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RESEARCH PAPER

Identification of an additional supraspinal component to the analgesic mechanism of action of buprenorphine

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Background and purpose: Buprenorphine displays attributes of opioids, but also some features distinct from them. We examined spinal and supraspinal signal transduction of buprenorphine-induced anti-nociception in mice compared with morphine and fentanyl.
Experimental approach: The opioid receptor antagonist naloxone, *Pertussis* toxin (PTX), G_z protein antisense and nociceptin/orphanin-FQ receptor agonist nociceptin, and antagonist, JTC-801, were injected supraspinally (intracerebroventricular) and spinally (intrathecal). Also the cell-permeable Ser/Thr protein phosphatase inhibitor okadaic acid was given supraspinally.
Key results: Spinal naloxone (20 μg) or PTX (1 μg) attenuated morphine, fentanyl and buprenorphine (s.c.) anti-nociception. Supraspinal naloxone or PTX attenuated morphine and fentanyl, but not buprenorphine anti-nociception. Spinal G_z protein antisense did not alter buprenorphine, morphine or fentanyl anti-nociception and supraspinal G_z-antisense did not alter morphine or fentanyl anti-nociception. However, supraspinal G_z-antisense (not random sense) reduced buprenorphine anti-nociception. Peripheral JTC-801 (1 $mg \cdot kg^{-1}$, i.p.) enhanced the ascending (3 $mg \cdot kg^{-1}$) and descending (30 $mg \cdot kg^{-1}$) portions of buprenorphine's dose–response curve, but only spinal, not supraspinal, nociceptin (10 $nmol \cdot L^{-1}$) enhanced buprenorphine anti-nociception. Intracereboventricular okadaic acid (0.001–10 pg) produced a biphasic low-dose attenuation, high-dose enhancement of buprenorphine(3 or 30 $mg \cdot kg^{-1}$, s.c.) anti-nociception, but did not affect morphine or fentanyl anti-nociception.
Conclusions and implications: Buprenorphine has an opioid component to its supraspinal mechanism of analgesic action. Our present results reveal an additional supraspinal component insensitive to naloxone, PTX and nociceptin/orphanin-FQ, but involving G_z protein and Ser/Thr protein phosphatase. These data might help explain the unique preclinical and clinical profiles of buprenorphine.
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Keywords: analgesia (antinociception); buprenorphine; mechanism of action; supraspinal

Abbreviations: AUC_{0-60}, area under the curve (0–60 min); B or BUP, buprenorphine; CT, cut-off time; DMSO, dimethyl sulfoxide; ED_{50}, effective dose (50% effect); F, fentanyl; i.c.v., intracerebroventricular; i.p., intraperitoneal; i.t., intrathecal; JTC-801, N-(4-amino-2-methylquinolin-6-yl)-2-(4-ethylphenoxymethyl) benzamide monohydrochloride; M, morphine; MPE, maximum possible effect; Nc, nociceptin; Nx, naloxone; NOP, nociceptin/orphanin-FQ; OA, okadaic acid; PL, pre-drug latency; PTX, *Pertussis* toxin; Ser/Thr, serine/threonine; Veh, vehicle; WAY-100635, N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl)-N-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride

Introduction

Buprenorphine {[5α,7α(*S*)]-17-(cyclopropylmethyl)-α-(1,1-dimethylethyl) - 4, 5 - epoxy - 18, 19 - dihydro - 3 - hydroxy - 6 - methoxy - α - methyl - 6, 14 - ethenomorphinan - 7 - methanol} (Bentley and Hardy, 1967; Bentley *et al.*, 1967) is a centrally acting analgesic that was first synthesized in the late 1960s. It is an analogue of the poppy-derived opiate alkaloid thebaine and possesses high binding affinity for opioid receptors (Villiger and Taylor, 1981; Rothman *et al.*, 1995; Huang *et al.*, 2001; Lutfy and Cowan, 2004), including those *in situ* in human brain (Greenwald *et al.*, 2003). Buprenorphine shares some of the general preclinical (Cowan, 1995) and clinical attributes of standard opioid agonists such as morphine and fentanyl (see Budd and Raffa, 2005), but differs by having slow receptor dissociation kinetics, a biphasic ('bell'- or 'inverted U'-shaped) dose–response relation in a few animal models (see Christoph *et al.*, 2005); clinical implications discussed in Raffa and Ding, 2007) and a ceiling effect on

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respiratory depression, but not analgesia, in humans (Dahan *et al.*, 2006). It has been suggested that morphine and buprenorphine induce anti-nociception through different transduction mechanisms (Wheeler-Aceto and Cowan, 1991).

Two equally plausible and firmly supported, but seemingly mutually exclusive, sets of results have emerged regarding the mechanism of buprenorphine's anti-nociceptive action. In one, low and high doses on buprenorphine's anti-nociceptive dose–response curve, even when biphasic, are attenuated by the opioid receptor antagonist naloxone (Rance *et al.*, 1980; Dum and Herz, 1981; Cowan, 1995), implying, by the law of mass action, a single (opioid) mechanism of action. In the other, the descending portion of buprenorphine's dose–response curve is eliminated by the nociceptin/orphanin-FQ (NOP) competitive receptor antagonist J-113397 {1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one} and is absent in NOP receptor [opioid receptor-like (ORL)1] knockout mice (Lutfy *et al.*, 2003), implying the crucial involvement of a NOP-mediated pathway.

As part of our ongoing study of buprenorphine, we investigated the supraspinal and spinal signal transduction of buprenorphine-induced anti-nociception in comparison with that of two standard opioids that do not share the attributes of buprenorphine, namely morphine and fentanyl. A combination of approaches described in the present report, including spinal and supraspinal administration of naloxone, *Pertussis* toxin (PTX), G_z protein subtype antisense and selective NOP receptor ligands, led to the unexpected identification of an additional supraspinal mechanism of buprenorphine-induced anti-nociception. To further explore the mechanism, we examined the possible involvement of serine/threonine (Ser/Thr) protein phosphatases. Protein kinases have been implicated in opioid tolerance, receptor internalization, neuropathic pain (Ahlgren and Levine, 1994; Liu and Anand, 2001; Wang and Wang, 2006) and possibly in respiratory depression (Groer *et al.*, 2007). In the present study, we measured the relative impact of the cell-permeable supraspinal protein phosphatase inhibitor okadaic acid (9,10-deepithio-9,10-didehydroacanthifolicin) (Li and Casida, 1992; Honkanen and Golden, 2002) on s.c. buprenorphine-, morphine- and fentanyl-induced anti-nociception. Okadaic acid binds the catalytic subunit of Ser/Thr phosphatases, thereby inhibiting enzymatic activity (Li and Casida, 1992; Honkanen and Golden, 2002). We report here that intracerebroventricular (i.c.v.) okadaic acid selectively affected buprenorphine-induced anti-nociception in a biphasic manner.

Methods

Animals

Animals were housed and treated in accordance with the recommendations and policies of the Institute of Laboratory Animal Resources (1996). The experiments adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain and were reviewed by an institutional animal care and use committee. Male albino mice 20–25 g (Charles River Laboratories) were used for all portions of the study. They were housed five per cage in a temperature-controlled room (22°C, 60% humidity) maintained on an automatic 12 h light–dark cycle. They had access to food and water *ad libitum*. The mice were acclimatized in the experimental room for 2 h before the test. Each mouse was used only once.

i.c.v. and intrathecal administration

The supraspinal (i.c.v.) injections were made into the right lateral cerebral ventricle according to the procedure described by Haley and McCormick (1957), using a 3.5 mm, 27 gauge hypodermic needle (B-D Yale; Rutherford, NJ) mated to a 10 μL Luer's-tip syringe (Hamilton; Reno, NV). The spinal (intrathecal, i.t.) injections were made into the subarachnoid space between L5 and L6 according to the procedure described by Hylden and Wilcox (1980), using a 30 gauge needle mated to a 10 μL Luer's-tip syringe. Each of the compounds or vehicle was injected in a total volume of 5 μL (i.c.v. or i.t.).

