receptor knock-out mice, as compared with the wild types (Majumdar et al., 2009). Therefore, the functional role of ^{125}I-[Tyr^{10}]N/OFQ(1-11) remains to be further clarified.

Several lines of evidence showed that Ro 64-6198 mimicked some but not other functions of N/OFQ *in vivo* and *in vitro* (Table 7), such as high dose of 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 anxiolytic action in rats but less effective in mice (Jenck et al., 2000), the increase in food intake but not the decrease in alcohol consumption (Economidou et al., 2006a), and the antiallodynic 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, of actions of N/OFQ. Taken together with the current findings with (+)-5a 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 and Lambert, 2004; Chiou et al., 2007).

2. Pharmacological characterization of two NOP receptor antagonists, Compound 24 and SB-612111, in vlPAG neurons.

2.1. Compound 24 possessed moderate antagonist potency at native NOP receptors in PAG.

The IC_{50} value (2.6 μM) of Compound 24 in inhibiting the effect of N/OFQ in vlPAG neurons is 17,000 times higher than that (0.15 nM) obtained in cloned receptors expressed in culture cells. The lower potency of Compound 24 observed in midbrain PAG slices might be due to the more penetration barriers in the brain tissue, compared with culture cells. However, the antagonistic potency of Compound 24 obtained in the mouse vas deferens tissue (pA2=8.24) (Fischetti et al., 2008) is still 450 times higher than that obtained in the brain tissue of our study. Although in both preparations (Table 11), the potencies of either the endogenous agonist, N/OFQ, or the peptide antagonists, UFP-101 and [Nphe^1]N/OFQ-(1-13)-NH_2 are at the same order (Chiou et al., 2002; Chiou et al., 2007). The reason for this discrepancy is not clear. Compound 24 has been shown to have good brain permeability in the mouse (Goto et al., 2006), suggesting it has high lipophilic property which might increase the non-specific bindings during penetrating the brain tissue.

2.2. Compound 24 exhibites moderate selectivity.

In vlPAG neurons, activation of MOP, but not DOP or KOP, receptors also results in membrane hyperpolarization (Chieng and Christie, 1994) due to GIRK channel activation. In this preparation, we have shown that N/OFQ-induced GIRK channel activation is mediated through NOP receptors from several studies using various specific antagonists (Chiou and Fan, 2002; Chiou et al., 2002b; Chiou et al., 2005). Compound 24 acted as an antagonist at these NOP receptors. Since Compound 24 also inhibited the GIRK channel current activated by DAMGO, a MOP receptor selective agonist, at concentrations higher than 3 μM, it is suggested that Compound 24 also acted as an MOP antagonist at higher concentrations. The inhibitory potency of Compound 24 at NOP receptors was only 3 folds higher than that at MOP receptors.

2.3. SB-612111 with potent antagonist activity at NOP receptor.

The pIC_{50} value (7.06) of SB-612111 obtained in the present study using rat vlPAG slices containing native NOP receptors is higher than pK_b or pA_2 values (8.16-9.7) obtained in studies using rodent vas deferens and guinea pig lieum preparations (Spagnolo et al., 2007), mouse cortex synaptosomes (Spagnolo et al., 2007) or cell cultures expressing NOP receptors (Camarda et al., 2009b; Spagnolo et al., 2007; Zaratin et al., 2004) (Table 12). The reason for this difference might be attributed to the more penetration barriers in brain tissue preparations, compared with cultured cells or

synaptosome preparations. Alternatively, it remains to be elucidated if the difference is due to the different sensitive between presynaptic NOP receptors, which were targeted in peripheral preparations and synaptosomes, and postsynaptic ones, which were studies in our preparations. Similar findings that higher potency of several NOP receptor ligands in cell culture expressing NOP receptors than in rodent tissues have been reported (Hashimoto et al., 2000). The potency of SB-612111 in our preparation is, however, higher than that ($pIC_{50:}$ 6.69) obtained in the CHO cells expressed with human NOP receptors, which were functionally assayed by the chimeric $G\alpha_{qi5}$ protein-mediated calcium mobilization (Camarda et al., 2009). This might be due to the chimeric protein $G\alpha_{qi5}$ was used since the binding affinity of SB-612111 in their study (Camarda et al., 2009) is lower than that using native G-proteins in CHO cells (Spagnolo et al., 2007).

2.4. SB-612111 is devoid of intrinsic activity.

SB-612111 did not exhibit any agonistic activity in our preparations at the concentration up to 1 μM. This is in line with previous reports in the culture cells, peripheral preparations and mouse cortical synaptosomes (Spagnolo et al., 2007). This characteristic of SB-612111, a pure NOP receptor antagonist without intrinsic activity, makes it valuable in elucidating the functional roles of endogenous N/OFQ *in vivo*

(Khroyan et al., 2009; Rizzi et al., 2007; Zaratin et al., 2004).

2.5. SB-612111 displays highly selectivity for NOP receptors.

In the present study, we also verified the selectivity of SB-612111 for NOP receptors in brain tissue preparations. In vlPAG neurons, activation of μ-opioid receptors, but not κ- or δ-opioid receptors, also results in membrane hyperpolarization by activation of GIRK channels (Chieng and Christie, 1994). Therefore, κ-opioid and δ-opioid receptors are unlikely the targets mediating GIRK channel activation. Furthermore, SB-612111 did not affect the GIRK current activated by DAMGO, a μ-opioid receptor selective agonist. Therefore, it is reasonably to conclude that the antagonism of SB-612111 is selective for NOP receptors among the opioid receptors in the vlPAG. This result extends previous findings that SB-612111 neither displayed any activity at recombinant μ-opioid receptors (Zaratin et al., 2004) and nor affected morphine-induced antinociception in the tail-flick assay (Khroyan et al., 2009).

2.6. Development of NOP receptor antagonists.

Several peptide and non-peptide NOP receptor antagonists have been developed since 1998. The characteristic of susceptible to peptidase hydrolysis in peptide antagonists limits their *in vivo* applications. Early developed non-peptide antagonists are

flawed by their non-specificity or by their residual agonist activity (Chiou et al., 2007). J-113397 was developed as a very potent and selective antagonist of NOP receptors without intrinsic activity (Chiou and Fan, 2002; Ozaki et al., 2000b). However, it was later found to have psychomimetic central activity (Koizumi et al., 2004) which is likely attributed to its affinity at σ receptors (Chiou et al., 2007). UFP-101 has widely determined as an ideal NOP receptor antagonist in a series of *in vitro* and *in vivo* studies (Calo' et al., 2005). Compound 24 has the merit of being a non-peptide NOP receptor antagonist and devoid of intrinsic activity. However, its non-specificity at higher concentrations might limit its usefulness in the studies investigating the functional roles of endogenous of N/OFQ. On the other hand, SB-612111, with the merits of non-peptide nature, good potency, specificity and devoid of intrinsic activity, is useful NOP receptor antagonist in investigating the physiological or pathological roles of endogenous N/OFQ. Currently, SB-612111 amd Compound 24 (product name is BAN ORL 24) are availableat Tocris Bioscience.

Conclusion

It is concluded that in vlPAG neurons, functional heterogeneity of NOP receptors can be revealed by Ro 64-6198 and (+)-5a Compound, but not by [Tyr^{10}]N/OFQ(1-11) or NOP receptor antagonsits. (+)-5a Compound affects the same subset of NOP receptors as Ro 64-6198 effects in vlPAG neurons. These neurons are mostly GABAergic and morphologically distinct from the (+)-5a Compound-insensitive ones. [Tyr^{10}]N/OFQ(1-11) precluded the effect of N/OFQ, suggesting it affects all N/OFQ-sensitive NOP receptors. Thus, the functions of different binding sites of N/OFQ in rodent brain remain to be clarified. Although neither Compound 24, nor SB-612111 can differentiate NOP receptor subsets in vlPAG neurons, we have proven Compound 24 is a competitive antagonist of NOP receptors with moderate potency and selectivity, but SB-612111 is a pure, potent and selective antagonist of NOP receptors.

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Tables and Figures

Table 1. Peptide NOP receptor agonists

Ligand	Ki† / EC_{50}‡ (nM)	References
	NOP	
N/OFQ	1†/1.44-14.5‡	(Guerrini et al., 1997; Reinscheid et al., 1995)
N/OFQ(1-11)	131†/10-138‡	(Mathis et al., 1997; Reinscheid et al., 1996)
N/OFQ(1-13)-NH_2	17.8‡	(Guerrini et al., 1997)
Cyclo[Cys^{10}, Cys^{14}]N/OFQ	0.12†/4.37‡	(Ambo et al., 2001)
[Arg^{14}, Lys^{15}]N/OFQ	0.32†/0.76‡	(Okada et al., 2000; Rizzi et al., 2002)
[pX]Phe^4N/OFQ(1-13)-NH_2	0.015-71†/0.1-1047‡	(Bigoni et al., 2002)
[(pF)Phe^4,Arg^{14},Lys^{15}]N/OFQ-NH_2 (UFP-102)	0.02†/0.067-2.69‡	(Carra et al., 2005)
[(pF)Phe^4,Aib^7,Aib^{11},Arg^{14},Lys^{15}]N/OFQ-NH_2	0.43-0.52‡	(Peng et al., 2006)
[(pF)Phe^4,Aib^7,Arg^{14},Lys^{15}]N/OFQ-NH_2 (UFP-112)	0.02†/0.02-4.6‡	(Arduin et al., 2007)
OS-461, OS-462, OS-500	0.43-0.9†/11-190‡	(Economidou et al., 2006b)
c[Cys(7,10)]N/OFQ(1-13)-NH_2	0.1†/0.08‡	(Kitayama et al., 2007)

OS-461: N-α-6-guanidinohexyl-ltyrosil-L-tyrosyl-L-arginyl-L-tryptophanamide

OS-462: N-α-6-guanidinohexyl-3,5-dimethyl-ltyrosyl-L-tyrosyl-N-[(R)-1-(2-naphthyl)ethyl]-L-argininamide

OS-500: N-α-6-guanidinohexyl-3,5-dimethyl-L-tyrosyl-3,5-dimethyl-L-tyrosyl-N-[(R)-1-(2-naphthyl)ethyl]-L-argininamide

(Modified from Chiou et al., 2007)

Table 2. Non-peptide NOP receptor agonists

Ligand	Ki† / EC_{50}‡ (nM)		References
	NOP	Other receptors / Transporters	
Buprenorphine	8.4‡	0.51‡ (MOP)	(Raynor et al., 1995; Wnendt et al., 1999)
Ro 64-6198	0.3-0.39†/0.32‡	36-47† (MOP); 89-214† (KOP) 1380-3787† (DOP)	(Jenck et al., 2000; McLeod et al., 2004)
NNC 63-0532	7.3†/305‡	209† (D2); 133† (D3); 140† (MOP); 405† (KOP)	(Guerrini et al., 2004; Thomsen and Hohlweg, 2000)
(+)-5a Compound	0.55†/65-605‡	537† (MOP); 309† (KOP); 2138† (DOP)	(Kolczewski et al., 2003; Liao et al., 2010)
W-212393	0.5†	76† (MOP); >1000† (KOP); >1000† (DOP) 11† (SERT)	(Teshima et al., 2005)
3-(4-piperidinyl)indoles[a]	18†/27200‡	500† (MOP); 1890† (KOP); >5000† (DOP)	(Bignan et al., 2006)
3-(4-piperidinyl)pyrrolo[2,3-b]pyridines[b]	4†/4200‡	390† (MOP); 2780† (KOP); >5000† (DOP)	(Bignan et al., 2006)
PCPB	0.12-2.1†	29† (DOP); 21† (MOP); 15† (KOP)	(Hirao et al., 2008a)
MCOPPB	0.09†	1.02† (MOP); 23† (KOP)	(Hirao et al., 2008b)
SCH 221510	0.3†/12‡	2854† (DOP); 65† (MOP); 131† (KOP)	(Varty et al., 2008)
SCH 225288	0.38†/1.35‡	21† (MOP); >39† (KOP); 773† (DOP)	(McLeod et al., 2009)
SCH 486757	4.6-6.7†	972† (MOP); 590† (KOP)	(McLeod et al., 2010)
HPCOM	1.44†/12‡	201† (MOP); >577† (KOP); >5000† (DOP)	(Hayashi et al., 2010)

SERT: serotonin transporter

Ro 64-6198: (1*S*,3*aS*)-8-(2,3,3*a*,4,5,6-Hexahydro-1*H*-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one

NNC 63-0532: (8-naphthalen-1-ylmethyl-4-oxo-1-phenyl-1,3,8-triaza-spiro[4.5]dec-3-yl)-acetic acid methyl ester

(+)-5a Compound: (3a*S*,6a*R*)-1-(*cis*-4-Isopropylcyclohexyl)-5'-methyl-2'-phenylhexahydrospiro[piperidine-4,1'-pyrrolo[3,4-*c*]pyrrole]

W-212393: 2-(3-[1-((1R)-acenaphthen-1-yl)piperidin-4-yl]-2,3-dihydro-2'-oxo-benzimidazol-1-yl)-N-methylacetamide

PCPB: 2-(3,5- dimethylpiperazin-1-yl)-1-[1-(1-methylcyclooctyl)piperidin-4-yl]-1*H*-benzimidazole

MCOPPB: 1-[1-(1-Methylcyclooctyl)-4-piperidinyl]-2-[(3R)-3-piperidinyl]-1H –benzimidazole

SCH 221510: 8-[bis(2-Methylphenyl)methyl]-3-phenyl-8-azabicyclo[3.2.1]octan-3-ol

SCH 225288: endo-3-[2-(aminomethyl)phenyl]-8-[bis(2-chlorophenyl)methyl]-8-azabicyclo-[3..2.1]octan-3-ol

SCH 486757: 8-[bis(2-chlorophenyl)methyl]-3-(2-pyrimidinyl)-8-azabicyclo-[3.2.1.]octan-3-ol

HPCOM:[1-[4-(2-[hexahydropyrrolo[3,4-c]pyrrol-2(1H)-yl]-1H-benzimidazol-1-yl)piperidin-1-yl]cyclooctyl]methanol

[a]The data of the best congener, compound 11, are shown. [b]The data of the best congener, compound 23, are shown.

