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RESEARCH PAPER

Activities of mixed NOP and μ-opioid receptor ligands

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Background and purpose: Compounds that activate both NOP and μ-opioid receptors might be useful as analgesics and drug abuse medications. Studies were carried out to better understand the biological activity of such compounds.
Experimental approach: Binding affinities were determined on membranes from cells transfected with NOP and opioid receptors. Functional activity was determined by [^{35}S]GTPγS binding on cell membranes and using the mouse vas deferens preparation *in vitro* and the tail flick antinociception assay *in vivo*.
Key results: Compounds ranged in affinity from SR14150, 20-fold selective for NOP receptors, to buprenorphine, 50-fold selective for μ-opioid receptors. In the [^{35}S]GTPγS assay, SR compounds ranged from full agonist to antagonist at NOP receptors and most were partial agonists at μ-opioid receptors. Buprenorphine was a low efficacy partial agonist at μ-opioid receptors, but did not stimulate [^{35}S]GTPγS binding through NOP. In the mouse vas deferens, each compound, except for SR16430, inhibited electrically induced contractions. In each case, except for N/OFQ itself, the inhibition was due to μ-opioid receptor activation, as determined by equivalent results in NOP receptor knockout tissues. SR14150 showed antinociceptive activity in the tail flick test, which was reversed by the opioid antagonist naloxone.
Conclusions and implications: Compounds that bind to both μ-opioid and NOP receptors have antinociceptive activity but the relative contribution of each receptor is unclear. These experiments help characterize compounds that bind to both receptors, to better understand the mechanism behind their biological activities, and identify new pharmacological tools to characterize NOP and opioid receptors.
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Abbreviations: CHO, Chinese hamster ovary; MC-CAM, methoclocinnamox; %MPE, percent maximal possible effect

Introduction

The NOP receptor (formerly ORL1) is a G-protein-coupled receptor of the opioid receptor family that was discovered and cloned by virtue of homology with the opioid receptors (Bunzow *et al*., 1994; Mollereau *et al*., 1994; Wang *et al*., 1994). NOP receptors are found in high levels throughout the brain, as well as in the spinal cord (Neal *et al*., 1999; Florin *et al*., 2000). The endogenous ligand for this receptor, nociceptin/orphanin FQ (now called N/OFQ), is a 17 amino-acid peptide that is a member of the family of opioid peptides (Meunier *et al*., 1995; Reinscheid *et al*., 1995). N/OFQ has sequence similarity to the opioid peptides, particularly dynorphin, but has an N-terminal sequence FGGF rather than YGGF, and consequently does not bind opioid receptors with high affinity (Gintzler *et al*., 1997). Likewise, the opioid peptides do not bind NOP receptors with high affinity (Adapa and Toll, 1997).

The available ligands for NOP receptors and their potential therapeutic uses have been recently reviewed (Zaveri, 2003). The initial NOP ligands reported were peptides based upon N/OFQ (Dooley and Houghten, 1996; Reinscheid *et al*., 1996) and hexapeptides derived from a combinatorial library (Dooley *et al*., 1997). The first reported antagonist [Phe$^1\psi$ (CH_2-NH)Gly^2]N/OFQ(1-13)NH_2([F/G]N/OFQ(1-13)NH_2) has antagonist activity in the mouse vas deferens (Guerrini *et al*., 1998), but was discovered to have full agonist activity when tested in Chinese hamster ovary (CHO) cells transfected with human ORL1 (Butour *et al*., 1998). It also acted similarly to N/OFQ in a variety of *in vivo* assays, including its action as an analgesic (Grisel *et al*., 1998; Candeletti *et al*., 2000). Subsequent studies have identified a number of high affinity

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and selective peptide agonists and antagonists that have been useful pharmacological tools to understand the physiological actions of NOP receptor activation (Calo *et al*., 2000, 2002; Rizzi *et al*., 2002; Carra *et al*., 2005). The initial non-peptide ligands were generally discovered via high-throughput screening in pharmaceutical companies. These are almost exclusively piperidine-based compounds and both agonists (Jenck *et al*., 2000; Rover *et al*., 2000) and antagonists (Kawamoto *et al*., 1999; Zaratin *et al*., 2004) have been reported.

Even though there is significant similarity among all of the opioid family receptors, including NOP receptors, classical opiates, even non-selective ones such as etorphine, bind relatively poorly to NOP receptors (Adapa and Toll, 1997). The one exception in the early NOP receptor studies was lofentanil, which has a K_i of approximately 24 nM for NOP receptors (Butour *et al*., 1997), even though the close analogue fentanyl has very low affinity (Zaveri *et al*., 2001). More recently, buprenorphine was found to have approximately 100 nM affinity for NOP receptors (Wnendt *et al*., 1999; Zaveri *et al*., 2001). Contrary to this observation, most of the NOP receptor-directed small molecules that have been reported have not had particularly high selectivity for NOP receptors. Even many of the 'selective' NOP receptor compounds in the literature have considerable activity at opioid receptors as well. For instance, J-113397, the first selective antagonist synthesized was reported, in the original publication, to be at least 600-fold selective for NOP over μ-, δ- and κ-opioid receptors (Kawamoto *et al*., 1999), but other publications have shown that both this compound (Zaratin *et al*., 2004) and another from that series (Zaveri *et al*., 2001) have far greater affinity at μ-opioid receptors than in the original publication (Kawamoto *et al*., 1999). Furthermore, Ro 64-6198, the selective agonist is 100-fold selective in binding studies (Jenck *et al*., 2000), but only approximately 10-fold selective for inhibition of cAMP accumulation (Dautzenberg *et al*., 2001).

We have synthesized and characterized several series of NOP ligands, many of which possess modest selectivity for NOP receptors (Zaveri *et al*., 2004). We have carried out structure–activity relationship studies in several of our series, and reported that small changes in the piperidine nitrogen substituent can modulate agonist–antagonist activities of the NOP ligands (Zaveri *et al*., 2005). Several ligands in our small molecule series also possessed significant affinity at μ- and some at κ-opioid receptors (Zaveri *et al*., 2004). We recently characterized one such non-selective NOP/μ-opioid agonist, SR16435 *in vivo* in models of acute antinociception and place preference (Khroyan *et al*., 2007).