Acetylcholine-induced abdominal irritant test

The procedure originally described by Collier *et al.* (1968) was used. Each mouse was injected i.p. with acetylcholine bromide (5.5 $mg \cdot kg^{-1}$; 0.25 mL per 25 g mouse), placed individually into an observation chamber (open-topped plastic box), and observed for the occurrence of a single behavioural response indicative of irritation to the injection of acetylcholine (wave of abdominal stretching and extension of at least one limb). Injection of vehicle (i.p.) never produced the behavioural response and i.p. administration of acetylcholine alone always produced the behavioural response. Test drug or vehicle was administered by the route and at time designated prior to the i.p. injection of acetylcholine. If a mouse displayed the behaviour during the 10 min observation period, it was immediately removed from the box; if it did not respond within the 10 min observation period, it was recorded as a non-responder.

Water tail-immersion/flick test

The procedure originally described by Janssen *et al.* (1963) with minor modifications was used. Mice were placed into restraining holders with their tails hanging freely and the distal portion of their tail was lowered into a temperature-controlled water bath (48°C or 55°C). The time between tail-immersion and removal ('flick') of the tail out of the water was recorded as tail-flick latency. A baseline control latency value was obtained for each mouse before drug administration. After drug administration, the procedure was repeated and reaction times were compared with pre-drug reaction times. Experimental values were recorded for each mouse at 20, 30, 40 or 60 min. The mice were removed immediately following a tail-flick or at the predetermined cut-off time (40 s for 48°C; 15 s for 55°C), established to prevent injury to the tail.

Quantification, statistics, nomenclature

For the acetylcholine-induced abdominal irritant test, anti-nociception was quantified as the per cent inhibition of

response (quantal measure) and was calculated for each dose as: % anti-nociception = 100 × (number of non-responders)/(number of mice in the group). For the water-immersion tail-flick test, anti-nociception was quantified as the per cent of maximum possible effect (%MPE) (graded measure) by using the formula: %MPE = 100 × (test latency – PL)/(CT – PL), where PL = the pre-drug latency and CT = the cut-off time. The results are reported as the mean %MPE ± SEM. In addition, for buprenorphine, the okadaic acid-induced change from baseline was determined and graphed as the area under the %MPE–time graph from 0 to 60 min post administration ($AUC_{0–60}$). The estimated ED_{50} value of each drug was calculated from the equation for the corresponding dose–response curve. Group means were compared using one-way ANOVA and, if $P < 0.05$, with a *post hoc* test. In all cases, a probability level of $P < 0.05$ was the criterion for statistical significance.

The nomenclature used here conforms to the *British Journal of Pharmacology* 'Guide to receptors and channels' (Alexander *et al.*, 2008).

Materials

Buprenorphine hydrochloride, morphine sulphate and fentanyl hydrochloride were gifts from the National Institute of Drug Abuse (Rockville, MD) to Ellen A. Walker, PhD (Temple University School of Pharmacy). Naloxone hydrochloride, yohimbine hydrochloride, PTX and okadaic acid (9,10-deepithio-9,10-didehydroacanthifolicin; mol wt 805) were purchased from Sigma-Aldrich Inc (St. Louis, MO). Nociceptin(1-13)NH_2, JTC-801 [*N*-(4-amino-2-methylquinolin-6-yl)-2-(4-ethylphenoxymethyl) benzamide mono-hydrochloride] and WAY-100635 {*N*-[2-[4-(2-methoxyphenyl) - 1 - piperazinyl] ethyl) - *N* - (2 - pyridinyl) cyclohexanecarboxamide trihydrochloride} were purchased from Tocris Bioscience (Ellisville, MI). Buprenorphine was dissolved in 0.1% lactic acid in sterile water. Morphine, fentanyl and naloxone were dissolved in saline. PTX, G_z antisense, nociceptin, yohimbine and WAY-100635 were dissolved in sterile water. Okadaic acid was dissolved in 1% Tween 80 (polysorbate 80) in ultrapure water. JTC-801 was dissolved in 10% DMSO (dimethyl sulfoxide). All doses are reported as the salt. Synthetic end-capped phosphorothioated G_z oligodeoxynucleotide antisense, 5′-GGGCCAGTAGCCCAATGGG-3′ (Standifer *et al.*, 1996) and random sense control, 5′-CCCTTATTTACTACTTTCGC-3′, were purchased from Boca Scientific Inc (Boca Raton, FL). The investigator was aware of the test treatments.

Results

Anti-nociception

Subcutaneous injection of buprenorphine, morphine or fentanyl induced dose-related anti-nociception in the abdominal irritant test and all three of the drugs attained 100% anti-nociception (Figure 1A). The order of potency was fentanyl (ED_{50} = 0.0004 mg·kg^{-1}) > buprenorphine (ED_{50} = 0.035 mg·kg^{-1}) > morphine (ED_{50} = 0.1 mg·kg^{-1}). The ED_{50} of buprenorphine was comparable to previous values (Huang *et al.*, 2001). The buprenorphine dose–response curve was not biphasic in this test.

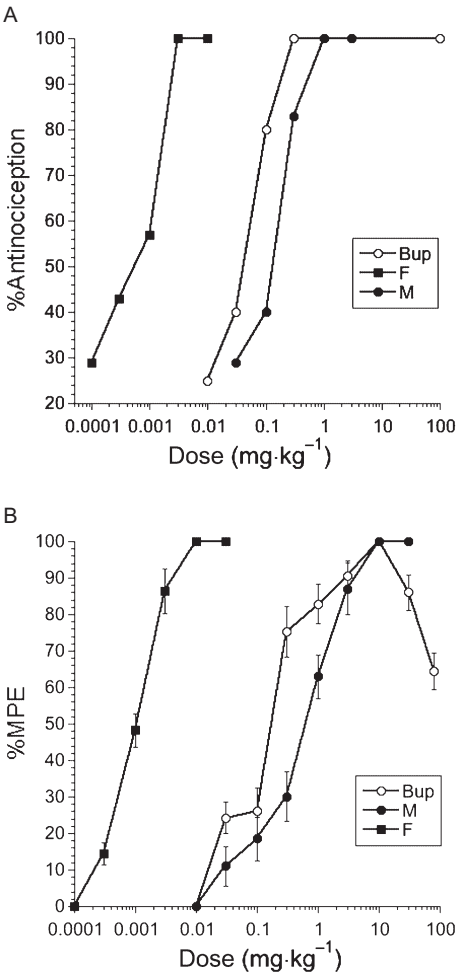

Figure 1 Anti-nociceptive dose–response curves for buprenorphine (Bup), morphine (M) and fentanyl (F) in the (A) abdominal constriction and (B) warm water (48°C) tail-dip/flick test (Raffa and Ding, 2007) in mice (mean ± SEM). Baseline latency = 7.6 ± 0.6 (Bup), 6.7 ± 0.7 (M) and 7.3 ± 0.4 (F) s. n = 4–8 mice per group. MPE, maximum possible effect.

S.c. administration of buprenorphine, morphine or fentanyl also induced dose-related anti-nociception in the 48°C water-immersion tail-flick test (Figure 1B). All three of the drugs attained 100% anti-nociception. At time of peak effect [determined in previous work (Raffa and Ding, 2007)], the order of potency was fentanyl (ED_{50} = 0.0011 ± 0.0003 mg·kg^{-1}) > buprenorphine (ED_{50} = 0.21 ± 0.04 mg·kg^{-1}) > morphine (ED_{50} = 0.55 ± 0.11 mg·kg^{-1}). The ED_{50} of buprenorphine was again comparable to previous values [e.g. Lutfy *et al.* (2003)]. The buprenorphine dose–response curve was biphasic in this test, increasing at doses below 10 mg·kg^{-1} and decreasing at doses above 10 mg·kg^{-1} (100% MPE at 10 mg·kg^{-1}). The morphine and fentanyl dose–response curves were monophasic below toxic doses, attaining 100% MPE at 10 and 0.01 mg·kg^{-1} respectively.