(Modified from Chiou et al., 2007)

Table 3. Peptide NOP receptor antagonists

Ligand	$pA_2^{\dagger}$ / $pIC_{50}^{\ddagger}$	pKi	pEC_{50}	Other Receptor	References
Ac-RYYRIK-NH_2/Ac-RYYRWK-NH_2	8.7-9.1†	8.1-8.3	8.1-9.3	—	(Dooley et al., 1997; Mason et al., 2001; Thomsen et al., 2000)
ZP-120 (Ac-RYYRWKKKKKKK-NH_2)	9.5†	9.6	8.88	—	(Kapusta et al., 2005; Rizzi et al., 2002a)
III-BTD	6.6-6.9†	—	—	MOP & DOP agonist	(Bigoni et al., 2000)
[$Phe^1\psi(CH_2\text{-}NH)Gly^2$]N/OFQ(1-13)-$NH_2$	6.7-7.6†/6.1‡	8.9-9.6	6.9-8.5	—	(Calo' et al., 2000c; Guerrini et al., 1998; Mason et al., 2001)
[$Nphe^1$]N/OFQ(1-13)-NH_2	6-8.45†	—	—	—	(Calo' et al., 2000b)
[$Nphe^1$,Arg^{14},Lys^{15}]N/OFQ-NH_2 (UFP-101)	6.9-9.1†	10.2	—	—	(Calo' et al., 2002; Chiou et al., 2005)
[$Nphe^1$,(pF)Phe^4,Aib^7,Aib^{11},Arg^{14},Lys^{15}]N/OFQ-NH_2	8.4-8.5†	—	—	—	(Peng et al., 2006)
Ac-RYYRIK-ol	8.46-8.52‡	8.85-9.4	9.09		(Gunduz et al., 2006)
Ac-RYYRIR-ol	7.99-8.2†	8.3-9.35	6.7-9.1	—	(Bojnik et al., 2010)

(Modified from Chiou et al., 2007)

Table 4. Non-peptide NOP receptor antagonists

Ligand	$pA_2^{\dagger}$ / $pIC_{50}^{\ddagger}$	pKi	pEC_{50}	Other Receptor	References
Naloxone benzoylhydrazone	5.7-6.9†	7.3-7.6	6.1-7	MOP partial agonist KOP agonist	(Bigoni et al., 2002; Chiou, 2000; Chiou, 2001)
TRK-820	—	6.42	—	KOP agonist; MOP antagonist	(Mizoguchi et al., 2003; Seki et al., 1999; Zaveri, 2003)
J-113397	8.2-8.9†/7.6-8.3‡	8.56	—	—	(Chiou and Fan, 2002; Ozaki et al., 2000a)
Trap-101 (Achiral analogue of J-113397)	7.75†	—	—	—	(Trapella et al., 2006)
JTC-801	5.59‡	7.35	—	—	(Yamada et al., 2002)
SB-612111	8.3†/7.06‡	9.48	—	—	(Zaratin et al., 2004)
Octahydrobenzimidazol-2-ones[a]	—	8	—	MOP antagonist	(Chen et al., 2004)
2-(1,2,4-oxadiazol-5-yl)-1H-indole[b]	7.3-7.4‡	—	—	—	(Sugimoto et al., 2006)
Compound 24	5.6-9.82‡	9.6	<6	MOP antagonist	(Goto et al., 2006; Liao et al., 2009)
Benzimidazole derivatives[c]	9.2‡	8.9	—	—	(Okamoto et al., 2008)
N-biarylmethyl spiropiperidine[d]	9.28‡	8.7	—	—	(Yoshizumi et al., 2008)
Arylpyrazole derivatives[e]	9.51‡	9.28	—	—	(Kobayashi et al., 2009)
4-aminoquinazoline[f]	7.77‡	8.85	—	—	(Okano et al., 2009)
GF-4	7.27-7.82†	7.46	—	—	(Volta et al., 2010)

TRK-820: (-)-17-cyclopropylmethyl-3,14b-dihydroxy-4,5'-epoxy-6b-[N-methyl-trans-3-(3-furyl)acrylamido]morphinan hydrochloride

J-113397: 1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one
SB-612111: (-)-*cis*-1-Methyl-7-[[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl]-6,7,8,9-tetrahydro-5*H*-benzocyclohepten-5-ol
Compound 24: 1-Benzyl-N-[3-spiro[isobenzofuran-1(3H),4'-piperidine]-1-yl] propyl-D-proline amide
GF-4: 1-[1-Cyclooctylmethyl-5-(1-hydroxy-1-methyl-ethyl)-1,2,3,6-tetrahydro-pyridin-4-yl]-3-ethyl-1,3-dihydro-benzoimidazol-2-one
[a]The data of the best congener, compound 23, are shown. [b]The data of the best congeners, compounds 39 and 67, are shown. [c]The data of the best congener, compound 28, are shown. [d]The data of the best congener, compound 37, are shown. [e]The data of the best congener, compound 31, are shown. [f]The data of the best congener, compound (1R-2S)-17, are shown.

(Modified from Chiou et al., 2007)

Table 5. Saturation analysis of ^{125}I-[Tyr^{14}]N/OFQ and ^{125}I-[Tyr^{10}]N/OFQ(1-11) binding in transfected CHO cells and mouse brain

Radioligand	CHO Cells	Mouse Brain	
	K_D (pM)	K_D (pM)	B_{max} (fmol/mg protein)
^{125}I-[Tyr^{10}]N/OFQ(1-11)	2700 ± 700	235 ± 7.8	43.2 ± 4.6
^{125}I-[Tyr^{14}]N/OFQ			
High affinity site	36.7 ± 1.6	3.8 ± 3.3	31.6 ± 17.8
Low affinity site	Not applicable	896 ± 636	233 ± 154

Binding with ^{125}I-[Tyr^{10}]N/OFQ(1-11) in CHO cells and mouse brain homogenates represents the means ± SEM of three independent experiments. Nonlinear regression analysis yielded a single site for ^{125}I-[Tyr^{10}]N/OFQ(1-11) in both tissue sources. The Bmax values in the CHO cells varied among preparations.

(Modified from Mathis et al., 1999)

Table 6. Binding affinity of N/OFQ derived peptides at ^{125}I-[Tyr^{10}]N/OFQ(1-11) and ^{125}I-[Tyr^{14}]N/OFQ binding sites in mouse brain and expressed NOP receptors in CHO cells.

Competition ligand	Mouse brain				CHO_{hNOP} cells			
	^{125}I-[Tyr^{10}]N/OFQ(1-11)		^{125}I-[Tyr^{14}]N/OFQ		^{125}I-[Tyr^{10}]N/OFQ(1-11)		^{125}I-[Tyr^{14}]N/OFQ	
	K_D (nM)	K_i (nM)	K_D (nM)	K_i (nM)	K_D (nM)	K_i (nM)	K_D (nM)	K_i (nM)
	0.235		0.0038 / 0.896 [a]		2.7		0.037	
N/OFQ		0.007		0.17		0.14		0.055
[Tyr^{14}]N/OFQ		0.007		0.65		0.049		0.05
N/OFQ(1-11)		8.7		262		41.4		125
[Tyr^{10}]N/OFQ(1-11)		1.6		79.8		-		-

[a] Showed two different affinity binding sites

(Modified from Mathis et al., 1999)

Table 7. Comparison of the effects of Ro 64-6198 and N/OFQ

Effect of N/OFQ	Effects of Ro 646198	References
Hyperalgesia	-	(Jenck et al., 2000)
Anxiolysis	± (Less effective in mice)	(Jenck et al., 2000)
Central regulation of urination	+	(Dayan et al., 2001)
Central Hypotension	-	(Dayan et al., 2001)
Central Bradycardia	-	(Dayan et al., 2001)
Central Sympathoinhibiiton	-	(Dayan et al., 2001)
↓ Mouse vas deference contraction	+ (Not blocked by NOP antagonists)	(Rizzi et al., 2001)
↓ Rat vas deference contraction	+	(Rizzi et al., 2001)
Anxiolytic-like effects	+	(Dautzenberg et al., 2001)
Memory impairment	+	(Higgins et al., 2002)
Reverse CRF-anorexia (low dose)	+	(Ciccocioppo et al., 2002)
Hyperphagia (high dose)	+	(Ciccocioppo et al., 2002)
No conditioning placement preference	+	(Le Pen et al., 2002)
Antitussive	+	(McLeod et al., 2002)
Hypolocomotion (high dose)	+	(Higgins et al., 2002; Kuzmin et al., 2004)
Hyperlocomotion (low dose)	-	(Kuzmin et al., 2004)
↓ Ethanol-induced CPP	+	(Kuzmin et al., 2003)
K^+ channel activation in rat PAG slices	± (Only in 1/3 of the recorded neurons)	(Chiou et al., 2004)

Neuropathic pain model Antinociceptive (i.t.)	i.t. (Less potent than N/OFQ) i.pl. (As potent as N/OFQ) s.c. (Ineffective, no hyperalgesia)	(Obara et al., 2005)
↓ Morphine-induced hyperlocomotion	↓ Morphine-induced hyperlocomotion (not NOP-mediated)	(Kotlinska et al., 2005)
↓ Alcohol consumption ↑ Food intake	↑ Alcohol intake at higher dose (via MOP, 1 mg/kg, i.p.) No effect at 0.3 mg/kg ↑ Food intake	(Economidou et al., 2006a)
Reduction of alcohol self-administration	Reduction of alcohol self-administration Prevent relapse-like alcohol drinking	(Kuzmin et al., 2007)
↓ Learning and memory	↓ Object recognition task	(Goeldner et al., 2008)
↓ SIA Hyperalgesia Hyperalgesia	↑ vocalization Tail-flick- ↓ SIA (0.3-3 mg/kg, i.p.) Hot-plate-↑ jump latency (0.3-3 mg/kg, i.p.) ↑ paw licking latency (3 mg/kg, i.p.) Shock threshold- no effect	(Reiss et al., 2008)
Antinociceptive	Thermo-water pain test- ↑ latency (0.001-0.01 mg/kg, i.v.)	(Podlesnik et al., 2011)
↓ Spatial learning and memory	+	(Kuzmin et al., 2009)
↓ Contextual fear learning	+	(Goeldner et al., 2009)
↓ Remifentanil self-administration	↓ Remifentanil self-administration (0.32 mg/kg, i.v.)	(Podlesnik et al., 2011)

CRF: Corticotropin-releasing factor
CPP: Conditioned place preference
SIA: Stress induced analgesia

Table 8. Comparison in the electrophysiological and morphometric properties between (+)-5a-insensitive and -sensitive neurons

Parameters	(+)-5a-insensitive	(+)-5a-sensitive
Membrane potential (mV)	-66.7 ± 0.4	-67.2 ± 0.5
Input resistance (MΩ)	329 ± 27	346 ± 33
Capacitance (pF)	59.7 ± 2.5	61.7 ± 2.9
Soma area (μm^2)	235.4 ± 56.7	364.3 ± 61.6
Number of primary dendrites	3.0 ± 0.3	4.4 ± 0.5*
Number of branching nodes	2.8 ± 0.3	7.0 ± 1.1**
Number of terminal tips	5.8 ± 0.4	11.5 ± 1.1***
Highest order of dendrites	2.8 ± 0.3	3.9 ± 0.3*
Total dendritic length (μm)	1476.7 ± 147.2	1732.8 ± 211.2

The electrophysiological properties were taken from 70 (+)-5a-insensitive and 78 (+)-5a-sensitive neurons. The recorded neurons either sensitive (n=8 from 6 rats) or insensitive (n=6 from 5 rats) to (+)-5a Compound were traced by biocytin-conjugated DAB staining and their 3-D morphology was reconstructed by Neurolucida and morphometrically analyzed by Neurolucida Explorer. Data are mean ± S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.005$ *vs.* the (+)-5a-insensitive group (Student *t*-test).

