Pain 132 (2007) 289–300

PAIN

www.elsevier.com/locate/pain

Oxycodone and morphine have distinctly different pharmacological profiles: Radioligand binding and behavioural studies in two rat models of neuropathic pain

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Received 12 September 2006; received in revised form 18 February 2007; accepted 19 March 2007

Abstract

Previously, we reported that oxycodone is a putative κ-opioid agonist based on studies where intracerebroventricular (i.c.v.) pre-treatment of rats with the κ-selective opioid antagonist, nor-binaltorphimine (nor-BNI), abolished i.c.v. oxycodone but not morphine antinociception, whereas pretreatment with i.c.v. naloxonazine (μ-selective antagonist) produced the opposite effects. In the present study, we used behavioural experiments in rat models of mechanical and biochemical nerve injury together with radioligand binding to further examine the pharmacology of oxycodone. Following chronic constriction injury (CCI) of the sciatic nerve in rats, the antinociceptive effects of intrathecal (i.t.) oxycodone, but not i.t. morphine, were abolished by nor-BNI. Marked differences were found in the antinociceptive properties of oxycodone and morphine in streptozotocin (STZ)-diabetic rats. While the antinociceptive efficacy of morphine was abolished at 12 and 24 weeks post-STZ administration, the antinociceptive efficacy of s.c. oxycodone was maintained over 24 weeks, albeit with an ~3- to 4-fold decrease in potency. In rat brain membranes irreversibly depleted of μ- and δ-opioid binding sites, oxycodone displaced [^{3}H]bremazocine (κ_2-selective in depleted membranes) binding with relatively high affinity whereas the selective μ- and δ-opioid ligands, CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2) and DPDPE ([D-Pen^{2,5}]-enkephalin), respectively, did not. In depleted brain membranes, the κ_{2b}-ligand, leu-enkephalin, prevented oxycodone's displacement of high-affinity [^{3}H]bremazocine binding, suggesting the notion that oxycodone is a κ_{2b}-opioid ligand. Collectively, the present findings provide further support for the notion that oxycodone and morphine produce antinociception through distinctly different opioid receptor populations. Oxycodone appears to act as a κ_{2b}-opioid agonist with a relatively low affinity for μ-opioid receptors.

Keywords: Oxycodone; Morphine; κ_2-opioid receptors; Chronic constriction injury (CCI); Streptozotocin (STZ); Painful diabetic neuropathy; Radioligand binding

Abbreviations: AUC, area under the curve; BIT, 2-(4-ethoxybenzyl)-1-diethylaminoathyl-5-isothiocyanatato-benzimidazole·HCl; CCI, chronic constriction injury; CNS, central nervous system; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; DA, Dark Agouti; DAMGO, D-Ala^2, *N*-Me-Phe^4,Gly-ol^5]-enkephalin; DPDPE, [D-Pen^{2,5}]-enkephalin; ED_{50}, dose that produces a half-maximal response; FIT, *N*-phenyl-*N*-[1-(2-(4-isothiocyanato)phenethyl)-4-piperidinyl]-propanamide·HCl; [^{3}H], tritiated; Hepes, 2-hydroxyethylpiperazine-*N*-2-ethane sulphonic acid; i.c.v., intracerebroventricular; i.t., intrathecal; LE, leu-enkephalin; nor-BNI, nor-binaltorphimine; PWT, paw withdrawal threshold; ΔPWT, normalized paw withdrawal threshold; ΔPWT AUC, area under the normalized paw withdrawal threshold versus time curve; %MAX ΔPWT AUC, percentage of the maximum possible ΔPWT AUC; s.c., subcutaneous; SD, Sprague–Dawley; U-69,593, (+)-(5α,7β,8β)-*N*-methyl-*N*-[7-(1- pyrrolidinyl)-1-oxaspiro[4.5]dec-8-ly]-benzeneacetamide.