Naloxone

Pretreatment (10 min) with naloxone (10 mg·kg^{-1} i.p.) had no significant effect alone in the 48°C water-immersion tail-flick

test ($P > 0.05$), but significantly antagonized ($P < 0.05$) the anti-nociception induced by 3 mg·kg^{-1} s.c. morphine (31.8 ± 2.4 vs. 74.4 ± 7.3% MPE at 20 min; 22.8 ± 1.6 vs. 45.5 ± 5.1% MPE at 40 min; and 13.6 ± 2.1 vs. 37.8 ± 4.1% MPE at 60 min), 0.003 mg·kg^{-1} s.c. fentanyl (35.1 ± 2.2 vs. 85.1 ± 5.7% MPE at 20 min; 26.2 ± 2.6 vs. 58.4 ± 4.4% MPE at 40 min; and 18.4 ± 3.2 vs. 42.4 ± 4.2% MPE at 60 min). Naloxone also antagonized anti-nociception induced by both 1 mg·kg^{-1} buprenorphine (a dose on the ascending portion of buprenorphine's dose–response curve) and 30 mg·kg^{-1} buprenorphine (a dose on the descending portion of buprenorphine's dose–response curve) (Figure 2A).

Spinal pretreatment with naloxone (20 µg, 10 min prior) significantly antagonized ($P < 0.05$) the anti-nociception induced by s.c. buprenorphine, morphine or fentanyl in the 48°C water-immersion tail-flick test (Figure 2B). In the same test, supraspinal (i.c.v.) naloxone (20 µg, 10 min prior) significantly antagonized ($P < 0.05$) the anti-nociception induced by s.c. morphine or fentanyl, but had no significant effect ($P > 0.05$) on the anti-nociception induced by 1 mg·kg^{-1} buprenorphine (Figure 2C). In other experiments, higher doses of naloxone (50 and 100 µg) did not antagonize buprenorphine at either 1 or 30 mg·kg^{-1} (data not shown).

Pertussis toxin

Pretreatment with PTX spinally [1 µg i.t., 48 h prior; based on Womer *et al.* (1997); Wheeler-Aceto and Cowan (1991) and verified in pilot study] had no effect of its own in the abdominal irritant test or 48°C tail-flick test, but significantly antagonized ($P < 0.05$) the anti-nociception induced by subcutaneous administration of buprenorphine (Figure 3A and C), morphine or fentanyl (Table 1).

Pretreatment with PTX supraspinally (1 µg i.c.v., 48 h prior) had no effect of its own in the abdominal irritant test or 48°C tail-flick test, but significantly antagonized ($P < 0.05$) the anti-nociception induced by s.c. morphine or fentanyl. However, identical PTX treatment supraspinally had no significant effect ($P > 0.05$) on s.c. buprenorphine-induced anti-nociception in the abdominal irritant or the 48°C tail-flick test (Figure 3B and D, Table 1).

G_z antisense

Pretreatment with i.t. G_z antisense or random sense [14.6 µg, 24 h prior; based on Raffa *et al.* (1994) and product instructions and verified in pilot study] had no effect of its own and had no significant effect ($P > 0.05$) on the anti-nociception induced by s.c. administration of buprenorphine, morphine or fentanyl in the 48°C tail-flick test (Table 2).

Pretreatment with i.c.v. G_z antisense or random sense (14.6 µg, 24 h prior) had no significant effect ($P > 0.05$) on the anti-nociception induced by s.c. morphine or fentanyl. In contrast, the same treatment with G_z antisense, but not random sense, significantly antagonized ($P < 0.05$) the anti-nociception induced by s.c. buprenorphine in the same test (Table 2).

Nociceptin and JTC-801

The NOP receptor antagonist JTC-801 (1 mg·kg^{-1}) (Shinkai *et al.*, 2000; Yamada *et al.*, 2002) had no significant effect of its

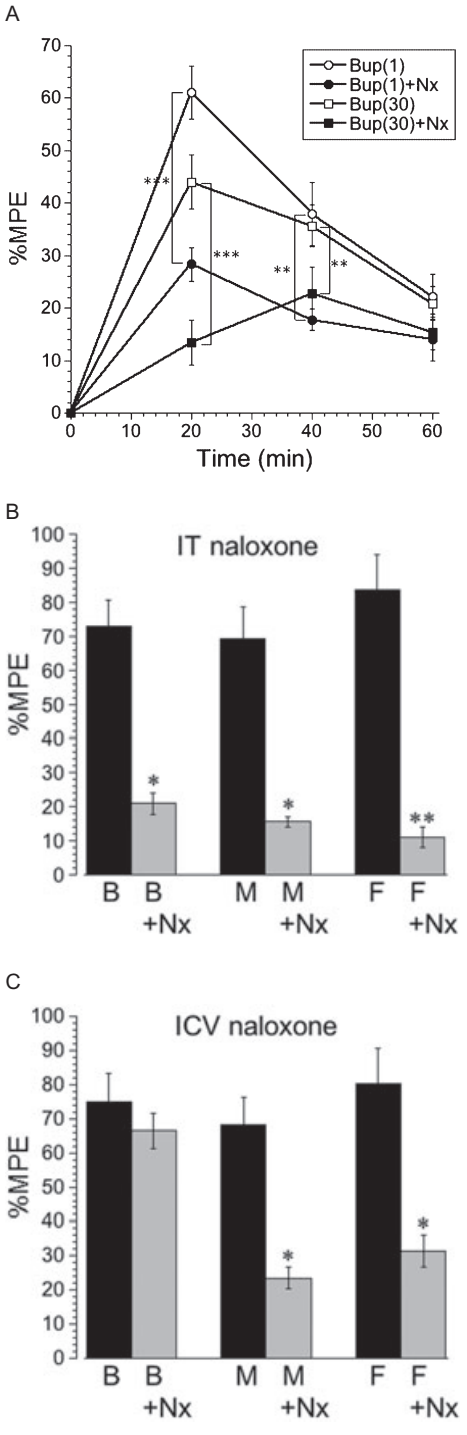

Figure 2 The effect of naloxone (Nx) administered: (A) i.p. (10 mg·kg^{-1}); (B) spinally (IT) (20 µg, 10 min prior); or (C) supraspinally (ICV) (20 µg, 10 min prior) on the anti-nociception induced by s.c. morphine (M) (3 mg·kg^{-1}); fentanyl (F) (0.003 mg·kg^{-1}); or buprenorphine (Bup or B) (1 and 30 mg·kg^{-1} in A; 1 mg·kg^{-1} in B and C). Baseline latencies with/without naloxone: (A) 5.6 ± 0.4 to 7.2 ± 1.1 s/5.9 ± 0.8 to 7.7 ± 0.6 s; (B) 8.3 ± 0.7/7.1 ± 0.6 s (B); 6.2 ± 0.8/7.4 ± 0.7 s (M); 8.1 ± 0.7/8.4 ± 0.3 s (F); (C) 6.2 ± 0.4/6.0 ± 0.8 s (B); 5.6 ± 0.7/5.4 ± 0.5 s (M); 5.4 ± 0.6/6.4 ± 0.6 s (F). Veh = vehicle. n = 6–8 mice per group. Mean ± SEM, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. ICV, intracerebroventricular; IT, intrathecal.