Table 9. Quantitative *in vitro* studies of N/OFQ(1-11) and [Tyr^{10}]N/OFQ(1-11)

			N/OFQ(1-11)			
Preparation	N/OFQ effect	Ki †/$EC_{50}^{\ddagger}$ (nM)	N/OFQ(1-11) effect	$Ki^{\dagger}/EC_{50}^{\ddagger}$ (nM)	Potency ratio ([b])	Reference
CHO_{hNOP}	Receptor binding	0.19†	Receptor binding	138†	726	(Reinscheid et al., 1996)
CHO_{hNOP}	Decrease of cAMP	1.05‡	Decrease of cAMP	>10‡	>9.5	(Reinscheid et al., 1996)
CHO_{hNOP}	Receptor binding	0.1†	Receptor binding	55†	550	(Rossi et al., 1997)
$HEK293_{hNOP}$	Decrease of cAMP	0.04‡	Decrease of cAMP	158‡	3950	(Lee and Ho, unpublished data)
Mouse brain homogenates	Competion with [^{125}I][Tyr^{14}]N/OFQ binding	0.1†	Competed with [^{125}I][Tyr^{14}]N/OFQ	131†	1310	(Mathis et al., 1997)

			[Tyr^{10}]N/OFQ(1-11)			
Preparation	N/OFQ effect	$Ki^{\dagger}/EC_{50}^{\ddagger}$ (nM)	[Tyr^{10}]N/OFQ(1-11) effect	$Ki^{\dagger}/EC_{50}^{\ddagger}$ (nM)	Potency ratio ([c])	Reference
CHO_{hNOP}	Receptor binding*	0.04†	Receptor binding	2.7†	9.3	(Mathis et al., 1999)
$HEK293_{hNOP}$	Decrease of cAMP	0.04‡	Decrease of cAMP	126‡	3150	(Lee and Ho, unpublished data)
Mouse brain	Receptor binding*	0.004†/0.9† ([a])	Receptor binding	0.235†	58.8	(Mathis et al., 1999)
Rat PAG slices	Stimulation of K^+ current	52‡	Stimulation of K^+ current	8980‡	173	The present study

* Saturation studies of [Tyr^{14}]N/OFQ binding

[a] Showed two different affinity binding sites; [b] N/OFQ(1-11)：N/OFQ; [c] [Tyr^{10}]N/OFQ(1-11)：N/OFQ

Table 10. Quantitative *in vivo* studies of N/OFQ(1-11)

Model	Animal	Route	N/OFQ effect	N/OFQ dose	Effect	Effective dose	Reference
Tail-flick assay	Mouse	i.t.	Analgesia	20 μg	Analgesia	20 μg	(King et al., 1997)
Tail-flick assay	Mouse	i.c.v.	Hyperalgesia	10-20 μg	Analgesia	10 μg	(Rossi et al., 1997)
Tail-flick assay	Mouse	i.c.v.	-	-	Analgesia	20 μg	(Mathis et al., 1998)
Morphine-withdraw syndrome	Rat	i.c.v.	-	-	Attenuatino	20 μg	(Kotlinska et al., 2004)
Capsaicin-induced nociception	Mouse	i.p.	Attenuation	160 pmol*	Attenuation	77 pmol*	(Sakurada et al., 2005)

*, ED_{50}

i.t., intrathecal

i.c.v., intracerebroventricular

i.p., intraplantar

Table 11. Quantitative *in vitro* studies of Compound 24

Preparation	N/OFQ effect	pEC_{50}	Compound 24 effect	pIC_{50}	Reference
Cell cultures					
CHO_{hNOP}	Stimulation of GTP-γS	-	Antagonist	9.8	(Goto et al., 2006)
CHO_{hNOP}	Stimulation of GTP-γS	8.95	Antagonist	9.98	(Fischetti et al., 2009)
CHO_{hNOP} ($G\alpha_{qi5}$)	Immediate Ca^{2+} transients	9.24	Antagonist	8.73	(Fischetti et al., 2009)
Peripheral preparations					
Mouse vas deferens	Inhibition of contraction	7.46	Antagonist	8.44	(Fischetti et al., 2009)
Rat vas deferens	Inhibition of contraction	-	Antagonist	8.28	(Fischetti et al., 2009)
Guinea Pig ileum	Inhibition of contraction	-	Antagonist	9.12	(Fischetti et al., 2009)
Brain preparations					
Rat PAG slices	Stimulation of K^+ current	7.3	Antagonist	5.6	(Liao et al., 2009)

Table 12. Quantitative *in vitro* studies of SB-612111

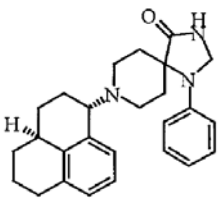
Preparation	N/OFQ effect	pEC_{50}	SB-612111 effect	$pA_2^{\dagger}/pIC_{50}^{\ddagger}$	Reference
Cell cultures					
HEK293	Inhibition of forskolin-induced luciferase expression	11.05	Antagonist	8.3†	(Zaratin et al., 2004)
CHO_{hNOP}	Stimulation of GTP-γS	9.2	Antagonist	9.7†	(Spagnolo et al., 2007)
CHO_{hNOP}	Decrease of cAMP	10.32	Antagonist	8.63†	(Spagnolo et al., 2007)
CHO_{hNOP}	Immediate Ca^{2+} transients	9.54	Antagonist	8.16†/6.69‡	(Camarda et al., 2009)
Peripheral preparations					
Rat vas deferens	Inhibition of contration	N.D.	Antagonist	8.2†	(Spagnolo et al., 2007)
Mouse vas deferens	Inhibition of contration	N.D.	Antagonist	8.5†	(Spagnolo et al., 2007)
Guinea pig ileum	Inhibition of contration	N.D.	Antagonist	8.4†	(Spagnolo et al., 2007)
Brain preparations					
Mouse cerebral cortex synaptosome	Decrease 5-HT release	7.85	Antagonist	8.45†	(Spagnolo et al., 2007)
Rat PAG slices	Stimulation of K^+ current	7.3	Antagonist	7.06‡	The present study

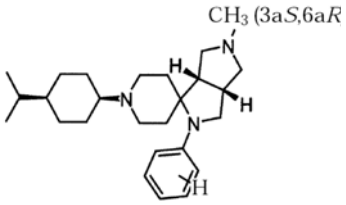
N.D. not determined

Agonists:

N/OFQ Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln

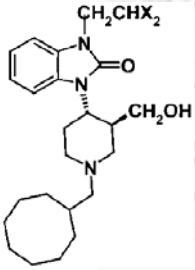
[Tyr^{10}]N/OFQ(1-11) Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Tyr-Ala

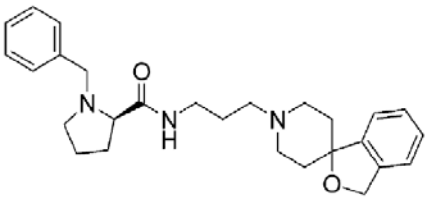
Ro 64-6198 

(+)-5a Compound 

Antagonists:

UFP-101 

J-113397 

Compound 24 

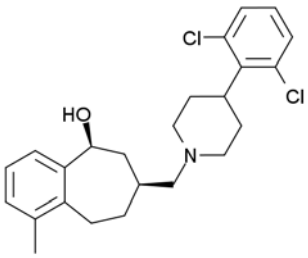
SB-612111 

Figure1. NOP receptor ligands.

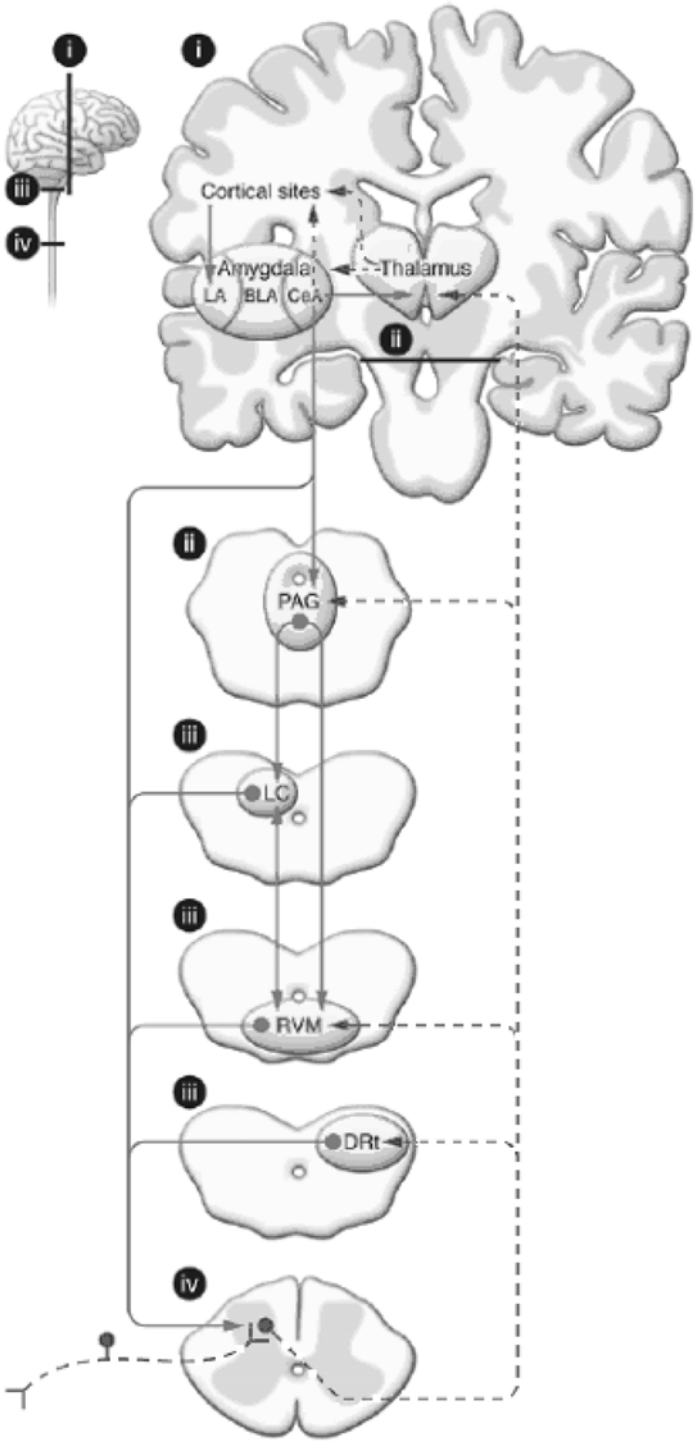


Figure 2. Schematic representation of pain modulating circuit. Ascending (dotted line) and descending (solid line) tracts are shown schematically. Areas labeled "i-iv" in the small diagram correspond with labeled details of the larger diagram. PAG, periaqueductal gray; LC, locus coeruleus; RVM, rostral ventromedial medulla; DRt, dorsal reticular nucleus; LA, lateral amygdala; BLA, basolateral amygdala; CeA, central nucleus of amygdale. Adapted from Ossipov et al., 2010.

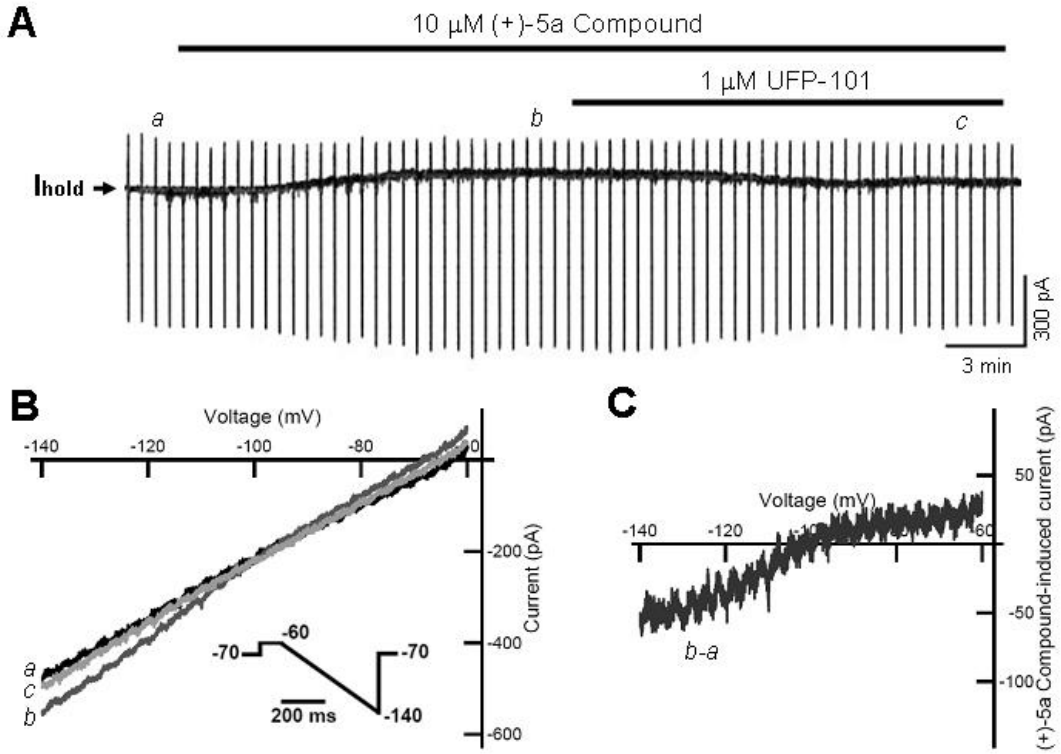


Figure 3. (+)-5a Compound induced G-protein coupled inwardly rectifying K^+ (GIRK) currents in rat ventrolateral periaqueductal gray (vlPAG) neurons in a manner antagonized by UFP-101, an NOP receptor antagonist. **A:** The chart recording of membrane currents evoked by hyperpolarization ramps, from -60 to -140 mV from the holding potential of -70 mV (inset), in a neuron treated with 10 μM (+)-5a Compound and further with 1 μM UFP-101. The holding current indicated by I_{hold} is the baseline of the recording traces. **B**: Current-voltage (I-V) curves of the neuron shown in A before (*a*) and after treatment with (+)-5a Compound (*b*) or with (+)-5a Compound plus UFP-101 (*c*). **C**: The I-V curve of (+)-5a Compound-induced current, obtained by subtracting the current in the control from that after treatment with (+)-5a Compound (*b*-*a*).