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0304-3959/$32.00
doi:10.1016/j.pain.2007.03.022

1. Introduction

Previous studies of the *in vivo* pharmacology of oxycodone and morphine in rats suggest that these two opioids mediate their intrinsic antinociceptive effects by distinctly different populations of CNS opioid receptors. For instance, intrathecal (i.t.) bolus doses of oxycodone have only ~2–7% of the potency of morphine for the relief of nociceptive pain in rats (Plummer et al., 1990; Pöyhiä and Kalso, 1992), mirroring the low potency of epidural oxycodone (~11%) relative to morphine for the relief of pain in post-operative patients (Backlund et al., 1997). However, following i.c.v. administration, oxycodone produces naloxone-sensitive dose-dependent antinociception in the rat with a potency ~44% that of i.c.v. morphine (Leow and Smith, 1994; Nielsen et al., 2000). In rats rendered tolerant to i.v. morphine, there is an absence of antinociceptive cross-tolerance to i.c.v. oxycodone (Nielsen et al., 2000). Furthermore, after i.c.v. administration in the rat, oxycodone produces a single antinociceptive phase with a rapid onset of peak effect (~5–7 min), in contrast to the slow onset of peak antinociception (~45–60 min) and the two antinociceptive phases evoked by i.c.v. morphine (Leow and Smith, 1994; Ross and Smith, 1997; Nielsen et al., 2000).

In vivo studies using selective opioid antagonists have found differences in the antinociceptive effects of each of i.c.v. oxycodone and morphine in the rat. Whereas i.c.v. pretreatment with the κ-selective antagonist, nor-binaltorphimine, (nor-BNI), abolished oxycodone's antinociceptive effects, i.c.v. naloxonazine (μ-selective) and naltrindole (δ-selective) did not (Ross and Smith, 1997). In the same study, morphine's antinociceptive effects were abolished by naloxonazine but not nor-BNI, a finding consistent with expectations for the prototypic μ-opioid agonist, morphine (Pasternak, 2001; Gaveriaux-Ruff and Kieffer, 2002).

At present, the opioid receptor binding profile of oxycodone is poorly defined. Using [^{3}H]DAMGO and rat brain homogenate, oxycodone bound with relatively low affinity to μ-opioid receptors ($K_i = 47.4 \pm 3.8$ nM, means ± SEM), in contrast to the high-affinity binding of morphine ($K_i = 1.2 \pm 0.13$ nM) (Chen et al., 1991). More recently, studies using [^{3}H]diprenorphine and membranes prepared from cells expressing cloned μ-, δ- and κ-opioid receptors also reported relatively low affinity binding for oxycodone ($K_i = 16.0 \pm 2.9$ nM) at the μ-opioid receptor compared with morphine ($K_i = 3.2 \pm 0.4$ nM) or DAMGO ($K_i = 0.21 \pm 0.03$ nM; Lalovic et al., 2006).

Although the use of opioids for the relief of neuropathic pain is controversial (Arner and Meyerson, 1988), more recent trials have reported significant benefit of oxycodone above placebo (Gimbel et al., 2003; Watson et al., 2003). Multiple studies have examined the anti-neuropathic properties of morphine in rodent models of neuropathic pain (Backonja et al., 1995; Kayser et al., 1995; Mao et al., 1995; Wegert et al., 1997; Suzuki et al., 1999), but the effect of oxycodone in such models remains poorly investigated. Hence, the aims of this study were 2-fold to: (i) compare the antinociceptive profiles of morphine and oxycodone in rats with a chronic constriction injury (CCI) of the sciatic nerve as well as in rats following streptozotocin (STZ)-induced diabetes and (ii) characterize the opioid receptor binding profile of oxycodone.