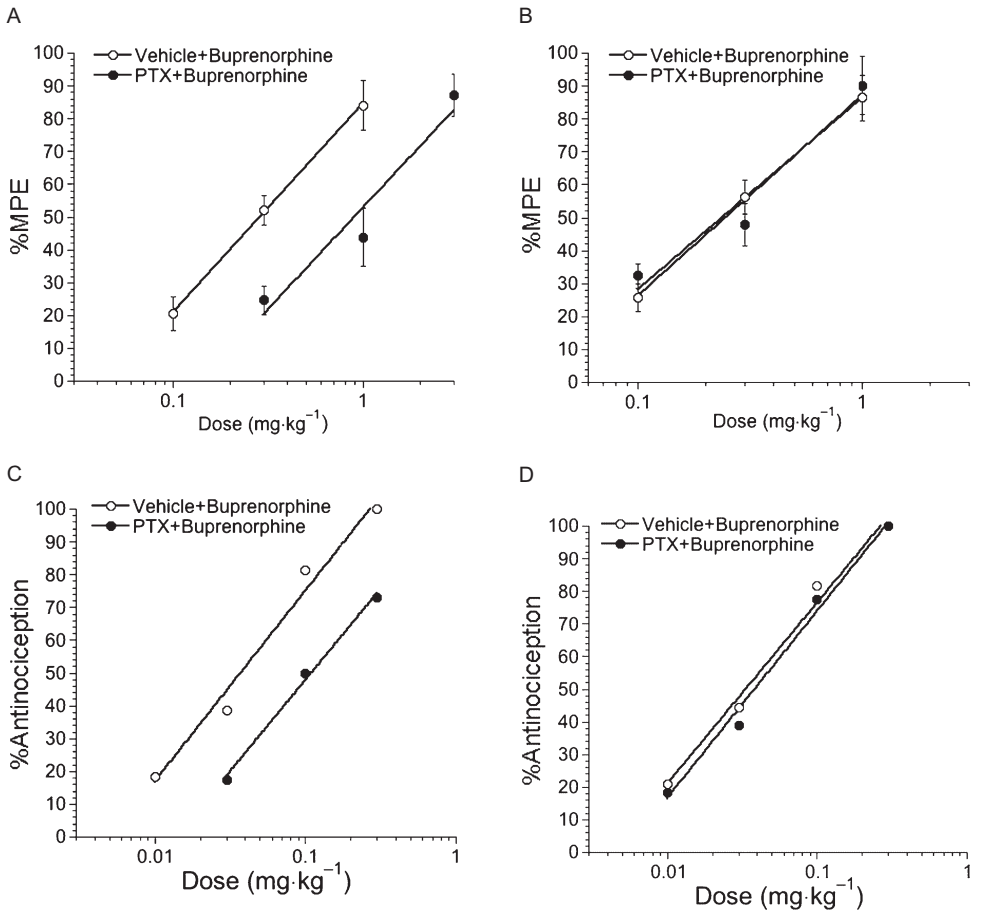

Figure 3 Buprenorphine (s.c.)-induced anti-nociceptive dose–response curves in the (A and B) warm water 48°C tail-dip/flick test and (C and D) abdominal irritant test. Vehicle or PTX was administered by spinal (i.t.) (baseline latency = 7.2 ± 0.4 s) (A and C) or supraspinal (i.c.v.) (baseline latency = 7.2 ± 0.4 s) route (B and D). I.t. baseline latencies with/without PTX = 6.7 ± 0.2/7.5 ± 0.4 s (Bup), 7.0 ± 0.3/6.9 ± 0.8 s (M), 6.5 ± 0.6/7.2 ± 0.7 s (F); i.c.v. baseline latencies with/without PTX = 7.4 ± 0.5/6.8 ± 0.5 s (Bup), 7.4 ± 0.6/8.2 ± 0.8 s (M), 6.3 ± 0.4/6.5 ± 0.4 s (F). Mean ± SEM, n = 8–10 mice per group. i.c.v., intracerebroventricular; i.t., intrathecal; MPE, maximum possible effect; PTX, *Pertussis* toxin.

Table 1 Comparison of s.c. ED_{50} values (mg·kg^{-1}) for morphine-, fentanyl- and buprenorphine-induced anti-nociception in mice pretreated with vehicle (veh) or with i.c.v. or i.t. PTX (1 μg, 48 h prior)

Test	*Route*	*Veh PTX*	*Morphine*	*Fentanyl*	*Buprenorphine*
TF (48°C)	i.c.v.	veh	0.53	0.0007	0.25
		PTX	1.24*	0.0018*	0.24
	i.t.	veh	0.63	0.0007	0.29
		PTX	1.56*	0.0015*	0.90*
MAIT	i.c.v.	veh	0.096	0.0004	0.034
		PTX	0.220*	0.001*	0.039
	i.t.	veh	0.091	0.0004	0.037
		PTX	0.248*	0.0011*	0.11*

n = 8–10 mice per group. Significant difference ($P < 0.05$) indicated by asterisk. Shaded cells indicate no difference ($P > 0.05$) from vehicle.
i.c.v., intracerebroventricular; i.t., intrathecal; MAIT, mouse abdominal irritant (5.5. mg·kg^{-1} acetylcholine i.p.) test; TF, warm water (48°C) tail-immersion/flick test; PTX, *Pertussis* toxin.

Table 2 Effects of G_z protein on anti-nociception (%MPE ± SEM) in the 48°C water tail-immersion/flick test in mice

Route	*Treatment*	*Morphine*	*Fentanyl*	*Buprenorphine*
i.t.	Control	78.5 ± 11.7	81.0 ± 9.7	70.2 ± 12.4
	G_z antisense	70.3 ± 8.8	79.8 ± 10.4	74.4 ± 7.9
i.c.v.	Control	69.0 ± 8.9	77.2 ± 9.1	76.3 ± 8.8
	G_z antisense	75.6 ± 9.4	82.6 ± 6.5	35.2 ± 6.6*

Mice were pretreated with G_z protein antisense or control (random sense) (14.6 μg) administered i.t. (baseline latency = 8.4 ± 0.4 s) or i.c.v. (baseline latency = 6.9 ± 0.5 s) 24 h before s.c. injection of approximate equi-anti-nociceptive dose of morphine, fentanyl or buprenorphine (3, 0.003 and 1 mg·kg^{-1} respectively). I.t. baseline latencies with/without G_z antisense = 7.5 ± 0.3/7.6 ± 0.3 s (Bup), 7.2 ± 0.4/6.8 ± 0.8 s (M), 6.8 ± 0.7/8.2 ± 0.6 s (F); i.c.v. baseline latencies with/without G_z antisense = 6.7 ± 0.4/5.9 ± 0.6 s (Bup), 6.0 ± 0.6/6.8 ± 0.5 s (M), 6.5 ± 0.5/6.8 ± 0.6 s (F). n = 6–8 mice per group. Significant difference ($P < 0.05$) from control indicated by asterisk and shaded cell.
i.c.v., intracerebroventricular; i.t., intrathecal.

own, but potentiated ($P < 0.05$) both a low dose (on the ascending limb) and high dose (on the descending limb) of the s.c. buprenorphine dose–response curve (3 and 30 mg·kg^{-1} respectively) in the 48°C tail-flick test (Figure 4).