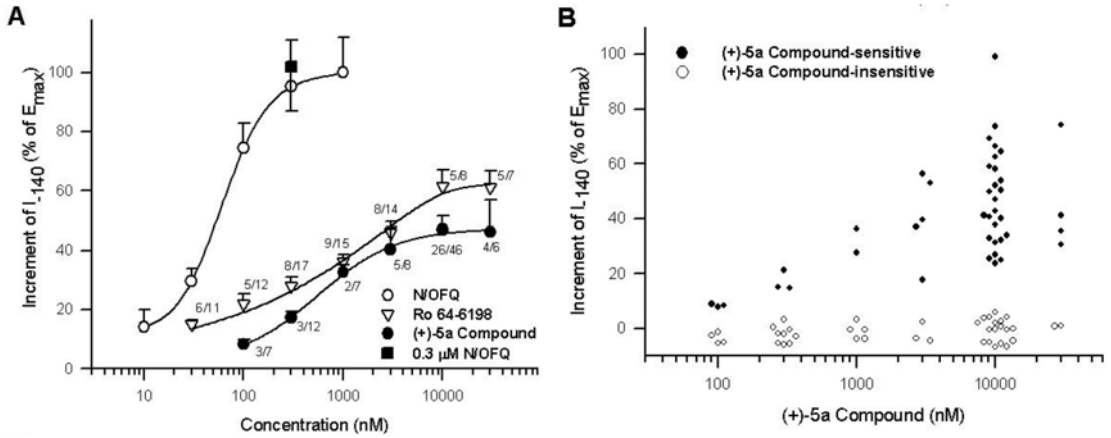


Figure 4. Concentration-dependent activation of the G-protein coupled inwardly rectifying K^+ (GIRK) currents induced by (+)-5a Compound, Ro 64-6198 and N/OFQ in ventrolateral periaqueductal gray (vlPAG) neurons. The ordinate is the increment of the membrane current at -140 mV (I_{-140}) induced by various NOP receptor agonists and was expressed as the percentage of the maximal increment (E_{max}) produced by N/OFQ, which was 39.4%±4% (n=26) increment and was obtained with 1 μM N/OFQ (Chiou et al., 2002). **A:** Concentration-response curves of N/OFQ (open circles and filled square), Ro 64-6198 (inverted triangles) and (+)-5a Compound (filled circles). The curves of N/OFQ and Ro 64-6198 were taken from our previous studies (Chiou et al., 2004; Chiou et al., 2002). The EC_{50} for (+)-5a Compound is 605±2 nM. The maximal effect produced by (+)-5a Compound is only 47% of that by N/OFQ. The numbers beside each point of the curve of Ro 64-6198 or (+)-5a Compound are the number ratios of Ro 64-6198- or (+)-5a Compound-sensitive neurons to the tested neurons. Data are mean±S.E.M. **B:** A scatter plot for the effect of (+)-5a Compound (0.1-30 μM) in each recorded neuron. Note that there is a sharp cut-off in the responses between (+)-5a Compound-sensitive (filled circles) and -insensitive neurons (open circules).

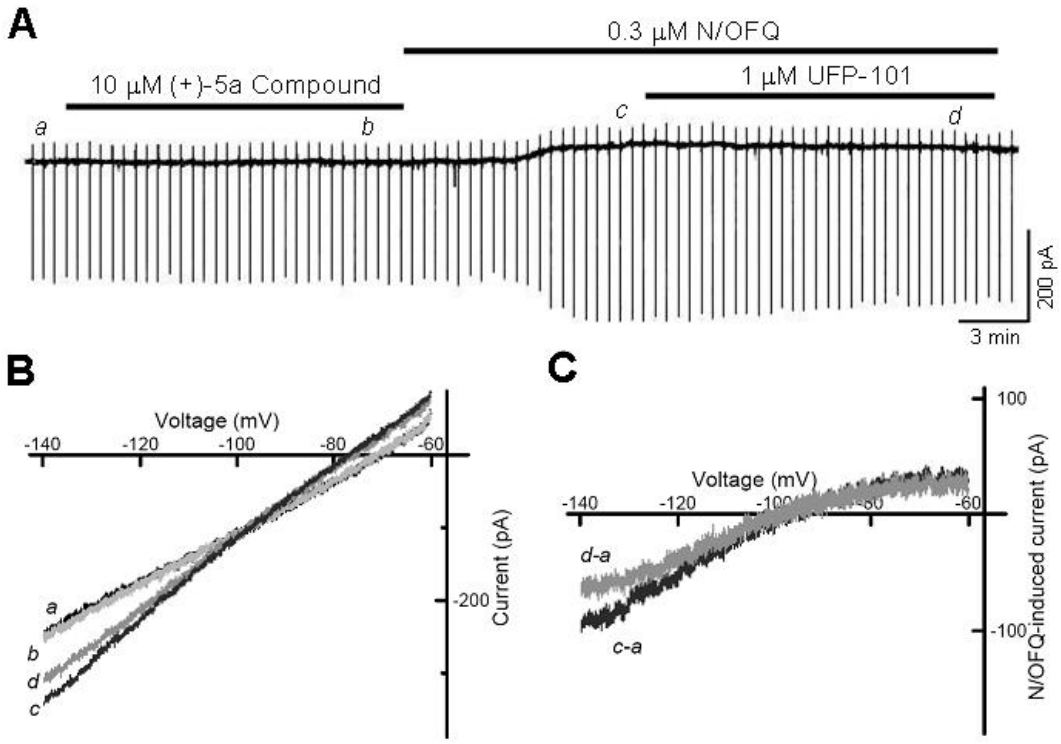


Figure 5. N/OFQ activated G-protein coupled inwardly rectifying K^+ (GIRK) channels in a (+)-5a Compound-insensitive neuron through NOP receptors. **A:** The chart recording of membrane currents in a ventrolateral periaqueductal gray (vlPAG) neuron which was insensitive to 10 μM (+)-5a Compound, but responsive to 0.3 μM N/OFQ. The effect of N/OFQ was reversed by 1 μM UFP-101. **B:** I-V curves of the membrane currents before (*a*) or after treatment with (+)-5a Compound (*b*), N/OFQ (*c*), or N/OFQ+UFP-101 (*d*). **C:** I-V curves of N/OFQ-induced currents in the absence (*c*-*a*) or presence of UFP-101 (*d*-*a*). In other 33 recorded neurons that were insensitive to (+)-5a Compound, N/OFQ had the same effect, which was blocked by UFP-101 in all neurons.

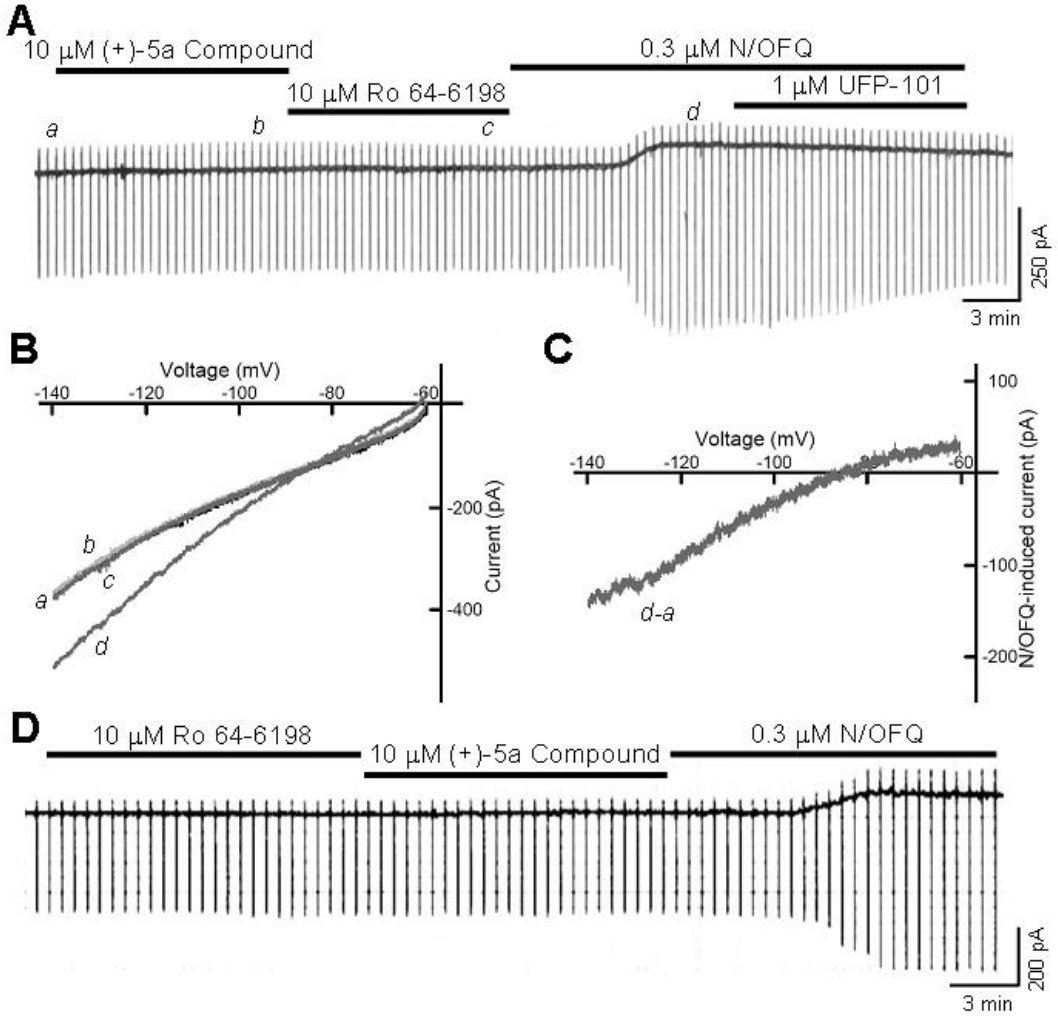


Figure 6. N/OFQ activated G-protein coupled inwardly rectifying K^+ (GIRK) channels through NOP receptors in the neurons insensitive to (+)-5a Compound, which were also insensitive to Ro 64-6198, and *vice versa*. **A:** The chart recording of membrane currents in a ventrolateral periaqueductal gray (vlPAG) neuron which was insensitive to 10 μM (+)-5a Compound and also insensitive to 10 μM Ro 64-6198, but responsive to 0.3 μM N/OFQ. The effect of N/OFQ was reversed by 1 μM UFP-101. **B**: I-V curves of the neuron shown in A before (*a*) and after treatment with (+)-5a Compound (*b*), Ro 64-6198 (*c*), or N/OFQ (*d*). **C**: The I-V curve of N/OFQ-induced current (*d*-*a*). **D:** The chart recording of membrane currents in a vlPAG neuron, which was insensitive to 10 μM Ro 64-6198 and also insensitive to 10 μM (+)-5a Compound, but responsive to 0.3 μM N/OFQ. The results were reproduced in other 8 and 4 neurons, respectively, in **A** and **D** conditions.