2. Materials and methods

2.1. Experimental animals

Ethical approval was obtained from the Animal Experimentation Ethics Committee of The University of Queensland. Adult male Sprague–Dawley (SD) rats and adult male albino guinea pigs were purchased from the Herston Medical Research Centre and the Central Animal Breeding House, respectively, at The University of Queensland (Brisbane, Australia). Adult male Dark Agouti (DA) rats were purchased from the Central Animal Breeding House, The University of Queensland (Brisbane, Australia) and from The Animal Resources Centre (Perth, Australia). Rats and guinea pigs were housed in a temperature controlled room (21 ± 2 °C) with a 12/12-h light/dark cycle; food and water were available *ad libitum*. DA rats were used for the behavioural experiments in STZ-diabetic rats where oxycodone was administered systemically, because they are genetically deficient in the enzyme (CYP2D1/2D2), which is required to catalyse the *O*-demethylation of oxycodone to oxymorphone (Yamamoto et al., 1998). This ensured that circulating concentrations of oxymorphone were very low (Huang et al., 2005), as is the case in humans after systemic oxycodone administration (Pöyhiä et al., 1992; Heiskanen et al., 1998; Lalovic et al., 2006). However, SD rats were used for i.t. studies, as this route of administration avoids systemic metabolism. Brains from SD and DA rats as well as guinea pigs were used to prepare membrane preparations for use in the radioligand binding studies.

2.2. Drugs and materials

Oxycodone hydrochloride was a generous gift from Tasmanian Alkaloids Pty Ltd (Hobart, Australia). Morphine hydrochloride was purchased from David Bull Laboratories (Melbourne, Australia). Morphine HCl and oxycodone HCl were dissolved in isotonic saline and stock solutions were stored at −20 °C until required. Sodium benzylpenicillin (Benpen™) vials containing 600 mg of powder, ketamine hydrochloride (Ketamav™-100) vials (100 mg/ml) and xylazine hydrochloride (Ilium Xylazil™-20) vials (20 mg/ml) were purchased from Abbott Australasia Pvt. Ltd (Sydney, Australia) and normal saline ampoules were obtained from Delta West Pvt. Ltd (Perth, Australia). Streptozotocin, citric acid and trisodium citrate were purchased from Sigma (Sydney, Australia). Sterile siliconized silk sutures (Dysilk™) were obtained from Dynek Pvt Ltd (Adelaide, South Australia). Single lumen polyethylene tubing (0.5-mm internal diameter) was purchased

from Critchley Electrical Products Pvt Ltd (Auburn, Australia). Blood glucose testing strips (Glucostix™) were purchased from The University of Queensland Campus Pharmacy (Brisbane, Australia) and a glucometer Precision Q.I.D™ (Medisense) kit was a generous gift from Dr. L. Brown (Department of Physiology and Pharmacology, The University of Queensland). Medical grade CO_2 and O_2 were purchased from BOC Gases Australia Ltd (Brisbane, Australia). [^{3}H]U69,593 ((+)-(5α,7β,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-ly]benzene-acetamide; 47.4 Ci/mmol), [^{3}H]DAMGO (48.9 Ci/mmol), [^{3}H]DPDPE-Cl ([D-Pen^{2,5}]-enkephalin; 48.6 Ci/mmol), [^{3}H]morphine (84.5 Ci/mmol) and [^{3}H]bremazocine (30 Ci/mmol) were purchased from the New England Nuclear Corporation (Boston, USA). Bremazocine hydrochloride, CTOP (D-Trp-Orn-Thr-Pen-Thr-NH_2), DAMGO, DPDPE, HEPES (2-hydroxyethylpiperazine-*N*-2-ethane sulphonic acid), naloxone hydrochloride, nor-binaltorphimine dihydrochloride (nor-BNI), Tris(hydroxymethyl) aminomethane (Tris) base, magnesium chloride, sodium chloride, sucrose, U69,593, and the Micro Protein Determination kit (Procedure # 690) were obtained from Sigma–Aldrich (Sydney, Australia). BIT (2-(4-ethoxybenzyl)-1-diethylaminoathyl-5-isothio-cyanatobenzimidaz- ole·HCl) and FIT (*N*-phenyl-*N*-[1-(2-(4-isothiocyanato)phenethyl)-4-piperidinyl]-propanamide · HCl) were a generous gift from Dr Kenner C. Rice of the National Institute on Drug Abuse, National Institutes of Health (Bethesda, USA). Optisafe® scintillation fluid and Whatman GF/B filter paper were purchased from The University of Queensland chemical store (Brisbane, Australia).