We hypothesized that small molecule NOP receptor ligands that have mixed NOP/μ-opioid activity may have an attractive profile with respect to pain and addiction (Khroyan *et al*., 2007). Because N/OFQ blocks both tolerance development and the reward associated with chronic morphine treatment, a compound with both μ and NOP agonist activities might be analgesic with low abuse potential and less tolerance development. Activation of both μ-opioid and NOP receptors can also work in the other direction, which is primarily opioid activity with some residual NOP receptor activity. For instance, buprenorphine has been reported to have some agonist activity at NOP receptors and it has been suggested that some of its behavioural activities may be due to NOP receptor binding at high doses (Bloms-Funke *et al*., 2000; Huang *et al*., 2001; Lutfy *et al*., 2003; Ciccocioppo *et al*., 2007). However, to fully understand the contribution of activation of each receptor to the overall *in vivo* antinociceptive profile, we studied these NOP/μ ligands in detail in transfected cells and native tissue preparations. We report the detailed *in vitro* evaluation of mixed NOP/μ-opioid receptor ligands at recombinant NOP and μ-opioid receptors in transfected cells and at native receptors in mouse vas deferens from wild-type and NOP-knockout animals. These assays are useful in characterizing these novel compounds, and in understanding the biological response of activation of NOP and opioid receptors. Our results show that even NOP receptor-selective compounds can have significant activity at the opioid receptors in a native tissue preparation, as well as *in vivo*. The results also have implications for the understanding of the antinociceptive activity of buprenorphine and other μ-opioid receptor partial agonists.

Materials and methods

Cell culture

All receptors were in CHO cells transfected with human receptor cDNA. The cells were grown in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum, in the presence of 0.4 mg ml−1 G418 and 0.1% penicillin/streptomycin, in 100-mm plastic culture dishes. For binding assays, the cells were scraped off the plate at confluence.

Receptor binding

Binding to cell membranes was conducted in a 96-well format, as described previously (Dooley *et al*., 1997; Toll *et al*., 1998). Cells were removed from the plates by scraping with a rubber policeman, homogenized in 50 mM Tris pH 7.5, using a Polytron homogenizer, then centrifuged once and washed by an additional centrifugation at 27 000 *g* for 15 min. The pellet was resuspended in Tris, and the suspension incubated with [^{3}H]DAMGO ([d-Ala^2, N-Me-Phe^4, Gly^5-ol]enkephalin) (51 Ci mmol^{-1}, 1.6 nM), [^{3}H]Cl-DPDPE ([d-Pen^2, d-Pen^5]enkephalin) (42 Ci mmol^{-1}, 1.4 nM), [^{3}H]U69593 (41.7 Ci mmol^{-1}, 1.9 nM) or [^{3}H]N/OFQ (120 Ci mmol^{-1}, 0.2 nM) for binding to, μ, δ, κ and NOP receptors, respectively. Non-specific binding was determined with 1 μM of unlabelled DAMGO, DPDPE, ethylketocyclazocine and N/OFQ, respectively. Total volume of incubation was 1.0 ml and samples were incubated for 60 min at 25 °C. The amount of protein in the binding assay was 15 μg. The reaction was terminated by filtration using a Tomtec 96 harvester (Orange, CT, USA) through glass fibre filters. Bound radioactivity was counted on a Pharmacia Biotech beta-plate liquid scintillation counter (Piscataway, NJ, USA) and expressed in counts per minute. IC_{50} values were determined using at least six concentrations of each compound and calculated using Graphpad/Prism (ISI, San Diego, CA, USA). K_i values were determined by the method of Cheng and Prusoff (1973).

[^{35}S]GTPγS binding

[^{35}S]GTPγS binding was conducted basically as described by Traynor and Nahorski (1995). Cells were scraped from tissue culture dishes into 20 mM HEPES, 1 mM EDTA, then centrifuged at 500 *g* for 10 min. Cells were resuspended in this buffer and homogenized using a Polytron Homogenizer. The homogenate was centrifuged at 27 000 *g* for 15 min, and the pellet resuspended in buffer A containing 20 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.4. The suspension was recentrifuged at 27 000 *g* and suspended once more in buffer A. For the binding assay, membranes (8–15 μg protein) were incubated with [^{35}S]GTPγS (50 pM), GDP (10 μM) and the appropriate compound, in a total volume of 1.0 ml, for 60 min at 25 °C. Samples were filtered over glass fibre filters and counted as described for the binding assays. Statistical analysis was conducted using the program Prism.

Electrically stimulated mouse vas deferens

All animal procedures were approved by the SRI International Institutional Animal Care and Use Committee.

Mice used were 30–35 g male Swiss Webster, male CD1-C57BL/6J-129 wild type ($NOP^{+/+}$) and knock out for the NOP receptor gene ($NOP^{-/-}$). Details of the generation and breeding of mutant mice have been reported previously (Nishi *et al*., 1997; Gavioli *et al*., 2003). The bioassay experiments were performed as previously described (Berzetei-Gurske *et al*., 1996; Calo *et al*., 1996). Mouse vas deferens tissues were suspended in 5 ml organ baths containing Mg^{2+}-free Krebs solution. The bath temperature was set at 33 °C. Tissues were stimulated through two platinum ring electrodes with supramaximal rectangular pulses of 1 ms duration and 0.05 Hz frequency. The resting tension was 0.3 g. The electrically evoked contractions were measured isotonically by means of Basile strain gauge transducers and recorded with a personal computer-based acquisition system (Autotrace; RCS, Florence, Italy).

After an equilibration period of about 1 h, the contractions induced by electrical field stimulation were stable. At this time, cumulative concentration–response curves were performed (0.5 log unit steps). When SR compounds were found to be inactive as agonists, concentration–response curves to N/OFQ were performed in their presence (15 min preincubation) for evaluating antagonist properties.

The agonist potencies are given as pEC_{50}, the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect. Antagonist potencies have been evaluated using the Gaddum Schild equation: $pK_B = \log((CR-1)/[\text{antagonist}])$ assuming a slope value equal to unity, where CR indicates the ratio between agonist potency in the presence and absence of antagonist. Data were analysed using nonlinear curve fitting with variable slope for derivation of pEC_{50} and E_{max} values (GraphPad Prism V4, San Diego, CA, USA). For pEC_{50} and pK_B values, the 95% confidence limits ($CL_{95\%}$) were given.