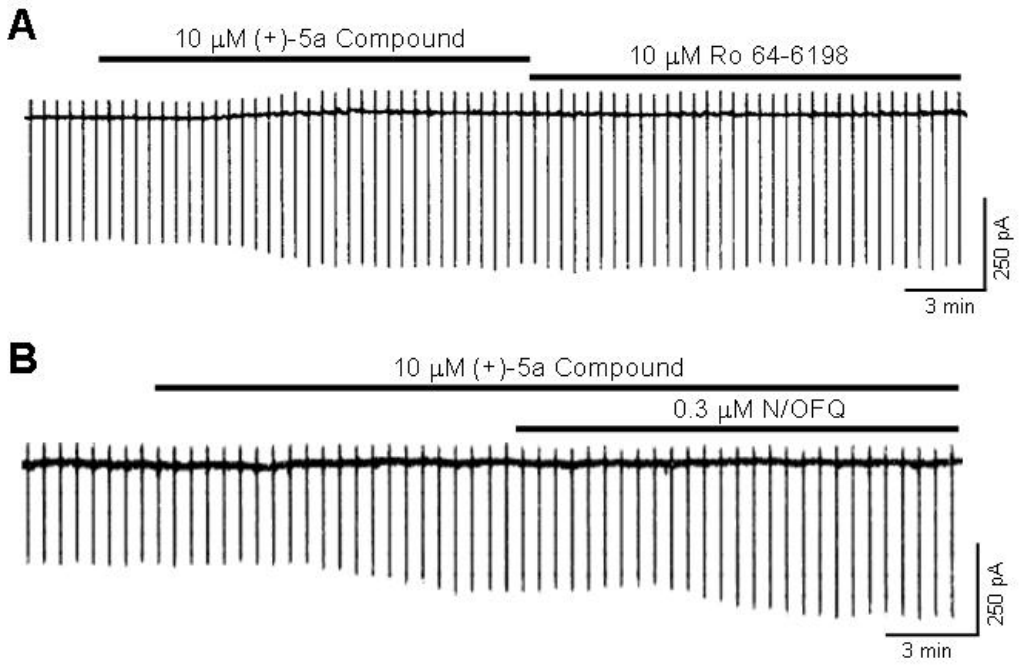


Figure 7. N/OFQ, but not Ro 64-6198, further increased G-protein coupled inwardly rectifying K^+ (GIRK) currents in (+)-5a Compound-sensitive neurons. Ro 64-6198, 10 μM (**A**) failed to, but N/OFQ, 0.3 μM did (**B**) further increase GIRK currents in neurons which were responsive to a pretreatment with (+)-5a Compound at the maximal effective concentration (10 μM). The results were reproduced in other 10 and 7 neurons, respectively, in **A** and **B** conditions.

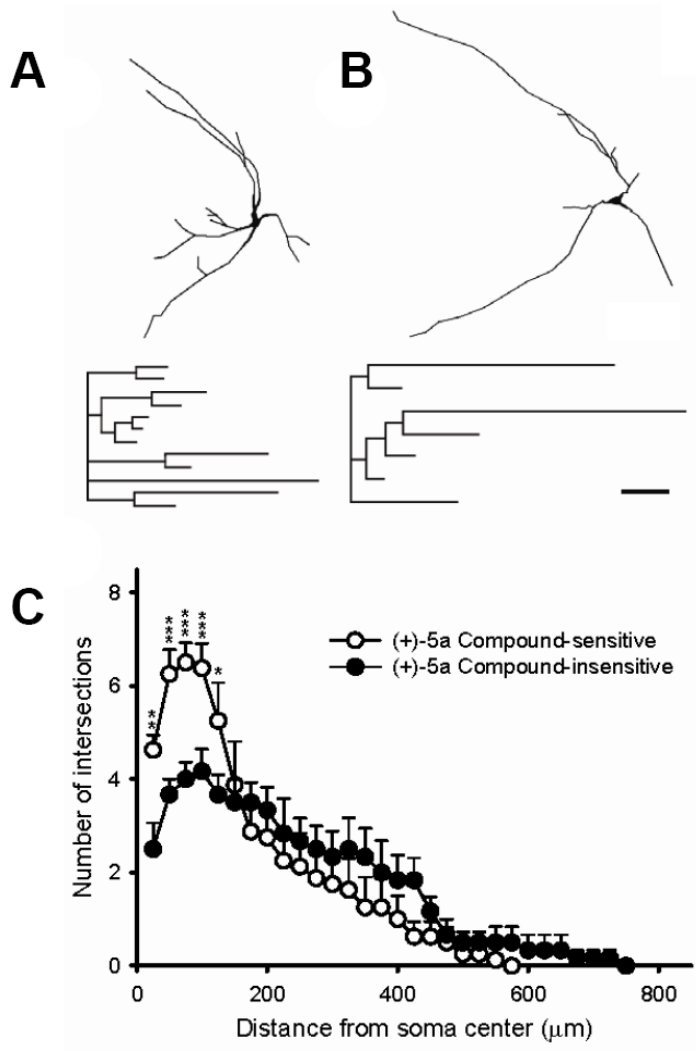


Figure 8. Morphology of (+)-5a Compound-sensitive and -insensitive ventrolateral periaqueductal gray (vlPAG) neurons. The morphology, dendrogram and dendritic complexity of (+)-5a Compound-sensitive (**A**) and -insensitive (**B**) vlPAG neurons. The morphology reconstructed by Neurolucida of a representive (+)-5a Compound-sensitive or –insensitive neuron. Their respective dendrograms are also shown in the lower panel. Scale bar: 100 μm. Dendritic complexities of (+)-5a Compound-sensitive (n=8) and -insensitive neurons (n=6) were estimated by Sholl analysis (**C**). Note that (+)-5a Compound-sensitive neurons had more intersections particularly in the proximal regions * p<0.05, ** p<0.01, *** p<0.005 *vs.* the (+)-5a-insensitive group (Student *t*-test).

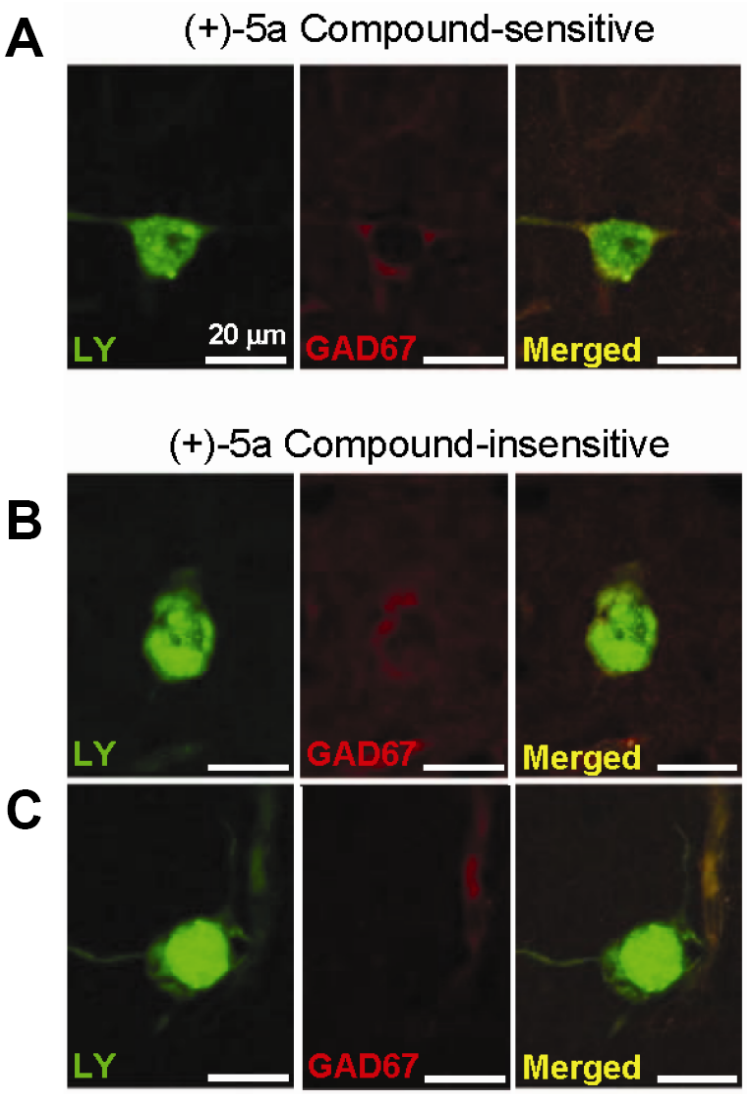


Figure 9. Glutamic acid decarboxylase-67 (GAD67) immunoreactivity of (+)-5a Compound-sensitive and -insensitive ventrolateral periaqueductal gray (vlPAG) neurons. The recorded neurons, either sensitive (**A**) or insensitive (**B**, **C**) to (+)-5a Compound, had been filled with Lucifer yellow (LY) after recording (left column) and were immunofluorescent stained with GAD67 (middle column). A colocalization of GAD67 immunoreactivity with Lucifer yellow shown in the merged images (right column) indicates that the recorded neuron is GABAergic. One neuron represents the 31 GAD67-immunoreactive neurons out of 40 (+)-5a Compound-sensitive neurons (**A**). One neuron represents the 14 GAD67-immunoreactive neurons out of 30 (+)-5a Compound-insensitive neurons (**B**). One neuron represents the 16 neurons without GAD67 immunoreactivity out of 30 (+)-5a Compound-insensitive neurons (**C**). Scale bars, 20 µm.

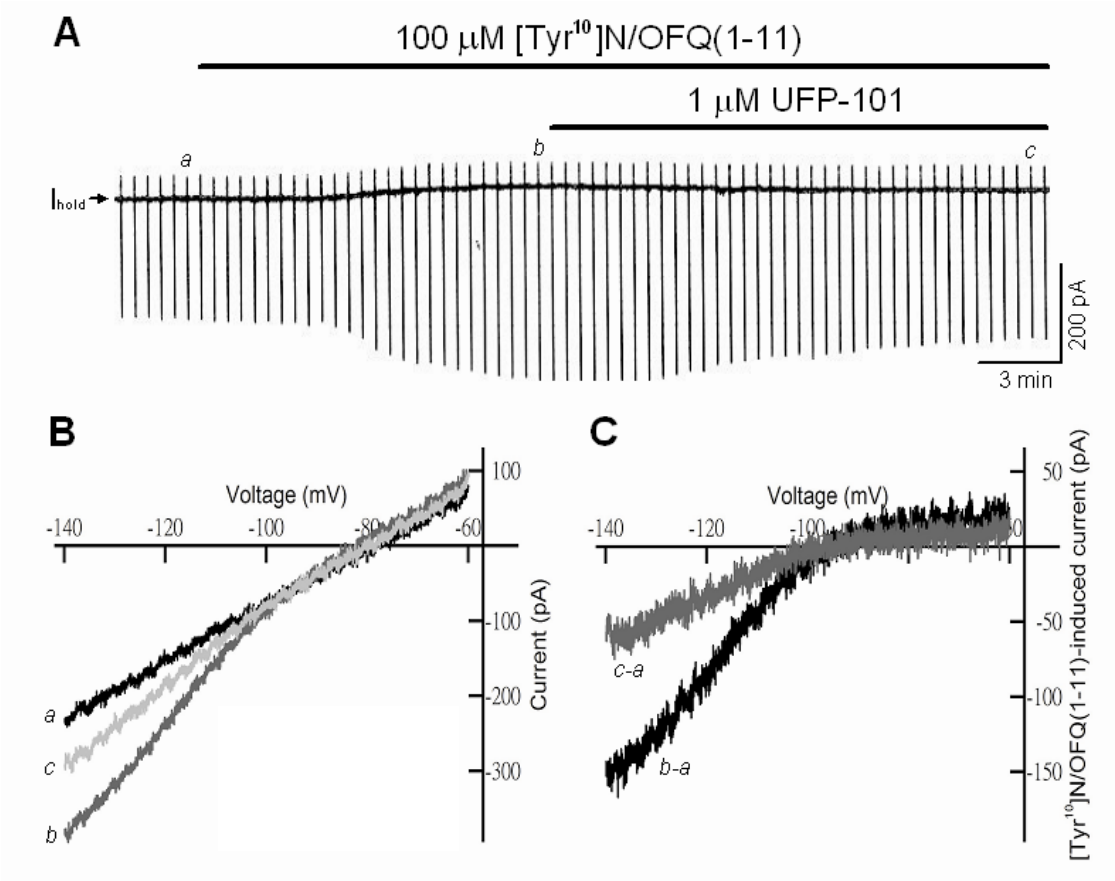


Figure 10. [Tyr^{10}]N/OFQ(1-11)-induced G-protein-coupled inwardly rectifying K^+ currents in rat vlPAG neurons in a manner antagonized by UFP-101, an NOP receptor antagonist. Membrane currents were evoked by hyperpolarization ramps from -60 to -140 mV at 0.2 mV/ms every 30 s from a holding potential of -70 mV (inset). **A:** The chart recording of the membrane currents of a vlPAG neuron treated with 100 μM [Tyr^{10}]N/OFQ(1-11) and further with 1 μM UFP-101. The holding current (I_{hold}) is the baseline of the recording traces. **B:** Current-voltage (I-V) curves of the membrane current in the control (*a*) or the presence of [Tyr^{10}]N/OFQ(1-11) (*b*) or [Tyr^{10}]N/OFQ(1-11) plus UFP-101. **C:** The I-V curves of the [Tyr^{10}]N/OFQ(1-11)-induced currents that were obtained by subtracting the current in the control from that during exposure to [Tyr^{10}]N/OFQ(1-11) in the absence (*b*-*a*) or presence (*c*-*a*) of UFP-101.