Nociceptin (10 nmol, 20 min prior) administered i.t. significantly ($P < 0.05$) increased 30 mg·kg^{-1} s.c. buprenorphine-induced anti-nociception (Figure 5A) in the 48°C tail-flick test, but nociceptin administered i.c.v had no effect on

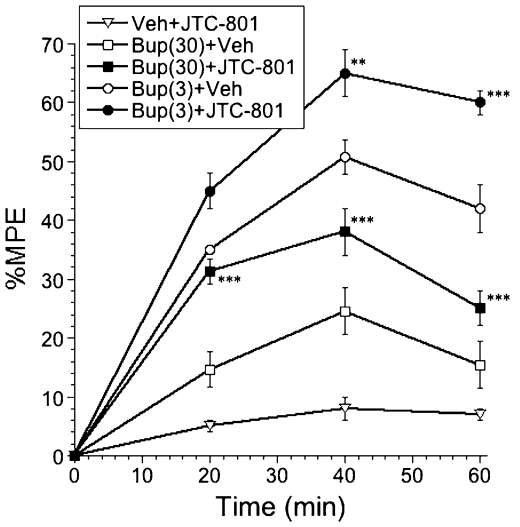

Figure 4 Enhancement of buprenorphine-induced anti-nociception in the 48°C tail-dip/flick test by the nociceptin/orphanin-FQ receptor antagonist JTC-801 (1 mg·kg^{-1}, i.p.) (baseline latency = 6.7 ± 0.4 s). Buprenorphine (Bup) was administered s.c. at either 3 or 30 mg·kg^{-1}. Baseline latencies = 5.0 ± 0.6 s to 5.6 ± 0.4 s. Veh = vehicle. Mean ± SEM, n = 6–8 mice per group. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with buprenorphine alone. MPE, maximum possible effect.

30 mg·kg^{-1} s.c. buprenorphine-induced anti-nociception in the same test (Figure 5B).

Yohimbine and WAY-100635

Buprenorphine-induced anti-nociception (3 or 30 mg·kg^{-1}, s.c.) in the warm water 48°C tail-dip/flick test was not altered by i.p. (3 or 30 mg·kg^{-1}) or i.c.v. (10 μg) administration of either an α-adrenoceptor antagonist (yohimbine) at a dose that inhibits anti-nociceptive doses of i.c.v. or i.t. propofol (Ge *et al.*, 2005) or a 5-HT$_{1A}$ antagonist (WAY-100635) at a dose that inhibits i.c.v. or i.t. trazodone (Zhang *et al.*, 2004) (data not shown).

Okadaic acid

Subcutaneous administration of approximately equipotent doses of buprenorphine (3 or 30 mg·kg^{-1}), morphine (10 mg·kg^{-1}) or fentanyl (0.01 mg·kg^{-1}) induced time-dependent anti-nociception in the 55°C water-immersion tail-flick test. Consistent with our earlier study (Raffa and Ding, 2007), at these doses, all three drugs attained the intended target of 40–60% MPE – allowing the detection of either an increase or a decrease in effect produced by okadaic acid. We used both 3 and 30 mg·kg^{-1} buprenorphine in subsequent study because its dose–response curve is biphasic in this test.

Subcutaneous administration of vehicle or okadaic acid alone (0.001, 0.01, 1 or 10 pg per mouse) had no significant effect on response latency in the 55°C tail-flick test (Figure 6A). I.c.v. administration of okadaic acid, over the same dose range did not alter ($P > 0.05$) the anti-nociception induced by either morphine or fentanyl (Figure 6B and C).

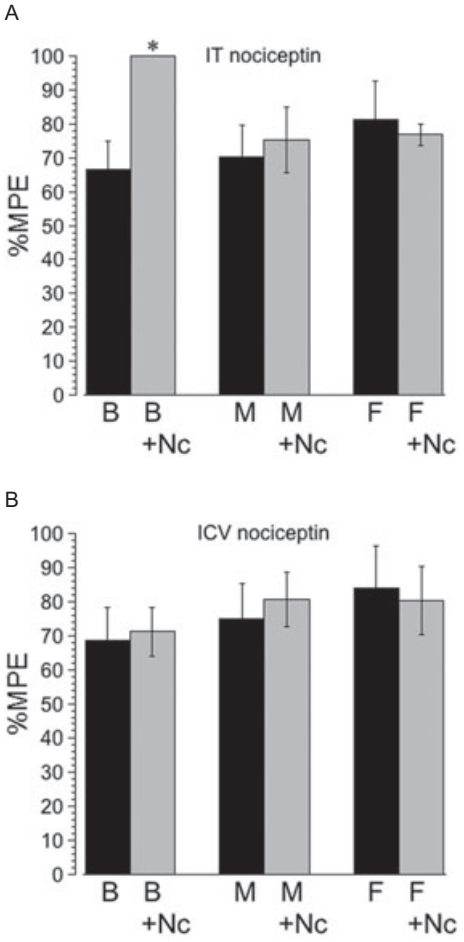

Figure 5 Enhancement of s.c. buprenorphine (30 mg·kg^{-1}) (B), but not morphine (3 mg·kg^{-1}) (M) or fentanyl (0.003 mg·kg^{-1}) (F), anti-nociception in the 48°C tail-dip/flick test by nociceptin (Nc) (10 nmol, 20 min prior) administered by (A) spinal (IT) (baseline latency = 8.1 ± 1.2 s), but not by (B) supraspinal (ICV) (baseline latency = 7.7 ± 0.7 s), route. I.t. baseline latencies with/without nociceptin = 7.8 ± 0.3/7.8 ± 0.8 s (Bup), 7.2 ± 0.7/7.2 ± 0.8 s (M), 6.7 ± 0.9/7.6 ± 0.8 s (F); i.c.v. baseline latencies with/without nociceptin = 8.0 ± 0.9/8.3 ± 0.4 s (Bup), 7.3 ± 0.7/6.1 ± 0.5 s (M), 5.5 ± 0.4/5.8 ± 0.7 s (F). Mean ± SEM, n = 6–8 mice per group. $^{*}P < 0.05$. i.c.v., intracerebroventricular; i.t., intrathecal; MPE, maximum possible effect.

I.c.v. administration of low doses of okadaic acid (0.003, 0.01 and 0.1 pg per mouse) significantly ($P < 0.05$) reduced the anti-nociception induced by 3 mg·kg^{-1} s.c. buprenorphine [the lowest dose of okadaic acid (0.001 pg per mouse) had no significant effect], whereas higher doses of okadaic acid (1 and 10 pg per mouse) significantly ($P < 0.05$) increased the anti-nociception induced by 3 mg·kg^{-1} s.c. buprenorphine in the 55°C tail-flick test (Figure 7A).

Likewise, i.c.v. administration of low doses of okadaic acid (0.003, 0.01 and 0.1 pg per mouse) significantly ($P < 0.05$) reduced the anti-nociception induced by 30 mg·kg^{-1} s.c. buprenorphine [the lowest dose of okadaic acid (0.001 pg per mouse) had no significant effect], whereas higher doses of okadaic acid (1 and 10 pg per mouse) significantly ($P < 0.05$) increased the anti-nociception induced by 30 mg·kg^{-1} s.c. buprenorphine in the 55°C tail-flick test (Figure 7B).