Antinociceptive activity in vivo *in mice*

Acute nociception was assessed in CD1 mice using the tail-flick assay with an analgesia instrument (Stoelting) that uses radiant heat. This instrument is equipped with an automatic quantification of tail-flick latency, and a 15-s cutoff to prevent damage to the animal's tail. During testing, the focused beam of light was applied to the lower half of the animal's tail, and tail-flick latency was recorded. Baseline values for tail-flick latency were determined before drug administration in each animal. The mean basal tail-flick latency was 6.25 ± 0.18 s (mean ± s.e.mean; $n = 32$).

Following baseline measures, animals received an injection (s.c.) of 30 mg kg^{-1} SR14150 alone or co-administered with 1 mg kg^{-1} naloxone and were tested for tail-flick latencies at 60 min after injection. Animals that were controls received an injection of vehicle, whereas positive controls received 15 mg kg^{-1} morphine prior to testing.

Statistical analyses

Antinociception (% maximum possible effect; %MPE) was quantified by the following formula:

$$\%\,\text{MPE} = 100 \times [(\text{test latency} - \text{baseline latency})/(15 - \text{baseline latency})].$$

If the animal did not respond prior to the 15-s cutoff, the animal was assigned a score of 100%.

Behavioural results were analysed using ANOVA with drug treatment (morphine or SR14150 alone or with naloxone) followed by Student–Newman–Keuls *post hoc* tests where appropriate. The level of significance was set at $P < 0.05$.

Materials

N/OFQ was prepared and purified as previously described (Guerrini *et al*., 1997) or else obtained from the NIDA drug supply programme, as were buprenorphine and pentazocine. SR14148, SR14150, SR16476 and SR16430 were prepared as hydrochloride salts and synthesized as described previously (Zaveri *et al*., 2004). Methoclocinnamox (MC-CAM) was synthesized by Husbands *et al*. (1998). All the other reagents were from Sigma Chemical Co. (St Louis, MO, USA) or E Merck (Darmstadt, Germany). Concentrated solutions (10 mM) of SR compounds, buprenorphine and pentazocine were made in dimethyl sulphoxide and kept at −20 °C until use. All the other substances were dissolved in physiological solution.

Results

Receptor binding

Binding affinities of N/OFQ, buprenorphine, pentazocine, MC-CAM and selected SR compounds (structures shown in Figure 1) at NOP and opioid receptors are shown in Table 1. Buprenorphine was chosen as a comparison because it also binds to both NOP and μ-opioid receptors. Pentazocine and MC-CAM were also investigated because these compounds are μ-opioid partial agonists that have similar antinociceptive profiles to buprenorphine, and have unknown NOP affinities or activities.

Binding studies demonstrated that each of the SR compounds had relatively high affinity for both NOP and

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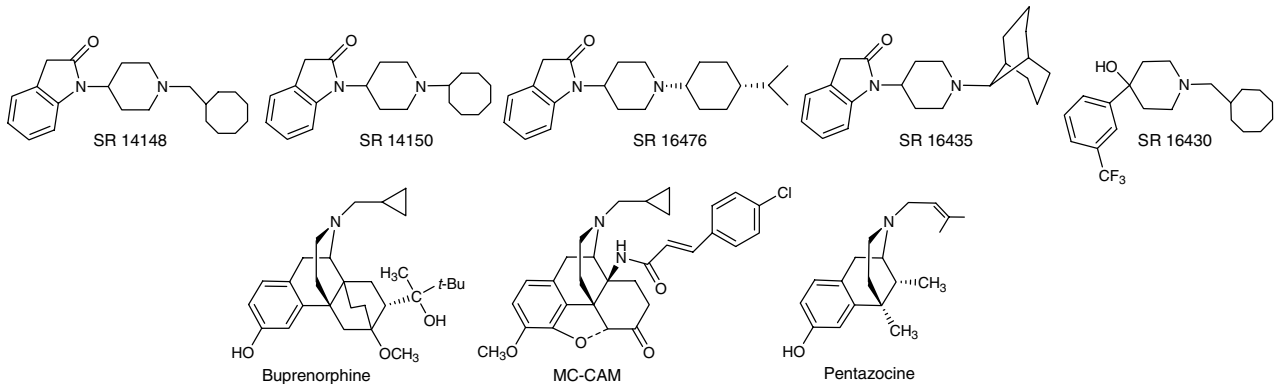

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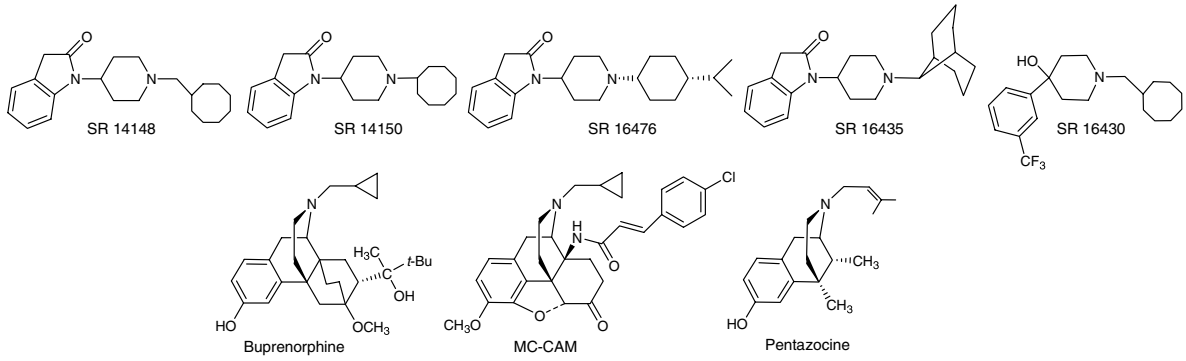

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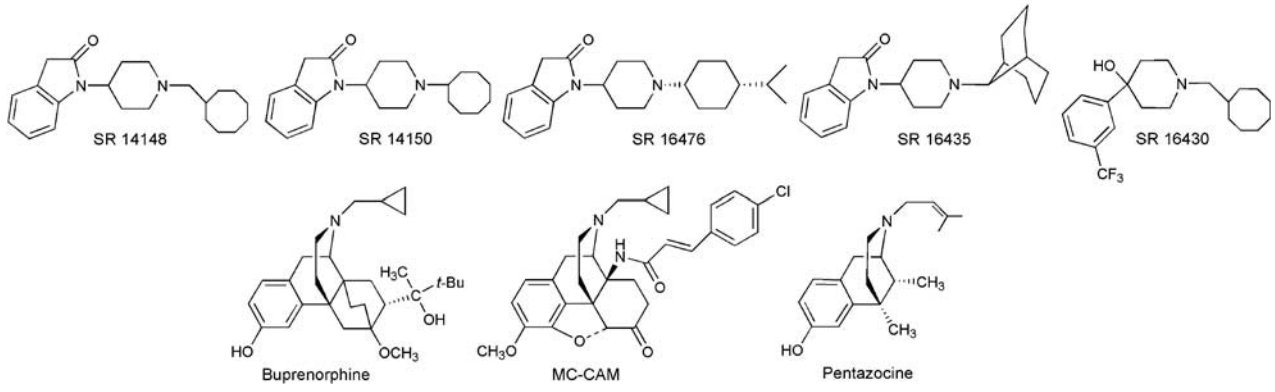

Figure 1 Structures of the compounds tested.