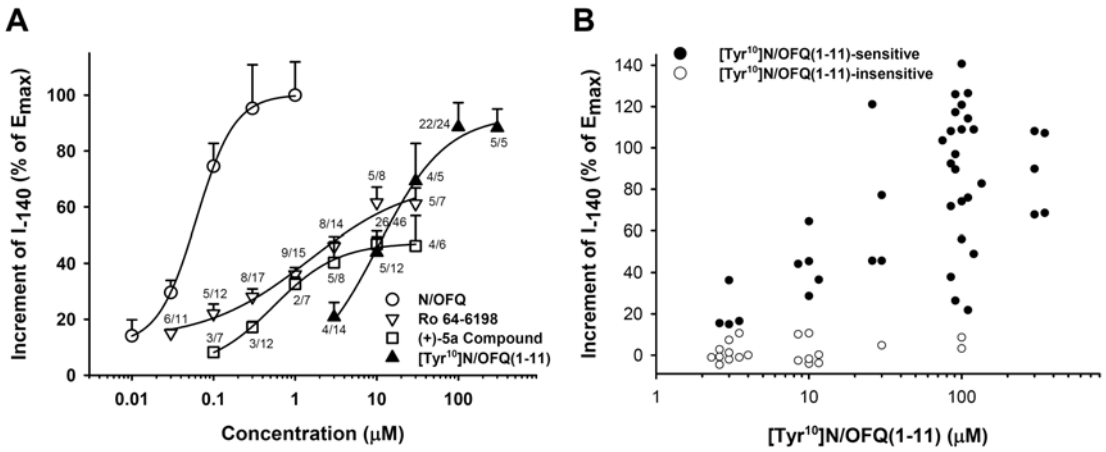


Figure 11. Concentration-dependent activation of GIRK currents induced by [Tyr^{10}]N/OFQ(1-11), (+)-5a Compound, Ro 64-6198 and N/OFQ in vlPAG neurons. The ordinate is the increment of the membrane current at -140 mV (I_{-140}) induced by various NOP receptor agonists and is expressed as the percentage of the maximal increment (E_{max}) produced by N/OFQ, which was 39.4%±4% (n=26) and was obtained with 1 μM N/OFQ (Chiou et al., 2002). **A:** The concentration-response curves of N/OFQ (open circles), Ro 64-6198 (open inverted triangles), (+)-5a Compound (open squares) and [Tyr^{10}]N/OFQ(1-11) (filled triangles). The curves of N/OFQ, Ro 64-6198 and (+)-5a Compound were taken from our previous studies (Chiou et al., 2004; Chiou et al., 2002; Liao et al., 2010). The EC_{50} for [Tyr^{10}]N/OFQ(1-11) is 9.0±0.9 μM.. The numbers next to each to each point of the curve of Ro 64-6198, (+)-5a Compound or [Tyr^{10}]N/OFQ(1-11) are the numerical ratios of Ro 64-6198-sensitive, (+)-5a Compound-sensitive or [Tyr^{10}]N/OFQ(1-11)-sensitive neurons to the tested neurons. Data are mean±S.E.M. **B:** A scatter plot for the effect of [Tyr^{10}]N/OFQ(1-11) (3-300 μM) in each recorded neuron.

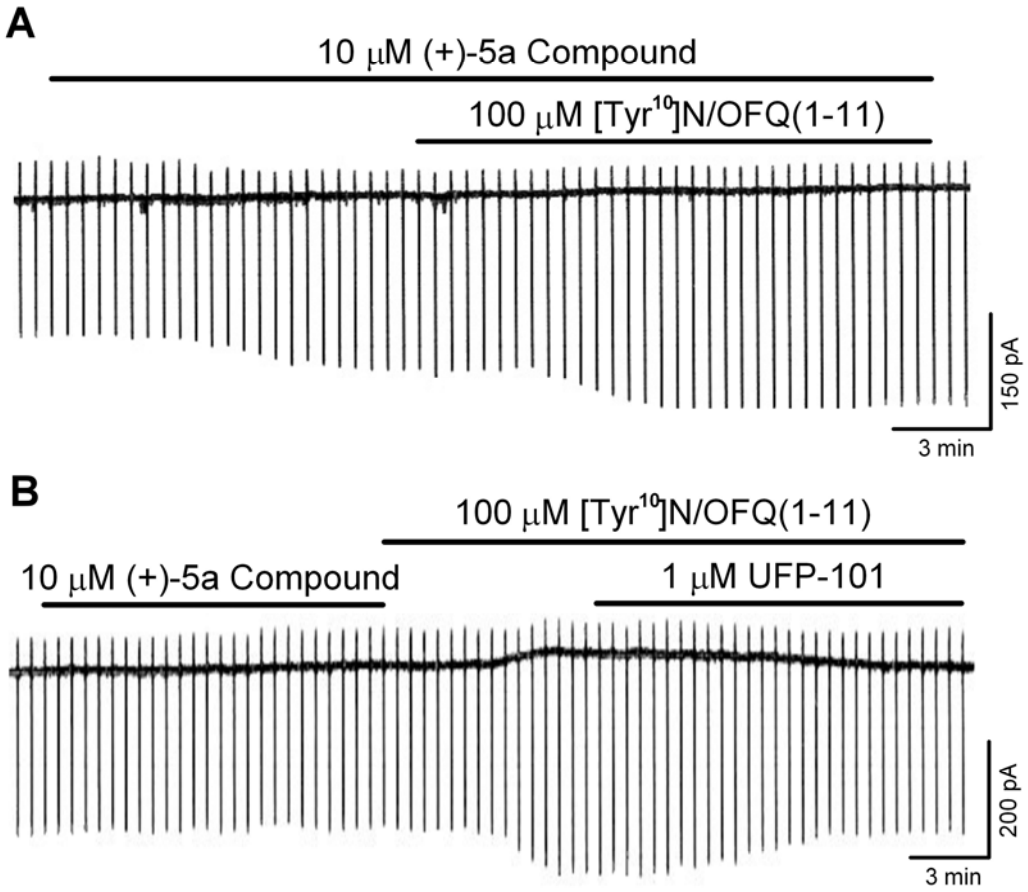


Figure 12. Effect of [Tyr^{10}]N/OFQ(1-11) in (+)-5a Compound-sensitive and -insensitive neurons. **A:** [Tyr^{10}]N/OFQ(1-11) did further increase GIRK currents in neurons which were responsive to a pretreatment with (+)-5a Compound at the maximal effective concentration (100 μM). **B:** The chart recording of membrane currents in a vlPAG neuron which was insensitive to 10 μM (+)-5a Compound, but responsive to 100 μM [Tyr^{10}]N/OFQ(1-11). The effect of [Tyr^{10}]N/OFQ(1-11) was reversed by 1 μM UFP-101. The results were reproduced in other 11 and 7 neurons, respectively.

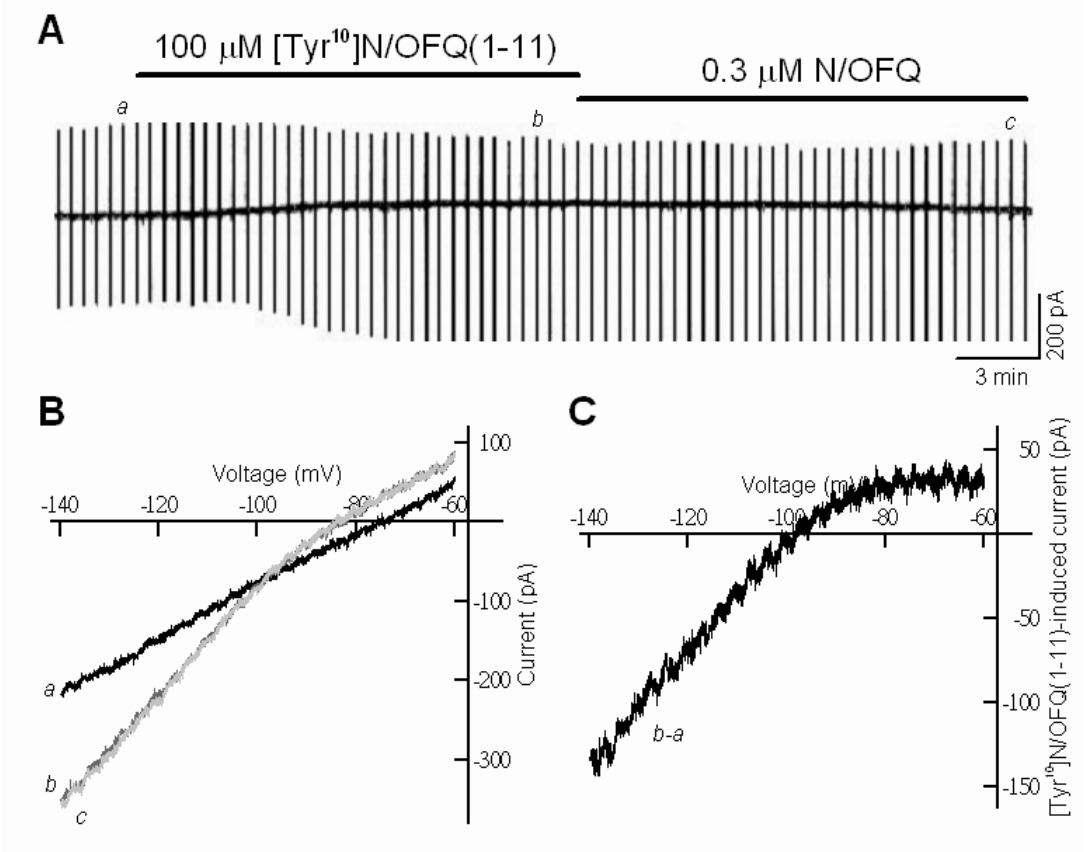


Figure 13. [Tyr^{10}]N/OFQ(1-11) precludes the effect of N/OFQ. N/OFQ did not further increase GIRK currents in [Tyr^{10}]N/OFQ(1-11)-sensitive neurons. Membrane currents were elicited and recorded as described in Fig. 10. **A:** The chart recording of the membrane current of a neurons treated with 100 μM [Tyr^{10}]N/OFQ(1-11) and further with 0.3 μM N/OFQ. **B:** I-V curves of the membrane current in the control (*a*) or the presence of [Tyr^{10}]N/OFQ(1-11) (*b*) or N/OFQ (*c*). **C:** The I-V curve of [Tyr^{10}]N/OFQ(1-11)-induced current that was obtained by subtracting the current in the control from that during exposure to [Tyr^{10}]N/OFQ(1-11) (*b*-*a*).

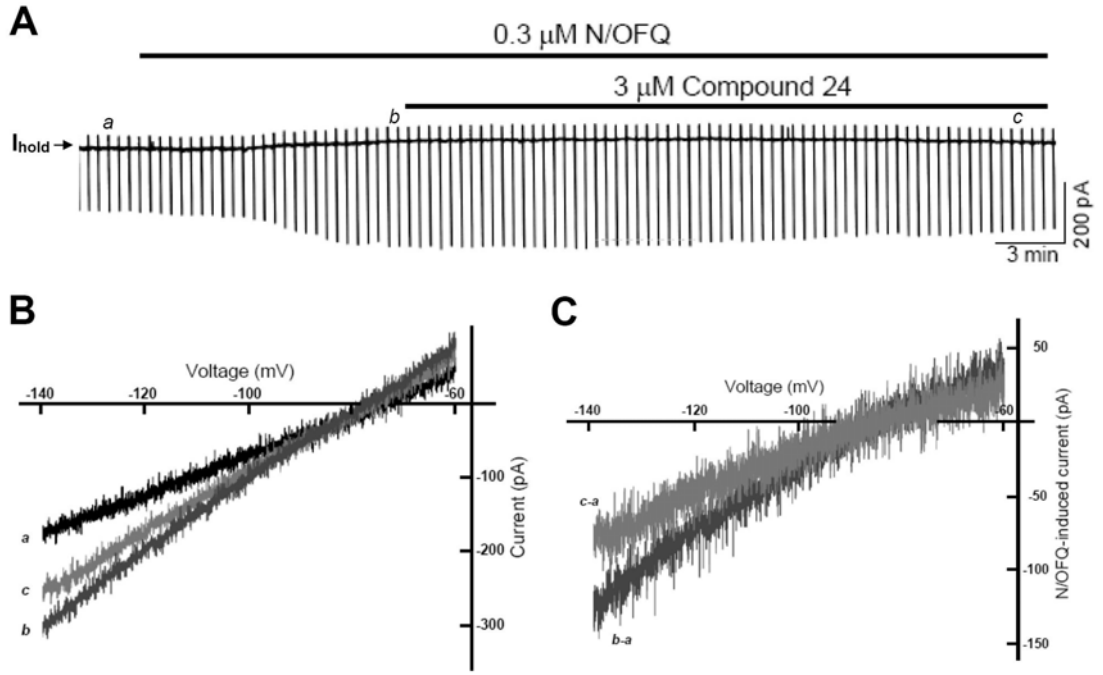


Figure 14. Compound 24 antagonized N/OFQ-induced GIRK current in vlPAG neurons. Membrane currents were evoked by hyperpolarization ramps from -60 to -140 mV at 0.2 mV/ms every 30 s from a holding potential of -70 mV (inset). **A:** The chart recording of the membrane currents of a vlPAG neuron treated with 0.3 μM N/OFQ and further with 3 μM Compound 24. The baseline of the traces resembles the holding current (I_{hold}) of the recorded neuron. **B:** Current-voltage (I-V) curves of the membrane current in the control (*a*) or the presence of N/OFQ (*b*) or N/OFQ plus Compound 24. **C:** I-V curves of the N/OFQ-induced currents that were obtained by subtracting the current in the control from that during exposure to N/OFQ in the absence (*b*-*a*) or presence (*c*-*a*) of Compound 24.