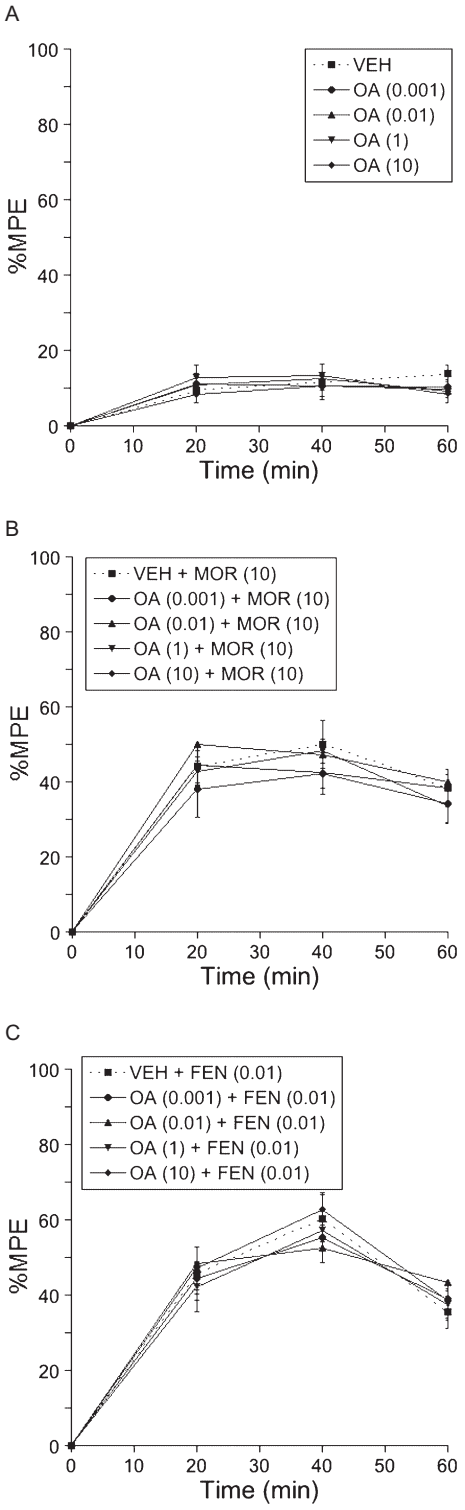

Figure 6 Lack of effect of vehicle (VEH, baseline latency = 1.1 ± 0.2 s) or intracerebroventricular okadaic acid (0.001–10 pg per mouse) (A) alone on % maximum possible effect (MPE) in the 55°C tail-immersion/flick test (mean ± SEM) or on anti-nociception induced by s.c. (B) morphine (10 mg·kg^{-1}) (MOR) (baseline latencies = 1.0 ± 0.3 s to 1.9 ± 0.3 s) or (C) fentanyl (0.01 mg·kg^{-1}) (FEN) (baseline latencies = 1.0 ± 0.3 s to 1.3 ± 0.6 s). n = 8–10 mice per group.

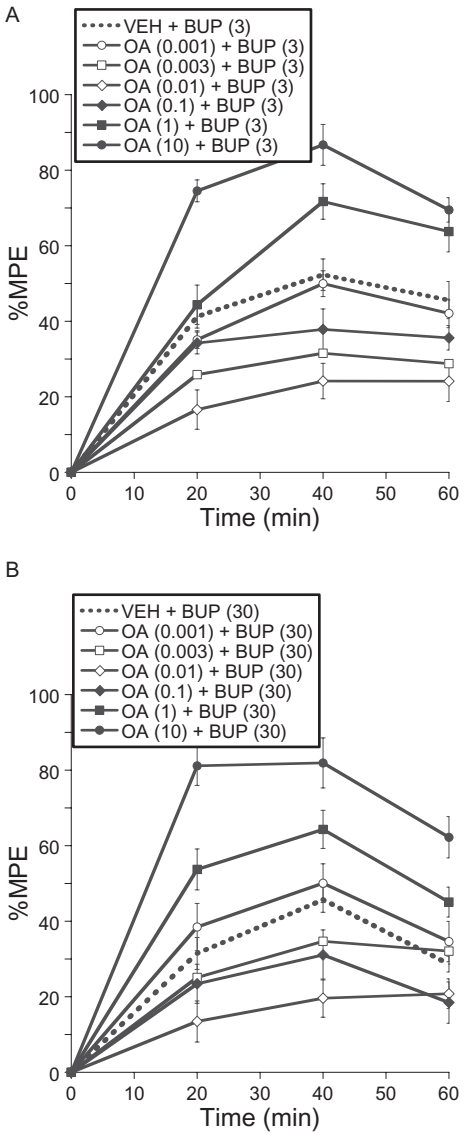

Figure 7 Dose-related effect of intracerebroventricular okadaic acid (0.001–10 pg per mouse) on anti-nociception induced by (A) 3 mg·kg^{-1}, s.c. buprenorphine (BUP) (baseline latencies = 1.3 ± 0.5 s to 1.7 ± 0.5 s) or (B) 30 mg·kg^{-1} s.c. buprenorphine (baseline latencies = 1.4 ± 0.4 s to 1.8 ± 0.5 s) in the 55°C tail-immersion/flick test (mean ± SEM). n = 8–10 mice per group. MPE, maximum possible effect; OA, okadaic acid; VEH, vehicle.

The biphasic nature of the effect of okadaic acid (low-dose attenuation, high-dose enhancement) on both 3 and 30 mg·kg^{-1} buprenorphine in the 55°C tail-flick test is displayed (incorporating AUC_{0-60}) in Figure 8.

Discussion

Buprenorphine has been classified as an opioid based on a large body of evidence, including high-affinity binding to μ opioid receptors; naloxone-sensitive anti-nociception/analgesia against a wide range of noxious stimuli in a variety of preclinical models and clinical settings; and significantly reduced anti-nociceptive effect in opioid receptor deficient (knockout) mice (e.g. Budd, 1981; Rothman *et al.*, 1995;

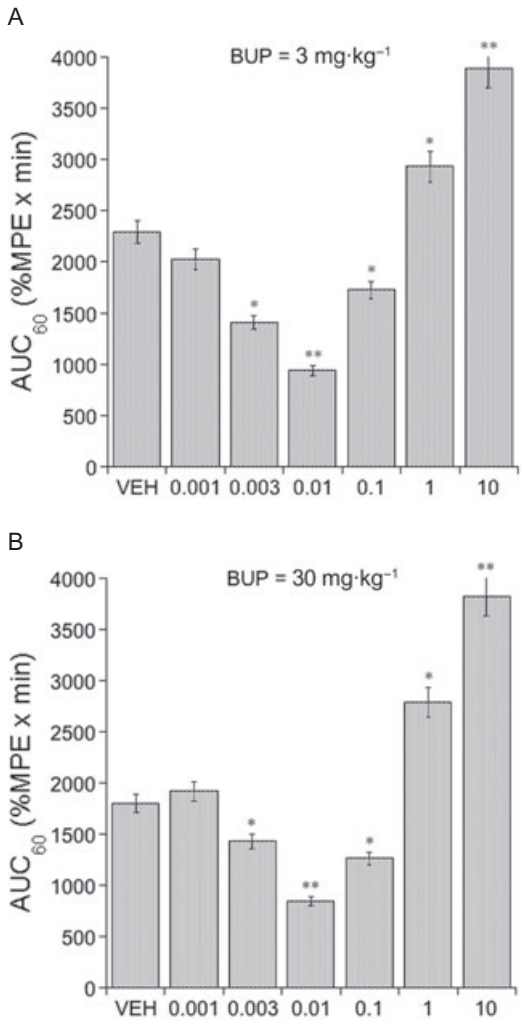

Figure 8 Dose-related biphasic effect of intracerebroventricular okadaic acid on anti-nociception induced by (A) 3 mg·kg^{-1} s.c. buprenorphine (BUP) or (B) 30 mg·kg^{-1} s.c. buprenorphine in the 55°C tail-immersion/flick test. The data have been re-calculated as the area under the anti-nociception curve from 0 to 60 min (AUC_{60}). Data shown are means ± SEM from 8–10 mice per group. (A) $F_{(6,63)} = 32.44$; (B) $F_{(6,63)} = 36.78$. *$P < 0.05$, **$P < 0.01$. MPE, maximum possible effect; VEH, vehicle.