μ-opioid receptors, and some had fairly high affinity for κ-receptors as well. The most selective compounds were SR14150 and SR16430, which were approximately 20- and 10-fold selective for NOP over μ-receptors. Buprenorphine was a non-selective opioid ligand, with high affinity at μ- and κ-receptors. It had moderate affinity at NOP receptors, but 50-fold lower affinity for NOP than the opioid receptors. MC-CAM and pentazocine also had high affinity for opioid receptors, both having less than 5 nM affinity at μ-receptors, but they had low (693 nM) and no affinity, respectively for NOP receptors.

Functional activity of these compounds was tested in two ways. Each was tested for stimulation of [^{35}S]GTPγS binding at each site in membranes derived from CHO cells transfected with the human receptor (Table 2). Some were also tested for functional activity in the mouse vas deferens assay to determine activity in a more physiological setting.

Functional activity in the [^{35}S]GTPγS-binding assay in transfected CHO cells

The results of the [^{35}S]GTPγS-binding assays are shown in Table 2. The compounds showed a range of functional profiles that varied from full agonist to antagonist at the NOP and opioid receptors. Buprenorphine, MC-CAM and pentazocine were each low-efficacy partial agonists at μ-receptors. In our hands, buprenorphine did not show any agonist activity at NOP receptors, in contrast to some

published data (Wnendt *et al*., 1999; Huang *et al*., 2001). MC-CAM did not show agonist activity at NOP receptors either, though this is not surprising considering its low affinity at this site. As we have shown previously, SR14148 and SR16430 were antagonists at NOP receptors (Zaveri *et al*., 2004; Khroyan *et al*., 2007). SR14148 (but not SR16430) had partial agonist activity at μ-receptors. SR14150, although 20-fold selective for NOP receptors in receptor binding, was only five-fold selective with respect to [^{35}S]GTPγS binding, and had partial agonist activity at each site, though higher efficacy at NOP receptors. SR16435 had high affinity for both NOP and μ-opioid receptors, and was a partial agonist with approximately equal potency and efficacy at the two sites. SR16476 also had high affinity at NOP and μ-opioid receptors; however, it was a full agonist at NOP receptors and partial agonist activity at μ-receptors.

Functional activity in the mouse vas deferens bioassay

N/OFQ inhibited the twitch response to electrical stimulation in a concentration-dependent manner showing high potency ($pEC_{50} = 7.48$) and maximal effects (−92% of control values). This effect of N/OFQ was rapid in onset, immediately reversible after washing of the tissues and could be repeated several times in the same tissue.

As shown in Table 3, buprenorphine, SR14148, SR14150, SR16435 and SR16476 all mimicked the inhibitory effect of N/OFQ showing similar maximal effects but different potencies. The following order of potency was obtained from these experiments: buprenorphine > N/OFQ > SR16476 > SR16435 > SR14150 > SR14148. Interestingly, the kinetics of action of the SR compounds were similar to that of N/OFQ (rapid onset, reversible and repeatable), whereas very different from that of non-peptide NOP ligand Ro 64-6198, which produced slow onset and non-reversible effects in this tissue (Rizzi *et al*., 2001).

SR16430, which is a 10-fold selective NOP antagonist, with no efficacy at μ-opioid receptors (Tables 1 and 2), did not modify the electrically induced twitch up to 1 μM; however, it produced a rightward shift of the concentration–response curve to N/OFQ without significantly affecting the maximal

Table 1 Binding affinities of compounds studied, at opioid and NOP receptors

Compounds	K_i *nM*			
	NOP	*μ*	*κ*	*δ*
N/OFQ	0.2 ± 0.04	437 ± 12	147 ± 3.4	2850 ± 512
Buprenorphine	77.4 ± 16	1.5 ± 0.8	1.5 ± 0.25	19.8 ± 0.7
MC-CAM	693 ± 57	4.74 ± 0.41	18.7 ± 2.1	5.22 ± 0.6
Pentazocine	>10 000	3.9 ± 0.7	2.2 ± 0.2	49.3 ± 15.1
SR14148	6.04 ± 0.42	14.4 ± 1.1	229 ± 23	>10 000
SR14150	1.39 ± 0.42	29.9 ± 2.1	42.7 ± 1.0	ND
SR16430	6.49 ± 1.39	60.9 ± 15	219 ± 18	2350 ± 195
SR16435	7.49 ± 0.78	2.70 ± 0.5	31.7 ± 4.82	>10 000
SR16476	3.96 ± 1.55	8.0 ± 0.97	148 ± 9	ND

Abbreviations: MC-CAM, methoclocinnamox; ND, not determined; N/OFQ, nociceptin/orphanin FQ.
Binding was carried out with membranes derived from Chinese hamster ovary (CHO) cells containing cloned human receptors, as described in Materials and methods. Values shown are means ± s.d. from at least two experiments conducted in triplicate. NOP, μ and κ values for SR16430 and SR16435 are from Khroyan *et al*. (2007).

Table 3 Potencies of compounds in the electrically stimulated mouse vas deferens

Compounds	*Agonist*		*Antagonist*
	pEC_{50} ($CL_{95\%}$)	E_{max} (%)	pK_B ($CL_{95\%}$)
N/OFQ	7.48 (7.41–7.56)	−92 ± 1	ND
Buprenorphine	7.67 (7.64–7.69)	−63 ± 4	ND
SR14148	6.26 (6.03–6.43)	−97 ± 5	7.12 (6.94–7.31)
SR14150	6.62 (6.45–6.79)	−97 ± 5	ND
SR16435	7.07 (7.03–7.10)	−80 ± 1	ND
SR16476	7.16 (7.06–7.26)	−99 ± 3	ND
SR16430	Inactive		6.68 (6.43–6.93)

Abbreviations: N/OFQ, nociceptin/orphanin FQ; ND, not determined because these compounds behave as agonists; inactive, inactive up to 1 μM.
The pEC_{50} and pK_B values are shown as means with the 95% confidence limits given in parentheses. E_{max} values (means ± s.e.mean) are expressed as percent inhibition of electrically induced twitches.
The antagonist property of SR16430 was tested using N/OFQ as agonist and the potency was assessed by testing the compound at 1 μM. For SR14148, the pK_B value was determined in the presence of 1 μM naloxone to block the μ-opioid receptor mediated activity. These data are means of at least four separate experiments.