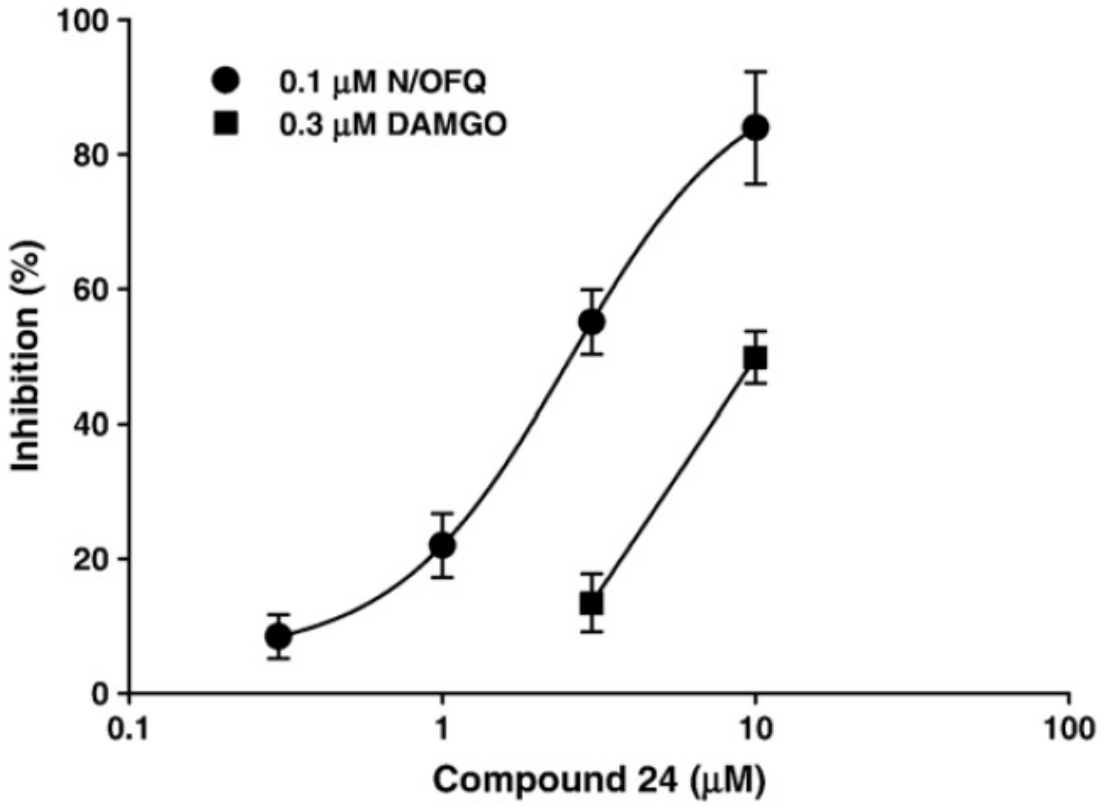


Figure 15. Concentration-response curves of Compound 24 in its inhibition of N/OFQ- or DAMGO-induced GIRK current. The concentration-response curve of Compound 24 in its inhibition of 0.1 μM N/OFQ-induced GIRK current (filled circles) was fitted by a logistic equation $I=I_{max}/[1+(D/IC_{50})^n]$, where I represents the percentage of the inhibition, I_{max} the maximal inhibition, D the concentration of Compound 24 and n the Hill coefficient. The inhibitory effect induced by Compound 24 on DAMGO-induced GIRK current was also shown (filled squares). In each neuron, N/OFQ or DAMGO was applied first and then Compound 24 was further added. The ordinate is the percentage of the inhibition induced by Compound 24, taking the effect of N/OFQ or DAMGO in each neuron as 100 %. n=4-8.

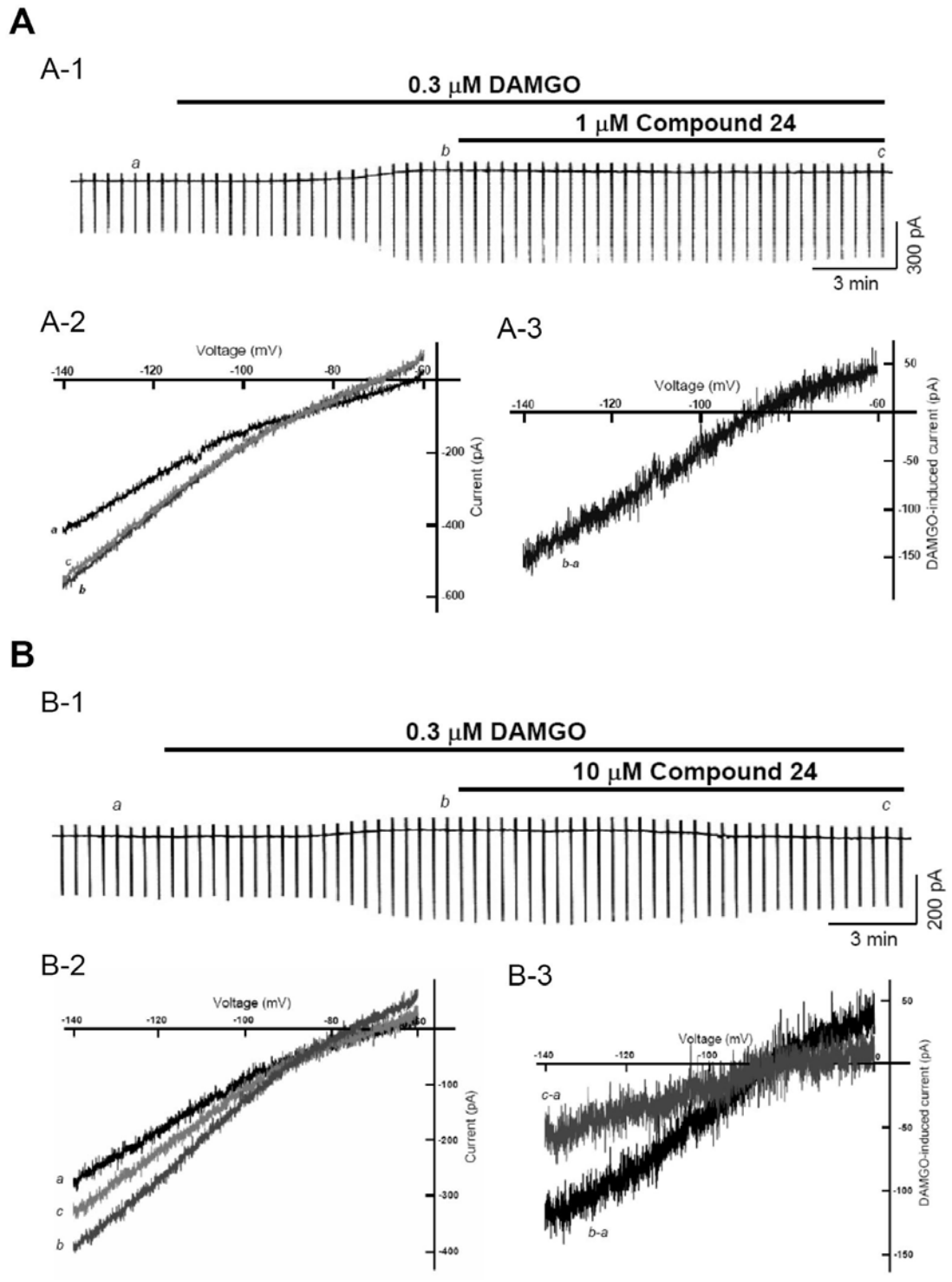


Figure 16. Effect of Compound 24 on DAMGO-induced GIRK current. Compound 24 did not affect the GIRK current induced by DAMGO at 1 μM (**A**) but reduced it at 10 μM (**B**). Membrane currents were elicited and recorded as described in Fig. 14. **A-1, B-1:** The chart recording of the membrane current of a neuron treated with 0.3 μM DAMGO and further with 1 μM (**A**) or 10 μM (**B**) Compound 24. **A-2, B-2:** I-V curves

of the membrane current in the control (*a*) or the presence of DAMGO (*b*) or DAMGO plus Compound 24 (*c*). **A-3, B-3:** The I-V curve of DAMGO-induced current that was obtained by subtracting the current in the control from that during exposure to DAMGO (*b*-*a*) or DAMGO plus Compound 24 (*c*-*a*). Note that the DAMGO-induced current was also characterized by inward rectification and reversed at potential near potassium equilibrium potential.

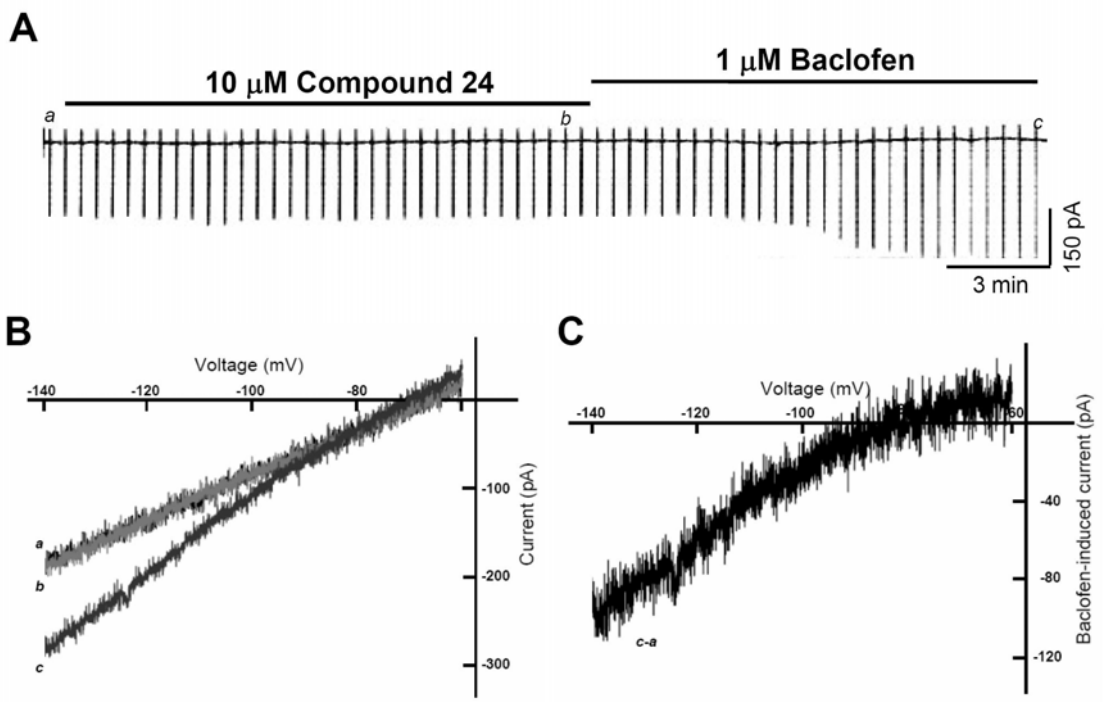


Figure 17. Compound 24 had no effect on membrane current *per se*. Membrane currents were elicited and recorded as described in Fig 14. **A:** The chart recording of the membrane current of a neurons treated with 10 μM Compound 24 or 1 μM baclofen. **B:** I-V curves of the membrane current in the control (*a*) or the presence of Compound 24 (*b*) or baclofen (*c*). **C:** The I-V curve of baclofen-induced current that was obtained by subtracting the current in the control from that during exposure to baclofen (*c*-*a*). Note that the baclofen-induced current was also characterized by inward rectification and reversed at potential near K^+ equilibrium potential.