2.3. Chronic constriction injury of the sciatic nerve and intrathecal cannulation procedures

Chronic constriction injury (CCI) of the sciatic nerve was produced according to the method of Bennett and Xie (1988) in adult male SD rats (222 ± 2.1 g, means ± SEM). CCI-surgery and i.t. cannulation were performed as previously described (Nielsen et al., 2005). Sham-operated SD rats underwent the same procedure, but the nerve was not ligated. Following CCI- or sham-CCI surgery, rats were housed singly for an 11-day period prior to i.t. cannulation, and for an additional 3 days before the commencement of testing procedures. An additional control group of SD rats that did not undergo either CCI- or sham-CCI surgery was also included in the study design.

2.4. Induction of diabetes with streptozotocin

Adult male DA rats (210 ± 4 g, means ± SEM) were anaesthetised as previously described (Nielsen et al., 2005), and a polyethylene cannula pre-filled with saline inserted into the right common jugular vein. Correct placement of the cannula was confirmed by the withdrawal of a small amount of blood, prior to the administration of an 85 mg/kg dose of streptozotocin in 0.1 M citrate buffer (pH 4.5) via the cannula. Water intake and blood glucose concentrations were subsequently monitored in individual rats; blood glucose levels were measured using either Glucostix™ or a Precision QID™ test kit. The presence of diabetes in DA rats by 10 days post-STZ injection was confirmed if water intake exceeded 100 ml/day and blood glucose concentrations were ⩾15 mM. DA rats not meeting these criteria were euthanized; the overall success rate for the induction of diabetes was 75%. By comparison, the water intake of control non-diabetic DA rats was approximately 20 ml/day and blood glucose concentrations were in the range 5–6 mM.

2.5. Opioid dosing regimens

2.5.1. Intrathecal opioid administration to SD rats with a chronic constriction injury (CCI) of the sciatic nerve

A 35 nmol dose of oxycodone or morphine was used in the study, as the antinociceptive effects of both opioids at this dose were found to be maximal and approximately equipotent in the contralateral hindpaw of CCI-rats in preliminary dose–response studies. Oxycodone, morphine, nor-BNI, and naloxone were administered in 0.9% saline. Groups of CCI- ($n = 6$), sham-CCI ($n = 4$), and non-operated ($n = 4$) SD rats received a single 10 μl injection of either oxycodone, morphine or 0.9% saline via the i.t. cannula, followed by a 20-μl flush with 0.9% saline. Opioid antagonists were administered by the same procedure; nor-BNI (10 nmol) or naloxone (30 nmol) was administered 24 h or 10 min prior to opioid administration, respectively. As nor-BNI produces μ-opioid receptor antagonist effects for up to 4 h following administration and its long-lasting κ-selective antagonist effects do not develop until later (Endoh et al., 1992; Wettstein and Grouhel, 1996), nor-BNI was administered 24 h prior to commencing the experimental procedures, in keeping with other studies where nor-BNI has been used as a κ-selective opioid antagonist (Friese et al., 1997; Schmidt et al., 2002; Clemente et al., 2004). Paw withdrawal thresholds (PWTs) in the rat hindpaws were quantified using calibrated von Frey filaments.

2.5.2. Opioid administration to STZ-diabetic DA rats

Single bolus doses (in 200 μl) of oxycodone, morphine or vehicle (0.9% saline) were administered to groups of STZ-diabetic DA rats under light CO_2/O_2 (50:50%) anaesthesia, by s.c. injection into the back of the neck using a 250 μl Hamilton syringe. Temporal changes in the antinociceptive potency of morphine and oxycodone were quantified at 3, 9, 12, and 24 weeks post-STZ administration using calibrated von Frey filaments. At the time of testing, rats were randomly assigned to a treatment group; a 3- to 4-day washout period was allowed between doses.