Table 2 Stimulation of [^{35}S]GTPγS binding to NOP, μ and κ receptors

Compounds	*NOP*		*μ*		*κ*	
	EC_{50} *(nM)*	*% stim*	EC_{50} *(nM)*	*% stim*	EC_{50} *(nM)*	*% stim*
N/OFQ	4.0 ± 0.1	100	>10 000	0	>10 000	0
Buprenorphine	>10 000	0	24.9 ± 14	17.7 ± 0.4	>10 000	0
MC-CAM	>10 000	0	17.8 ± 11	7.7 ± 2.2	>10 000	0
Pentazocine	ND	—	39.5 ± 0.3	20.2 ± 3.4	27.5 ± 7.8	39 ± 14
SR14148	>10 000	0	239 ± 43	25.9 ± 6.1	>10 000	0
SR14150	20.8 ± 3.1	54.2 ± 0.9	98.8 ± 12.5	23.4 ± 3.2	276 ± 75.8	37.6 ± 3.4
SR16430	>10 000	0	>10 000	0	>10 000	0
SR16435	28.7 ± 0.6	45.0 ± 5	29.5 ± 10	30 ± 0.5	>10 000	0
SR16476	26.5 ± 4.3	100 ± 15	73.5 ± 5.3	49.7 ± 0.5	>10 000	0

Abbreviations: MC-CAM, methoclocinnamox; ND, not determined; N/OFQ, nociceptin/orphanin FQ; % stim, percent stimulation.
Binding was carried out with membranes derived from Chinese hamster ovary (CHO) cells containing cloned human receptors, as described in Materials and methods. Values shown are means ± s.d. from at least two experiments conducted in triplicate. EC_{50} values determined on compounds for which there is less than 20% stimulation are somewhat inaccurate due to the poor stimulation. Values for SR16430 and SR16435 are from Khroyan *et al*. (2007).

agonist response (Figure 2). The pK_B value of SR16430 calculated from this series of experiments was 6.68.

SR14148, also a NOP antagonist but with μ partial agonist activity, inhibited the twitch response, albeit with low potency, as did the NOP full agonist SR16476 (Figure 3a). This inhibition of twitch response was primarily due to their activity at μ-opioid receptors, as it is also present in NOP−/− tissue and the values of potency and maximal effect of SR14148 and SR16476 were superimposable on those obtained in tissues from wild-type mice (Figure 3b). SR14148 was further evaluated as an NOP receptor antagonist in the presence of naloxone to prevent opioid agonist effects of the compound. When 1 μM naloxone was added to the buffer and concentration–response curves to N/OFQ were performed in the absence and presence of SR14148 (1 μM), it resulted in a parallel rightward shift of the concentration–response curve to N/OFQ, without significantly affecting the maximal agonist response (Figure 4). The pK_B value of SR14148 calculated from these experiments was 6.94. This demonstrates that, at the concentrations tested, SR14148 could exhibit its NOP antagonist activity, although the overall effect of the compound in this native receptor preparation is due to its opioid receptor activity.

The inhibitory effects of NOP/μ agonists SR14150 and SR16476 in the mouse vas deferens were further evaluated in the presence of the selective peptide NOP receptor antagonist UFP-101 (10 μM) as well as with naloxone (1 μM). As shown in Figure 5, the inhibitory effects elicited by both compounds were only slightly sensitive to UFP-101, whereas they were antagonized by naloxone to a greater extent. SR14150, which is 20-fold selective for NOP receptors but is a partial agonist at this site, appears to rely upon μ-opioid receptors to inhibit the electrically induced contractions in the mouse vas deferens, although the effect was completely abolished in the presence of naloxone (Figure 5a). However, SR16476, which has full agonist activity at the NOP receptor, maintained significant agonist activity in the vas deferens even in the presence of 1 μM naloxone (Figure 5b), indicating

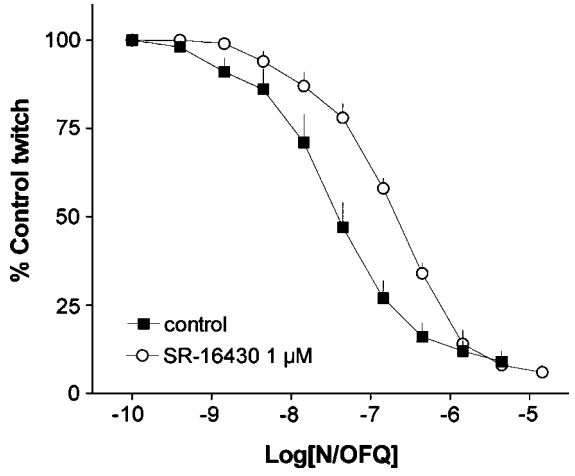

Figure 2 Concentration–response curve for N/OFQ inhibition of electrically induced contractions of the mouse vas deferens obtained in the absence and presence of 1 μM SR16430. The values are means ± s.e.mean of four separate experiments.

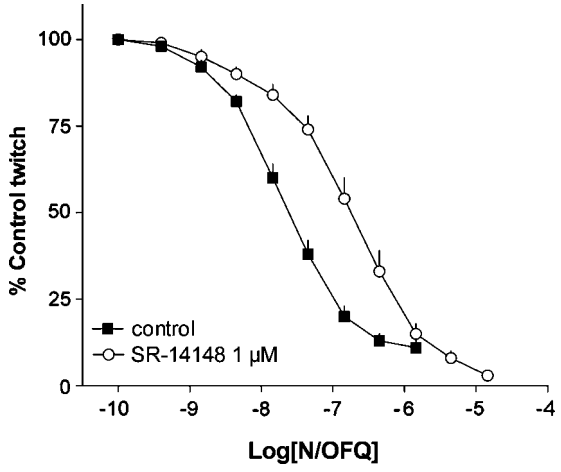

Figure 4 Concentration–response curve for N/OFQ inhibition of electrically induced contractions of the mouse vas deferens obtained in the absence and presence of 1 μM SR14148. Naloxone (1 μM) was added to the buffer. The values are means ± s.e.mean of three separate experiments.