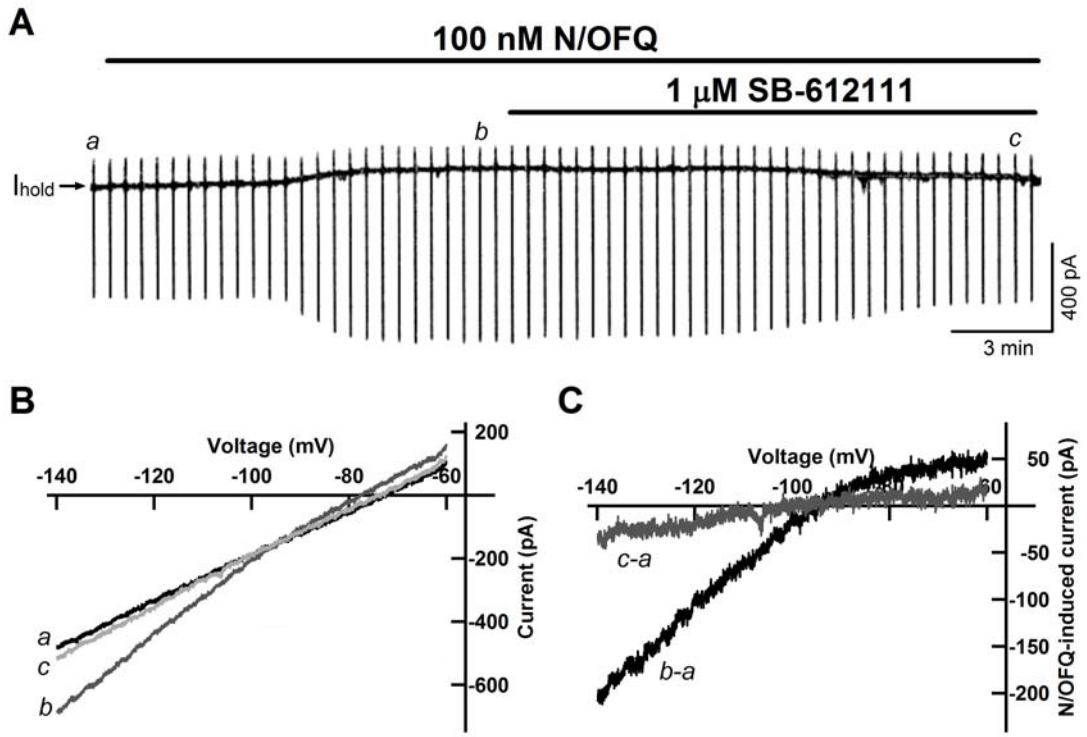


Figure 18. SB-612111 antagonized N/OFQ-induced GIRK currents in vlPAG neurons. **A:** The chart recording of membrane currents evoked by 400 ms hyperpolarization ramps, from -60 to -140 mV with the holding potential of -70 mV (inset), in a vlPAG neuron treated with 100 nM N/OFQ and followed by 1 μM SB-612111. The holding current indicated by I_{hold} is the baseline of the recording traces. **B:** Current-voltage (I-V) curves of the membrane currents in the control (*a*) or the presence of N/OFQ (*b*) or N/OFQ plus SB-612111 (*c*). **C:** I-V curves of the N/OFQ-induced currents that were obtained by subtracting the current in the control from that during exposure to N/OFQ in the absence (*b*-*a*) or presence (*c*-*a*) of SB-612111.

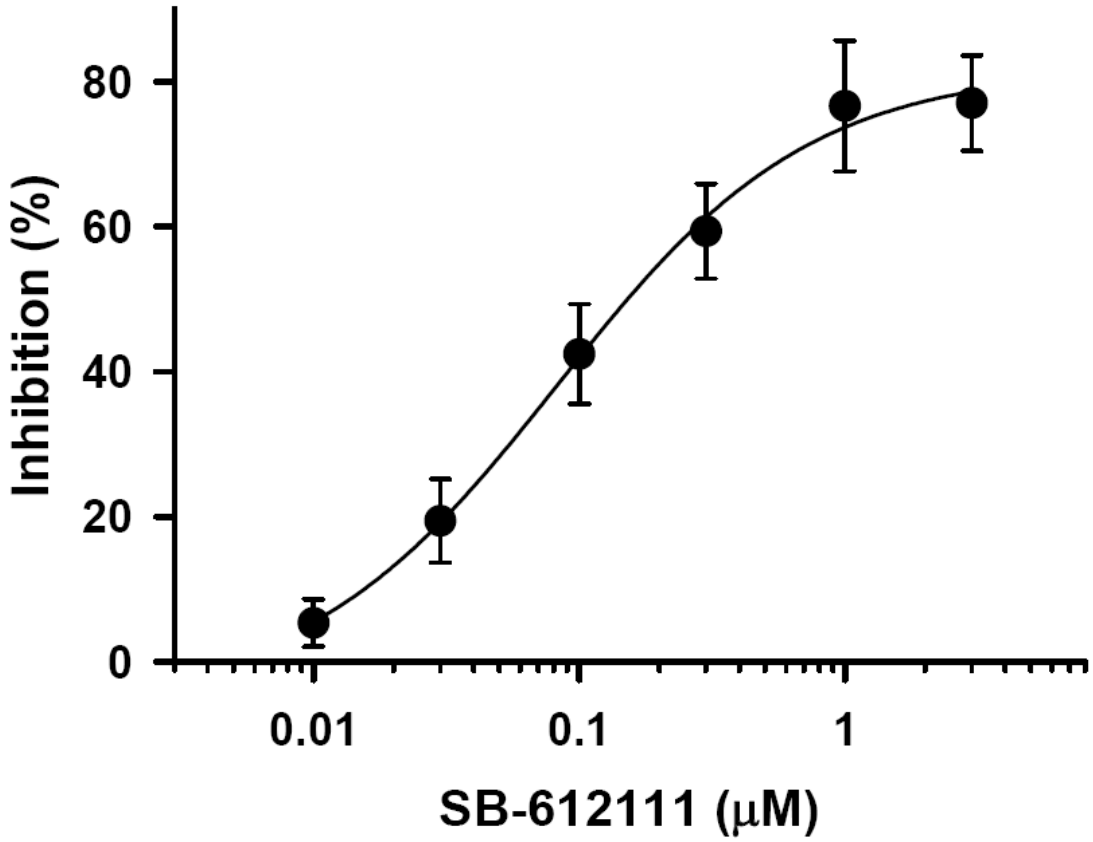


Figure 19. Concentration-inhibition curves of SB-612111 against N/OFQ-induced GIRK currents. The ordinate is the percent inhibition induced by SB-612111 against 100 nM N/OFQ-induced GIRK currents at -140 mV. In each neuron, N/OFQ was applied first and then SB-612111 was further added. The current induced by 100 nM N/OFQ in each neuron was taken as 100%. The concentration-inhibition curve of SB-612111 was fitted according to the logistic equation $I=I_{max}/[1+(D/IC_{50})^n]$, where I is the percentage of inhibition, I_{max}, the maximal inhibition, D, the concentration of SB-612111, IC_{50}, the half maximal inhibitory concentration, and n, the Hill coefficient. n=4-8.

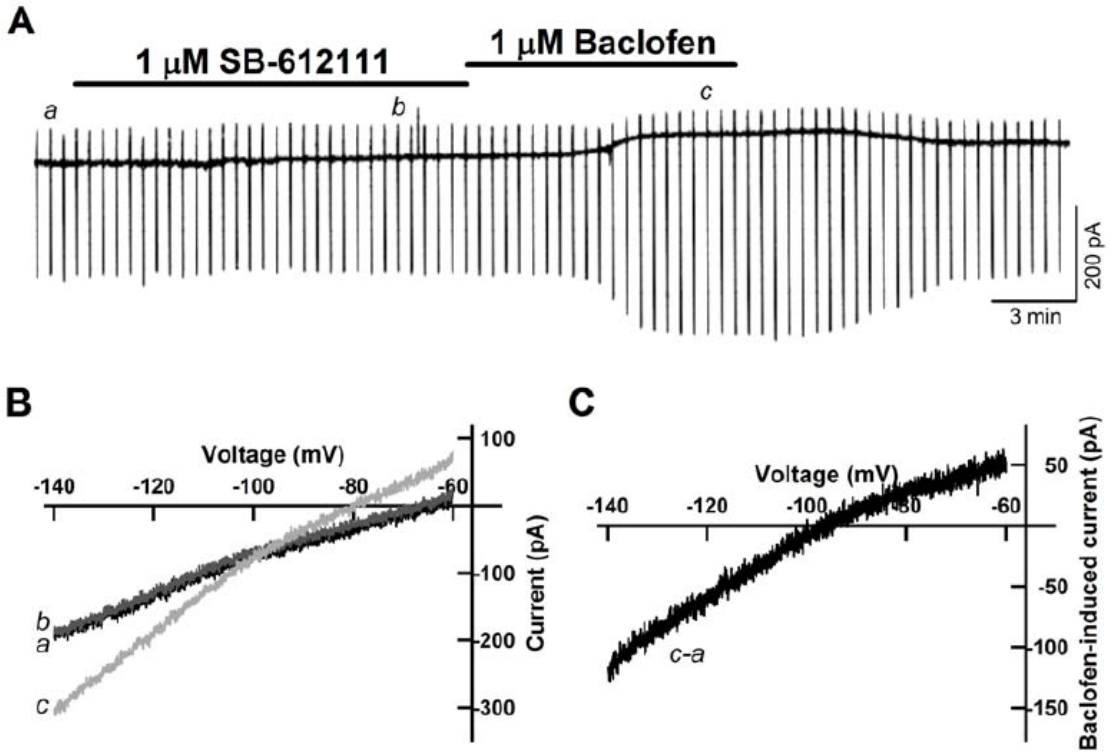


Figure 20. SB-612111 had no effect on membrane current *per se*. Membrane currents were elicited and recorded as that presented in Fig 18. **A:** The chart recording of membrane currents in a neuron treated with 1 μM SB-612111 and then with 1 μM baclofen. **B:** I-V curves of the membrane current in the control (*a*) or the presence of SB-612111 (*b*) or baclofen (*c*). **C:** The I-V curve of baclofen-induced current was obtained by subtracting the current in the control from that during exposure to baclofen (*c*-*a*). Note that in the neuron not affected by SB-612111, baclofen induced a GIRK current, which is characterized by inward rectification and reverses at the potential near the equilibrium potential of K^+ ions.

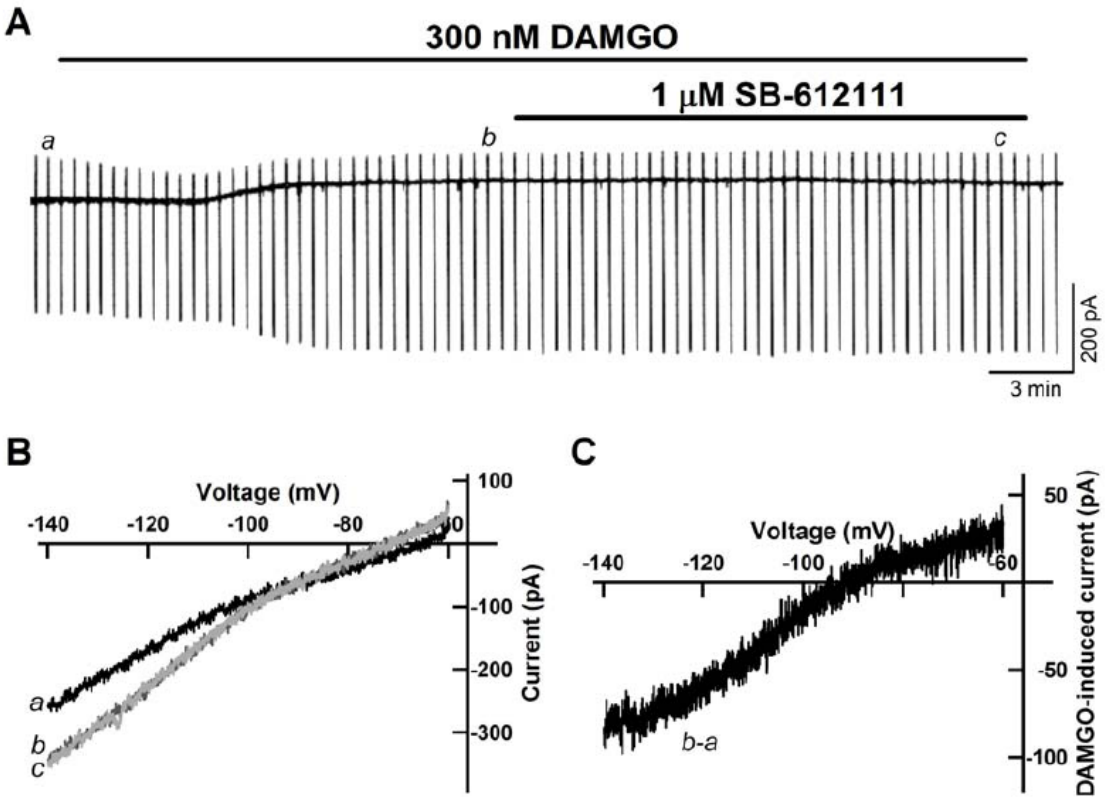


Figure 21. SB-612111 did not affect DAMGO-induced GIRK current. Membrane currents were elicited and recorded as that presented in Fig. 18. **A:** The chart recording of membrane currents of a neuron treated with 300 nM DAMGO and further with 1 μM SB-612111. **B:** I-V curves of the membrane current in the control (*a*) or the presence of DAMGO (*b*) or DAMGO plus SB-612111 (*c*). **C:** The I-V curve of DAMGO-induced current that was obtained by subtracting the current in the control from that during exposure to DAMGO (*b*-*a*). Note that DAMGO-induced current was also characterized by inward rectification and reversed at the potential near the equilibrium potential of K^+ ions.