Morgan *et al.*, 1999; Huang *et al.*, 2001; Gilbert and Franklin, 2002; Lutfy *et al.*, 2003; Ide *et al.*, 2004; Neilan *et al.*, 2004; Christoph *et al.*, 2005; Yamamoto *et al.*, 2006 and reviewed in Cowan and Lewis, 1995; Budd and Raffa, 2005). Buprenorphine's affinity for NOP receptors is three orders of magnitude lower than that for μ opioid receptors. Depending on the test system, buprenorphine is characterized as an agonist at μ receptors, antagonist at δ receptors, low-efficacy agonist or antagonist at κ receptors and an agonist (partial or full) at NOP receptors (see Budd and Raffa, 2005). At the same time, buprenorphine has been described as a 'unique' opioid based on equally compelling evidence, including characterization *in vivo*; slow receptor binding kinetics (particularly in regard to dissociation); lower than expected intrinsic activity at opioid receptors (in GTPγS and adenylate cyclase assays) (Traynor, 2004); and a 'ceiling' for respiratory depression but no ceiling for analgesia (e.g. Kosterlitz *et al.*, 1975; Martin *et al.*, 1976; Villiger and Taylor, 1981; Boas and Villiger, 1985; Cowan and Lewis, 1995; Traynor and Nahorski, 1995; Blake *et al.*, 1997; Yu *et al.*, 1997; Selley *et al.*, 1998; Toll *et al.*, 1998; Lee *et al.*, 1999; Budd and Raffa, 2005; Dahan *et al.*, 2006 and reviewed in Cowan and Lewis, 1995; Budd and Raffa, 2005). Several explanations have been proposed for buprenorphine's unique preclinical and clinical features, such as high-dose 'auto-inhibition', special receptor kinetics, exceptional binding locations, active metabolite and interaction with a pro-nociceptive system (Dum and Herz, 1981; Sadée *et al.*, 1982; Lee *et al.*, 1999; Lutfy and Cowan, 2004). As none has proved completely satisfactory, or led to seemingly contradictory theories, we decided to investigate further buprenorphine's mechanism(s) of anti-nociceptive action using a variety of techniques within the same laboratory.

A critical question for any explanation of buprenorphine's characteristics involves whether it is susceptible to antagonism by naloxone. Early studies demonstrated that peripheral administration of naloxone produces a parallel rightward displacement of buprenorphine's dose–response curve – both the ascending and descending portions in models in which both are present (Dum and Herz, 1981; Cowan, 1995). More recent studies suggest the involvement of the NOP receptor (Lutfy *et al.*, 2003; Ide *et al.*, 2004), for which naloxone antagonism is less clear (Mogil and Pasternak, 2001). The present findings confirm that peripheral administration of naloxone antagonizes both portions of buprenorphine's dose–response curve when both (ascending and descending) are present – but demonstrates that supraspinal (i.c.v.) administration of naloxone is without effect against s.c. administration of buprenorphine in two different anti-nociceptive tests (tail-flick and abdominal irritant). Yamamoto *et al.* (2006) also found that, in the rat formalin test, i.c.v. buprenorphine produced anti-nociception and that i.p. buprenorphine was antagonized by i.p. naloxone, but not by i.c.v. naloxone. Oligomerization of opioid receptors might in some way influence buprenorphine's overall anti-nociceptive action, but these are naloxone-sensitive (George *et al.*, 2000) and thus do not account for the naloxone-insensitive portion of buprenorphine's action addressed in the current work. Therefore, these results provide evidence, at the receptor level, of an additional supraspinal mechanism of action for buprenorphine.

Opioid receptors primarily transduce agonist ligand binding-induced signal via PTX-sensitive G protein-mediated pathways at spinal and supraspinal locations (Parolaro *et al.*, 1990; Pasternak, 1993; Raffa *et al.*, 1994; Garzón *et al.*, 2000). PTX, a protein derived from *Bordetella pertussis*, catalyses ADP-ribosylation of a cysteine side chain on α-subunits of $G_{i/o}$ proteins. This is a covalent modification that locks the G protein in a form that cannot be activated by ligand-receptor complex formation. Buprenorphine-induced anti-nociception involves both spinal and supraspinal sites, but whether the anti-nociception is mediated through the same PTX-sensitive or insensitive pathways at both spinal and supraspinal sites is a critical question. The present findings demonstrate that supraspinal administration of PTX is without effect against buprenorphine at doses that antagonize morphine and fentanyl and all three of the drugs when PTX is administered spinally. These results provide further evidence, at the second messenger level, of an additional supraspinal mechanism of action for buprenorphine.

The lack of supraspinal PTX antagonism of buprenorphine prompted us to investigate the PTX-insensitive G protein G_z (Ho and Wong, 1998), based on its known coupling to the NOP receptor (Chan *et al.*, 1998). Spinal administration of G_z antisense had no effect on buprenorphine, morphine or fentanyl anti-nociception, but supraspinal antisense (but not control) significantly reduced the anti-nociception induced by buprenorphine – but not by morphine or fentanyl. These results imply the involvement of a G_z-mediated mechanism in the supraspinal component of buprenorphine's mechanism of action, consistent with the results of Sánchez-Blázquez *et al.* (2001) using a different antisense sequence (5′-C*G*TGATCTCACCCTTGCTCTCTGCCGGGCCA*G*T-3′), and is further considered below. The G_z antisense results by themselves do not exclude opioid receptors, as there is evidence of opioid receptor coupling to G_z proteins (Garzón *et al.*, 1998; Karim and Roerig, 2000; Tso *et al.*, 2000), but the results are consistent with the demonstrated naloxone-insensitivity and give some direction towards the actual pathway.

The possible involvement of the NOP receptor was investigated because the descending portion of buprenorphine's dose–response curve, when present, has been reported to be eliminated by peripheral administration of the NOP receptor antagonist J-113397 and in NOP receptor knockout mice (Lutfy *et al.*, 2003). We confirm, using a different NOP receptor antagonist (JTC-801) (Yamada *et al.*, 2002), that peripheral administration of an NOP receptor antagonist potentiates buprenorphine-induced anti-nociception. This appears to be due to an action at the spinal level, as spinal administration of nociceptin was additive with or potentiated buprenorphine-induced anti-nociception, whereas supraspinal administration had no effect, despite having a pro-nociceptive effect of its own. This is consistent with the report that the spinal anti-nociceptive action of blocking NOP receptor signalling prevails over the supraspinal pro-nociceptive effects in the formalin test (Rizzi *et al.*, 2006).

Our findings support the proposal (Lutfy *et al.*, 2003) that the descending portion of buprenorphine's dose–response curve might be due to activation of NOP receptors, particularly as NOP receptor signalling involves PTX-insensitive G_z (Chan *et al.*, 1998). Nociceptin, although possibly anti-nociceptive spinally, is inactive, hyperalgesic or functionally anti-opioid supraspinally (see Vanderah *et al.*, 1998 and references therein). Buprenorphine has affinity for and intrinsic activity at the NOP receptor (Wnendt *et al.*, 1999; Huang *et al.*, 2001). Buprenorphine's significantly lower affinity for the NOP receptor than for μ-opioid receptors (285 vs. 0.08 nmol·L^{-1}) or a difference in relative receptor concentration could account for the influence of the NOP receptor only at higher doses of buprenorphine. NOP receptor activation through G_z inhibits cAMP accumulation and affects other intracellular second messenger processes (Mogil and Pasternak, 2001) and might affect recycling of opioid receptors or complexes. However, this might not be the entire explanation, as the synthetic mixed μ-opioid/NOP receptor partial agonist SR 16435 [1-(1-(bicyclo[3.3.1]nonan-9-yl)piperidin-4-yl)indolin-2-one] does not produce a biphasic anti-nociceptive dose–response curve (Khroyan *et al.*, 2007). Thus the details remain to be elucidated.

A major new finding of the present work is that there is a supraspinal component of buprenorphine-induced anti-nociception that is not mediated via the usual (naloxone- and PTX-sensitive) opioid or by NOP receptors – as anti-nociception cannot be simultaneously unaffected by nociceptin, augmented by an NOP receptor antagonist, and blocked by antisense to G_z. This 'non-traditional' pathway was neither yohimbine-sensitive nor WAY-100635-sensitive, eliminating the α-adrenoceptor or 5-HT_{1A} pathways as possibilities.