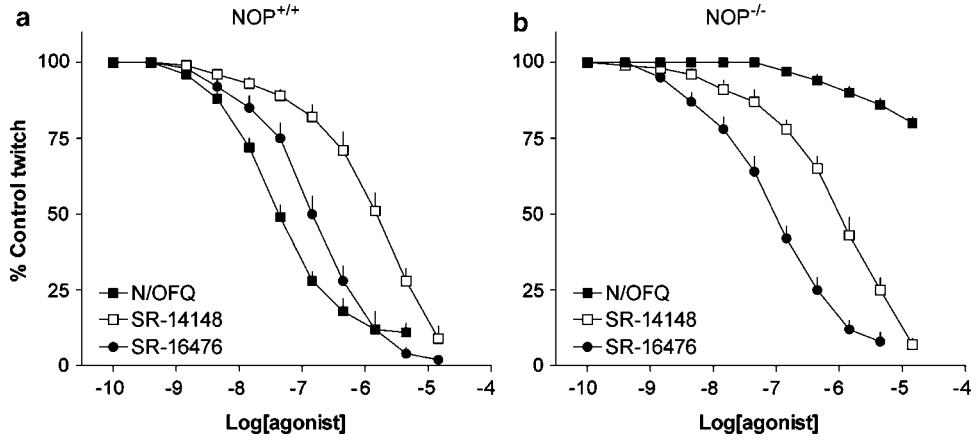

Figure 3 Effect of N/OFQ, SR14148 and SR16476 in the electrically stimulated mouse vas deferens from $NOP^{+/+}$ (**a**) and $NOP^{-/-}$ (**b**) mice. N/OFQ had pEC_{50} of 7.49 in the $NOP^{+/+}$ mice and was inactive in the tissue from $NOP^{-/-}$ animals. SR14148 had pEC_{50} values of 5.92 and 6.02, and SR16476 had pEC_{50} values of 6.85 and 7.08 in the $NOP^{+/+}$ and $NOP^{-/-}$ mice, respectively. The values are means ± s.e.mean of three separate experiments.

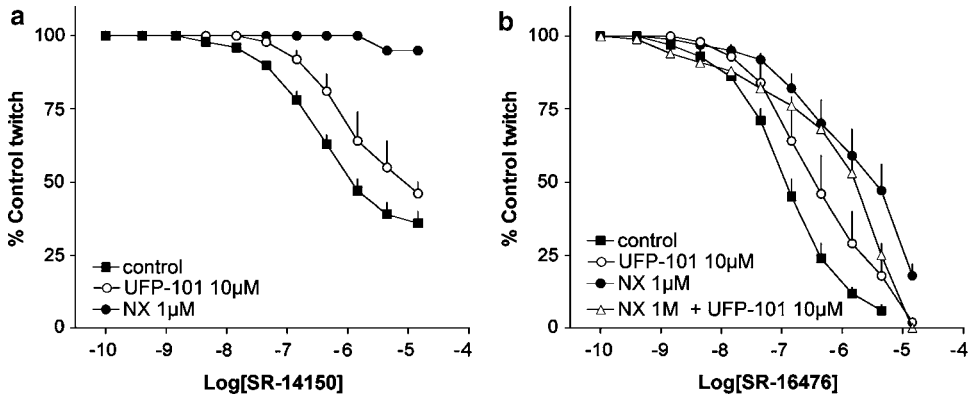

Figure 5 Concentration–response curve for SR14150 (**a**) and SR16476 (**b**) inhibition of electrically induced contractions of the mouse vas deferens obtained in the absence and presence of UFP-101 (10 μM) and naloxone (1 μM). The values are means ± s.e.mean of three separate experiments.

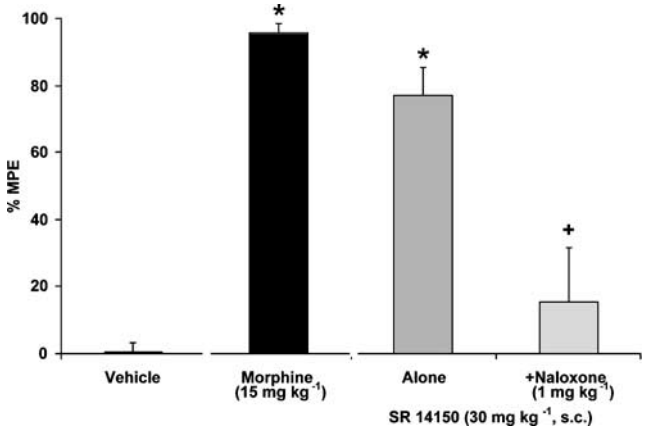

Figure 6 The effect of naloxone (1 mg kg^{-1}) on SR14150-induced antinociception in the tail flick assay *in vivo* at 60 min post-injection time. Data are mean %MPE (± s.e.mean). An asterisk (*$P<0.05$; Student–Newman–Keuls test) represents a significant difference from vehicle control, whereas a plus sign (+$P<0.05$; Student–Newman–Keuls test) represents a significant difference from SR14150 alone.

significant NOP receptor-mediated inhibition of twitch. In fact, even in the presence of both antagonists, the response to SR16476 was not further inhibited, suggesting that this compound may be inhibiting contractions at higher concentrations through another unknown receptor.

Effects of SR14150 on tail-flick latency and reversal by co-administration of naloxone

The effects of SR14150 alone or co-administered with naloxone on tail-flick latency are shown in Figure 6. The overall ANOVA indicated that there was a significant effect [$F(3,43)=39.4$, $P<0.05$]. As expected, our positive control morphine produced a significant increase in tail-flick latency relative to controls ($P<0.05$). The 30 mg kg^{-1} dose of SR14150 produced levels of antinociception, similar to those produced by morphine. Naloxone reversed the antinociceptive effects of SR14150 and brought the %MPE back to control levels, indicating that the analgesic effects of SR14150 were mediated by agonist activity at μ-opioid receptors.