Groups of DA rats received morphine at the following doses: 2.0–8.0 mg/kg at 3 weeks ($n = 6$), 2.0–8.0 mg/kg at 9 weeks ($n = 7$), 1.8–14.2 mg/kg at 12 weeks ($n = 4$–6), and 5.5–14.2 mg/kg at 24 weeks ($n = 6$). The dose of morphine was capped at 14.2 mg/kg, as preliminary assessment of larger doses (18 mg/kg) at 12 weeks post-STZ showed that neuroexcitatory behaviours including intermittent mild myoclonus of the face and limbs, and biting of the wire mesh testing cage were produced. Additionally, groups of DA rats received oxycodone at the following doses: 0.9–4.5 mg/kg at 3 weeks ($n = 6$), 0.9–4.5 mg/kg at 9 weeks ($n = 6$–9), 1.5–9.0 mg/kg at 12 weeks ($n = 4$–6), and 1.8–9.0 mg/kg at 24 weeks ($n = 6$). A

cohort of weight-matched control non-diabetic DA rats (n= 36, 210 ± 4 g, means ± SEM) was also studied, in which individual rats were administered one of three s.c. bolus doses of either morphine (0.8–6.0 mg/kg), oxycodone (0.7–2.3 mg/kg) or vehicle (0.9% saline).

2.6. Assessment of paw withdrawal thresholds (PWTs)

Mechanical allodynia/hyperalgesia, the distinguishing feature of neuropathic pain, was quantified using calibrated von Frey filaments as previously described (Nielsen et al., 2005). Calibrated filaments that produced a buckling weight of 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 g were used to assess PWTs. A score of 20 g was given to animals that did not respond to any of the von Frey filaments.

In the STZ-diabetic rat study, PWT values were determined prior to the induction of diabetes with STZ, at 1-week post-STZ administration and just prior to each opioid testing session. Similarly, baseline PWTs were determined in control non-diabetic DA rats immediately prior to opioid administration. In both studies, baseline PWTs were determined prior to opioid administration from three consecutive readings taken ~5 min apart; baseline PWTs were determined for both hindpaws. After opioid administration, a single PWT measurement was taken for each hindpaw at the following post-dosing times: 0.25, 0.5, 0.75, 1, 1.5, 2 and 3 h.

2.7. Preparation of rodent brain membranes for in vitro radioligand binding studies

Following decapitation of animals, rat and guinea-pig brains were placed individually into ice-cold (4 °C) 0.32 M sucrose solution prior to cerebellum removal. Brain tissue was homogenized for 1 min in ice-cold (4 °C) Tris–HCl (50 mM Tris, 5 mM $MgCl_2$, pH 7.4) buffer (10 ml/g wet weight of tissue) followed by centrifugation (40,000g, 10 min, 4 °C) and re-suspension in fresh ice-cold Tris–HCl buffer. This was repeated and the resuspended membranes were stored frozen at −80 °C until required.

2.8. Depletion of μ- and δ-opioid receptors in rat brain homogenates

Irreversible acylation of μ-and δ-opioid receptors to effectively deplete these two receptor populations in rat brain homogenate was undertaken using the reagents, BIT and FIT, respectively, according to the method of Rothman et al. (1990a,b, 1992). Briefly, aliquots (100 μl) of rat brain homogenate suspended in Tris–HCl buffer (pH 7.4) containing sodium chloride (100 mM) were incubated with BIT (1 μM) and FIT (1 μM) for 60 min at 0 °C. Following incubation, homogenates were centrifuged and washed twice, then re-suspended in Tris–HCl buffer (50 mM, pH 7.4). 'Depleted' rat brain membrane preparations were stored at −80 °C, until required.

The protein content of brain membrane preparations was determined according to the method of Lowry et al. (1951) or using a Sigma® Micro Protein Determination kit (Procedure # 690); a method based on that of Lowry et al. (1951) and Ohnishi and Barr (1978).

2.9. Radioligand binding assays

Radioligand binding assays for each of the major classes of opioid receptors (μ, δ and κ) were performed using rat, or guinea-pig brain membrane preparations in the case of κ_1-opioid receptor binding, according to previously published methods (Rothman et al., 1985, 1990a,b, 1992) with minor modifications. Due to the reportedly low expression of κ_1-opioid receptors in rat brain tissue and much higher selective binding (~10:1) in guinea-pig brain preparations (Tiberi and Magnan, 1989), guinea-pig brain membrane preparations were used for radioligand binding experiments with [^{3}H]U69,593.