We pursued the new mechanism by comparing the effect of the Ser/Thr protein phosphatase inhibitor okadaic acid on buprenorphine-, morphine- and fentanyl-induced anti-nociception. Ser/Thr protein phosphatases, such as PP1, PP2 (ion-independent subtype A, Ca^{2+}-dependent subtype B and Mg^{2+}-dependent subtype C) and PP4 through PP7 (Cohen, 1997), are the most common protein phosphatases present in the mammalian CNS (Price and Mumby, 1999). Okadaic acid inhibits protein phosphatases PP2A and PP4 with IC_{50} values ≈ 0.1–0.3 nmol·L^{-1}, PP1 and PP5 with about 10–100-fold less potency, and PP2B, PP2C and PP7 with low potency or not at all (Bialojan and Takai, 1988; Haystead *et al.*, 1989; Honkanen, 1993; Li *et al.*, 1993; Chen *et al.*, 1994; Hastie and Cohen, 1998; Huang and Honkanen, 1998; Honkanen and Golden, 2002). The major finding was demonstration of an okadaic acid-sensitive supraspinal component of buprenorphine-induced anti-nociception in the mouse tail-immersion/flick test that is different from both morphine- and fentanyl-induced anti-nociception. It is important to put the present results in context and in perspective. First, a few studies have examined the effect of okadaic acid on morphine-induced anti-nociception. The results have been mixed, possibly due to differences between male and female animals (Moncada *et al.*, 2003; Ocaña *et al.*, 2007) or between pressure and heat stimuli (Maeda *et al.*, 2005). The results of the present study are consistent with the studies that used male mice and heat stimulus: okadaic acid does not alter morphine-induced anti-nociception in morphine-naïve mice (Bernstein and Welch, 1998; Gabra *et al.*, 2007). Second, okadaic acid has physiological actions in addition to inhibition of Ser/Thr protein phosphatases, and the nature and selectivity of the actions are dose-dependent (see discussions in Bernstein and Welch, 1998; Moncada *et al.*, 2003; 2005; Maeda *et al.*, 2005; Gabra *et al.*, 2007; Ocaña *et al.*, 2007). We intentionally used a very wide range of doses of okadaic acid (0.001–10 pg per mouse) that span multiple actions and make no claim that our results are limited to inhibition of Ser/Thr protein phosphatases. The critical feature of the present results is that a difference was demonstrated between buprenorphine and the other opioids (morphine and fentanyl). Whether or not the mechanism exclusively involves Ser/Thr protein phosphatases remains to be elucidated, but would not change the finding of a difference. Third, some of the studies found that okadaic acid produced different effects in morphine-naïve and morphine-tolerant mice (Bernstein and Welch, 1998; Gabra *et al.*, 2007; Ocaña *et al.*, 2007). The present study examined the effect of okadaic acid in naïve animals. Whether or not okadaic acid would produce a similar differential effect on buprenorphine's supraspinal anti-nociceptive action in buprenorphine-, morphine- or fentanyl-tolerant animals is of significant interest. Fourth, our previous results suggested the lack

involvement of (naloxone- or PTX-sensitive) opioid or NOP receptors in buprenorphine's additional supraspinal anti-nociceptive mechanism. The fact that okadaic acid attenuates the anti-nociception induced by clonidine (an α_2-adrenoceptor agonist) and baclofen (a $GABA_B$ receptor agonist) (Moncada *et al.*, 2005) suggests that the additional supraspinal mechanism of buprenorphine-induced anti-nociception in the tail-immersion/flick test is non-opioid.

We believe that the results lead to two separate, but compatible, conclusions – that there is both an opioid component and a non-opioid component to the supraspinal anti-nociceptive mechanism of action of buprenorphine. Buprenorphine is already known to have an opioid component to its supraspinal mechanism of anti-nociceptive action, as anti-nociception induced by i.c.v. buprenorphine is antagonized by i.c.v. naloxone (Yamamoto *et al.*, 2006). It is possible that peripheral or spinal effects of systemic buprenorphine mask the supraspinal opioid effect and this would explain why a supraspinal opioid component was *not* antagonized by i.c.v. administration of naloxone or PTX in the present study. As systemic buprenorphine anti-nociception *was* attenuated by i.c.v. administration of G_z antisense and okadaic acid, this latter mechanism clearly contributes to the supraspinal anti-nociceptive action of buprenorphine. This additional supraspinal component of buprenorphine does not negate the coexistence of an opioid component.

These findings suggest several further studies: to determine if the effect extends to other noxious stimuli and other (acute, phasic and chronic) nociceptive and neuropathic pain models; to determine if it is inhibition of Ser/Thr protein phosphatases – or some other mechanism – that accounts for the results with okadaic acid; to test the effect of okadaic acid on buprenorphine in tolerant [buprenorphine, morphine, fentanyl, and other (non-opioid)] animals; to attempt to elucidate the (G_z-sensitive) pathway; and examine the end point of respiratory depression.

We believe that these data harmonize some earlier explanations of buprenorphine's 'unique' (Budd and Raffa, 2005) characteristics (Table 3). For example, the ability of peripheral administration of naloxone to shift the entire buprenorphine anti-nociceptive dose–response curve (ascending and descending, when biphasic) was confirmed in the present study and is now seen to result from an action at the spinal level. Thus, the shift of the entire biphasic curve by naloxone does not preclude the existence of a less-dominant, naloxone-insensitive supraspinal anti-nociceptive action of buprenorphine. A biphasic curve is also consistent with this construct – a dominant spinal site/mechanism and less-dominant supraspinal site/mechanism. The elimination of the descending portion of buprenorphine's anti-nociceptive dose–response curve in ORL1 KO mice (Lutfy *et al.*, 2003) can then be explained as the result of the elimination of the dominant spinal *pro*-nociceptive effect of buprenorphine binding to NOP receptors at high doses [buprenorphine's affinity at opioid receptors is much greater than is its affinity at NOP receptors (Huang *et al.*, 2001)] without excluding a supraspinal anti-nociceptive mechanism that does not involve NOP – discovered in the present study. Finally, the well-documented 'ceiling' effect on respiratory depression displayed by buprenorphine (Dahan, 2005; Dahan *et al.*, 2005; 2006), combined with its ability to produce fully effective analgesia, might be a manifestation of its non-opioid (or at least non-μ-opioid) transduction at the supraspinal level.

Table 3 Comparison of features of buprenorphine's actions and suggested explanations provided by the present study

Attribute	*Present study*
Buprenorphine displays a biphasic (inverted 'U'-shape) dose–response curve in some anti-nociceptive tests	A biphasic curve is consistent with the findings in the present study of a dominant spinal site/mechanism of anti-nociception and less-dominant supraspinal site/mechanism
Naloxone antagonizes all phases of buprenorphine's anti-nociceptive dose–response curve	In light of findings of the present study, antagonism by peripheral administration of naloxone is primarily spinal and does not exclude a naloxone-insensitive supraspinal anti-nociceptive action of buprenorphine
The high-dose descending portion of a biphasic curve is eliminated by opioid receptor-like (ORL)1 knockout	Based on the present study, elimination of the high-dose descending portion can be explained as due to elimination of a dominant spinal pro-nociceptive action without excluding a supraspinal anti-nociceptive action not involving ORL1
Buprenorphine displays a ceiling effect in respiratory depression but not in analgesia	The present study reveals a naloxone-independent supraspinal mechanism contributes to buprenorphine anti-nociception, whereas opioid-induced respiratory depression is μ-opioid-mediated

In summary, the present study found an additional mechanism to buprenorphine's supraspinal analgesic mechanism of action. Specifically, the results demonstrate a naloxone-, PTX- and NOP-insensitive, G_z protein-sensitive, transduction pathway involved in supraspinal buprenorphine-, but not morphine- or fentanyl-induced anti-nociception. This pathway was further characterized by the finding of a biphasic effect (low-dose attenuation, high-dose enhancement) of okadaic acid on buprenorphine, but not morphine or fentanyl. These findings might help explain buprenorphine's favourable distinguishing clinical attributes compared with other opioids.

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Conflict of interest statement

R.B.R. is a paid consultant of several pharmaceutical companies, including Grünenthal GmbH, but receives no royalty (cash or otherwise) from sales of any products.

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