Discussion

N/OFQ and other NOP-active compounds have very complicated pharmacological profiles. Initially, when N/OFQ was injected i.c.v., it was found to be nociceptive or block opioid- and stress-induced analgesia (Meunier *et al*., 1995; Reinscheid *et al*., 1995; Mogil *et al*., 1996). Conversely, when administered intrathecally, N/OFQ is analgesic or potentiates the antinociceptive activity of morphine (Xu *et al*., 1996; Tian *et al*., 1997). Because NOP receptor activation is inhibitory (Moran *et al*., 2000), NOP must be blocking opioid pathways in the brain and blocking pain pathways in the spinal cord. The involvement of an endogenous NOP tone in pain processing is also unclear. Although selective peptide antagonists, when administered i.c.v., are potent analgesics (Di Giannuario *et al*., 2001; Calo *et al*., 2002), with the exception of the moderately potent and poorly selective JTC-801 (Yamada *et al*., 2002; Suyama *et al*., 2003), small molecule antagonists do not seem to have antinociceptive activity in models of acute pain (Ozaki *et al*., 2000; Zaratin *et al*., 2004). The biological actions of NOP activation or blockade are also complicated when it comes to regulation of opioid actions. For instance, both N/OFQ and small molecule NOP antagonists have been shown to block the development of morphine tolerance (Lutfy *et al*., 2001; Zaratin *et al*., 2004). Moreover, genetic experiments suggest that a functioning NOP system is required for development of tolerance to opioids (Ueda *et al*., 2000; Chung *et al*., 2006).

Because the *in vivo* actions of compounds active at NOP receptors are complicated, it is important to have as thorough an *in vitro* profile of new ligands as possible, particularly because initial experiments can be misleading. The first publication of an NOP 'antagonist' was a mouse vas deferens study of [F/G]N/OFQ(1-13)NH_2 (Guerrini *et al*., 1998). In the mouse vas deferens, this compound is an antagonist, with no ability to inhibit electrically induced contractions. Nevertheless, when tested in CHO cells, it was found to be a quite efficient agonist with respect to

inhibition of cAMP accumulation (Butour *et al*., 1998). It is also a partial agonist for stimulation of [^{35}S]GTPγS binding (Burnside *et al*., 2000), and in fact, is as efficacious as N/OFQ for inhibition of morphine antinociception *in vivo* (Grisel *et al*., 1998; Candeletti *et al*., 2000). We have found similar and somewhat surprising results for some of the SR compounds tested. Although SR14148 is an antagonist at NOP receptors, and weak at stimulating [^{35}S]GTPγS binding to μ-receptor containing CHO cells, it still produces virtually 100% inhibition of contractions of the mouse vas deferens, almost certainly due to its μ-receptor agonist activity. More surprisingly, SR14150 is a high-affinity partial agonist at NOP receptors, and it inhibits contractions of the mouse vas deferens. Even though it is 20-fold selective for NOP receptors, and only produces approximately 20% stimulation of μ-receptors in the [^{35}S]GTPγS assay, the inhibition of electrically stimulated twitch response in the mouse vas deferens is due to its μ-receptor partial agonist activity and not its NOP receptor agonist activity, since it is almost completely abolished by naloxone and poorly inhibited by UFP-101 (Figure 5). These results in the mouse vas deferens suggest that SR14150 could also potentially have μ-agonist activity *in vivo*. In fact, we have confirmed this by demonstrating that SR14150 had antinociceptive activity in the tail-flick assay in mice and that this activity was blocked by naloxone (Figure 6). However, some mixed NOP/μ-opioid receptor agonists are capable of producing behavioural effects mediated by both NOP and μ-opioid receptors. SR16435 has antinociceptive activity due to μ-opioid receptor binding, but is sedative due to activation of NOP receptors (Khroyan *et al*., 2007).The data with the mixed NOP/μ ligands in the mouse vas deferens demonstrates that in this native tissue preparation, NOP activity is manifested only when the compound is a full agonist (100% efficacy in GTPγS-binding assay) but not when the NOP ligand is a partial agonist (compare SR16476 and SR14150). These results are perhaps predictive of the overall *in vivo* effects of such mixed ligands, as we have demonstrated with SR16435 (Khroyan *et al*., 2007) and with SR14150 (Figure 6). These results also suggest that the NOP receptor reserve in the mouse vas deferens may be significantly less than in the transfected CHO cells. Thus, only compounds that have full agonist activity at NOP show NOP-related effects in the mouse vas deferens, not reversible by naloxone (that is SR16476), but NOP partial agonists such as SR14150 that also have μ partial agonist activity only show μ-related activity, completely reversible by naloxone.

It is possible that a beneficial profile for an analgesic compound would be a mixed NOP/μ-opioid receptor agonist. Because NOP receptor agonists attenuate both tolerance development and reward, we have hypothesized that a compound that binds to and activates both NOP and μ-opioid receptors would be analgesic with reduced tolerance development and reduced addiction liability. We examined this hypothesis with the mixed agonist SR16435, as we reported previously (Khroyan *et al*., 2007). This compound has approximately equal affinity at both receptors and partial agonist activity at both receptors. Although SR16435 is analgesic and has somewhat reduced tolerance development, it was equally rewarding as morphine in the place-conditioning paradigm in mice. However, the NOP receptor full agonist Ro 64-6198 has been demonstrated to block morphine-conditioned place preference, as well as alcohol-conditioned place preference and self-administration, when added systemically (Shoblock *et al*., 2005; Kuzmin *et al*., 2007). Therefore, it is possible that SR16435 maintains its rewarding nature because it is only a partial agonist at NOP receptors. Our current results with the mouse vas deferens and our previous *in vivo* observations with SR16435 strongly suggest that to obtain effective NOP receptor modulation of the μ-receptor-mediated tolerance and reward, full NOP receptor agonist activity is required. We are currently examining SR16476 and other full NOP receptor agonists/μ-opioid receptor partial agonists, for analgesic activity and potential to induce reward. These studies will provide evidence as to whether NOP receptor full agonist activity, together with μ-receptor activity can produce analgesics with reduced addiction liability.

Results presented here may also answer some of the questions regarding the antinociceptive profile of buprenorphine, a potent μ-receptor partial agonist that has been reported to have NOP receptor activity at higher concentrations. There is some evidence that the mixed nature of buprenorphine is responsible for some of its *in vivo* properties with respect to both pain relief and addiction. Lutfy *et al*. (2003) have demonstrated that buprenorphine is considerably more potent in the tail-flick assay in the presence of the NOP receptor antagonist J-113397. Furthermore, at high signal intensity, buprenorphine displays an inverted U-shaped dose–response curve in the tail-flick assay, and the descending portion is not apparent in NOP receptor-knockout mice (Lutfy *et al*., 2003). These authors have suggested that the analgesic activity of buprenorphine is attenuated due to activation of NOP receptors at higher concentrations of buprenorphine. Similarly, Ciccocioppo *et al*. (2007) have demonstrated that buprenorphine potentiates alcohol consumption in alcohol-preferring rats at low doses but attenuates alcohol consumption at higher doses. As with the analgesic activity, the alcohol-attenuating actions of buprenorphine are blocked by an NOP receptor antagonist (Ciccocioppo *et al*., 2007). Once again, because we know that NOP receptor agonists block alcohol self-administration and morphine and alcohol place preference (Murphy *et al*., 1999; Ciccocioppo *et al*., 2000, 2004), the most straightforward explanation is that NOP receptors mediate the alcohol-attenuating actions of high doses of buprenorphine.

On the other hand, some of our current data would argue against that hypothesis. First of all, in our hands, buprenorphine showed at least 50-fold higher affinity at μ-opioid receptors than at NOP receptors (Table 1) and the inhibition of analgesia occurs at concentrations barely above those required for analgesia (Lutfy *et al*., 2003). For instance, Lutfy *et al*. show that in the NOP receptor-knockout mice there is higher antinociceptive activity than in the wild type even at the lowest analgesic dose of buprenorphine (0.3 mg kg^{-1}). For alcohol consumption, there is a more reasonable 100-fold difference in potentiating (0.03 mg kg^{-1}) and inhibiting (3 mg kg^{-1}) doses of buprenorphine, presumably μ-opioid and NOP-receptor mediated actions, respectively (Ciccocioppo *et al*., 2007). It is certainly possible that buprenorphine's

mechanism of action is different with respect to pain versus alcohol consumption. It is also possible that buprenorphine is particularly effective at activating NOP receptors, but this does not seem to be the case either. Buprenorphine was reported to be highly efficacious at NOP receptors in a MAP kinase assay in CHO cells, but at three orders of magnitude higher concentrations than is required for activation by N/OFQ (Lutfy *et al*., 2003). Buprenorphine has also been demonstrated to have NOP receptor agonist activity by two other groups (Wnendt *et al*., 1999; Huang *et al*., 2001). Liu Chen and colleagues have even shown partial NOP agonist activity using the [^{35}S]GTPγS-binding assay in CHO cells obtained from our laboratory (Huang *et al*., 2001). However, in our experiments, buprenorphine is an NOP receptor antagonist with no agonist activity (Table 2). Furthermore, in a study of [^{35}S]GTPγS binding in brain membranes, which tend to have lower receptor reserve (and therefore compounds tend to have lower efficacy than in the artificial system of transfected cells), buprenorphine was an antagonist at NOP receptors and had K_e for inhibition of N/OFQ activity of approximately 2000 nM (Lester and Traynor, 2006).

There are other reasons to question the involvement of NOP receptors in the inverted U-shaped profile of buprenorphine. First of all, buprenorphine is a partial agonist at μ-opioid receptors. In fact, other partial agonists are also known to have inverted U-shaped dose–response curves. In particular, both MC-CAM and pentazocine are partial μ-opioid receptor agonists (Table 2) and can both display similar inverted U-shaped dose–response curves in the tail-flick assay (Cowan *et al*., 1977; Husbands and Lewis, 2003). In fact, MC-CAM has biological properties very similar to those of buprenorphine and was considered, along with buprenorphine, as a drug abuse medication (Husbands and Lewis, 2003). Moreover, MC-CAM has low affinity and pentazocine has no affinity for NOP receptors. Therefore, neither could mediate an agonist response at NOP receptors, and their inverted U-shaped dose–response curves for antinociceptive activity are certainly not due to NOP receptor activation. Finally, both SR16435 and SR14150 have far higher affinity and efficacy at NOP receptors than buprenorphine, both are analgesic in the tail-flick test in mice, sensitive to naloxone, but not NOP receptor antagonists, and neither showed an inverted U-shaped dose–response curve at the stimulus intensity used in those experiments (Figure 6 and Khroyan *et al*., 2007). The involvement of NOP receptors in the antinociceptive actions of these compounds is not clear, but NOP activation does not seem to be attenuating the μ-opioid-mediated antinociceptive activity.

These arguments suggest other hypotheses for the effect of NOP receptor antagonists and knockout mice on buprenorphine's antinociceptive activity are needed. One such hypothesis is that opioid agonists might release endogenous N/OFQ and this endogenous N/OFQ could activate NOP receptors in brain and thus attenuate the opioid analgesic activity. It is possible that high-efficacy agonists are not affected by release of endogenous N/OFQ, at least not to the point where we can measure them in a standard tail-flick assay. This would be consistent with the fact that the morphine dose–response curve for antinociceptive activity is unchanged in NOP-knockout mice (Mamiya *et al*., 2001). Buprenorphine, on the other hand, as a partial agonist, may be more susceptible to the anti-opiate actions of endogenous N/OFQ. MC-CAM has even lower efficacy at μ-opioid receptors than buprenorphine (Table 2) and has a pattern of *in vivo* agonist effectiveness that is less than buprenorphine, which in turn is less than morphine (Woods *et al*., 1995). At higher signal intensity, such as 55 °C water, many partial agonists lose agonist activity (Smith *et al*., 1999). It would be interesting to see if the analgesic activity of MC-CAM and other partial agonists such as pentazocine or butorphanol was potentiated by NOP receptor antagonists or in NOP receptor-knockout mice. If so, it would suggest an NOP receptor tone in the antinociceptive pathway and would lead to the question, why NOP receptor antagonists are not better analgesics.

In conclusion, we have tested a series of compounds with both NOP and μ-opioid receptor activity, including the opioid analgesic buprenorphine. This set of compounds binds to the two receptors with varying affinities and efficacies. *In vitro*, mouse vas deferens bioassays demonstrated that assays on transfected cells could sometimes be misleading with respect to the actions of uncharacterized compounds and suggest that the antinociceptive activity of some of these compounds is probably due to activation of μ-opioid receptors. This was confirmed with the 20-fold selective NOP agonist, SR14150, whose antinociceptive activity was reversed by naloxone. Nevertheless, compounds with affinity for both NOP and μ-opioid receptors may have a useful profile as analgesics with reduced tolerance development and reduced addiction liability.

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Conflict of interest

The authors state no conflict of interest.

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