Radioligand binding studies performed with rat brain membrane preparations were incubated at 25 °C for 1 h. Binding studies performed with 'depleted' rat brain membrane preparations were incubated at 4 °C for 4 h to increase the specific binding of the radioligand. Radioligand binding assays that used guinea-pig membrane preparations were incubated for 1 h at 37 °C (Rothman et al., 1992). Three independent assays containing duplicate replicates at each unlabelled ligand concentration were performed by incubating 100 μl aliquots of brain membrane preparation (100 mg wet weight of tissue per ml) with 50 μl of the appropriate radioligand (1 μl/l ml from stock solutions of [^{3}H]DAMGO (1.32 nM), [^{3}H]DPDPE (0.9 nM), [^{3}H]U69,593 (0.9 nM), [^{3}H]morphine (1 nM) or [^{3}H]bremazocine (3.43 nM), and one of nine different concentrations (range 100 pM–10 μM) of the unlabelled opioid ligand of interest in 50 mM Hepes–Tris buffer (pH 7.4; 500 μl total volume). Binding assays undertaken in 'depleted' rat brain membrane preparations used [^{3}H]bremazocine as the radioligand and the unlabelled ligands were CTOP, DPDPE, morphine, bremazocine, and oxycodone in the presence or absence of leu-enkephalin (LE). Non-specific binding and total binding were determined in every assay.

Following incubation, the assays were terminated using a Brandell™ Cell harvester loaded with Whatman GF/B filter paper presoaked in 1% polyethyleneimine to retain the radioligand. After three washes with ice-cold Hepes–Tris buffer (pH 7.4), individual filters were placed in scintillation fluid (4 ml) for 12 h prior to liquid scintillation counting using a Packard™ β-counter (Tricarb 2700 TR) with a 66% counting efficiency for tritium.

2.10. Data analysis

Data analysis and statistical comparisons were performed using GraphPad Prism™ software. Displacement curves were generated for radioligand binding assays by plotting the counts per minute for total specific binding at each concentration of unlabelled ligand versus the unlabelled ligand concentration. The radioligand binding data were fit by one- or two-site competition binding models, and the inhibition binding constants, K_i (nM) values, were estimated using nonlinear regression. The F-test was used to determine whether competition binding curves, derived from the displacement of bound radioligands by unlabelled ligands, were best described by either a one- or two-site binding model. The statistical significance criterion was $p < 0.05$.

For the CCI behavioural studies, the paired Wilcoxon test was used to compare baseline pre-dosing ipsilateral and contralateral hindpaw PWT values for CCI, sham-CCI, and non-operated SD rats. PWTs were subsequently normalized

by subtraction of the baseline pre-dosing PWT values. The area under the normalized PWT versus time (0–3 h) curve (ΔPWT AUC) was determined by trapezoidal integration. Between-group ΔPWT AUC values were subsequently compared by Kruskal–Wallis analysis of variance and Dunn's multiple comparison test for individual pairwise comparisons. The statistical significance criterion was $p < 0.05$.

For the STZ-diabetic DA rat studies, ΔPWT AUC values were subsequently converted to a percentage of the maximum possible AUC value for control non-diabetic rats (%MAX ΔPWT AUC values) using the following formula:

$$\%\text{MAX } \Delta\text{PWT AUC} = \frac{\Delta\text{PWT AUC}}{\text{MAX } \Delta\text{PWT AUC}} \times 100$$
$$\text{where MAX } \Delta\text{PWT AUC} = 25.875\ \text{g} \cdot \text{h}$$

The % MAX ΔPWT AUC values for morphine and oxycodone were plotted versus the respective drug dose to produce individual dose–response curves. ED_{50} doses (means ± SEM) were estimated by non-linear regression. The estimation of ED_{50} values was facilitated by the inclusion of theoretical maximum and minimum % MAX ΔPWT AUC values. The Mann–Whitney test was used to compare the ED_{50} values for morphine and oxycodone at 3, 9, 12 and 14 weeks post-STZ administration with their respective ED_{50} values in the weight-matched control non-diabetic rats. The statistical significance criterion was $p < 0.05$.

3. Results

3.1. Behavioural studies in CCI-rats administered i.t. opioids

In CCI-rats, means (±SEM) baseline PWT values were significantly lower ($p < 0.05$) for the ipsilateral hindpaw (5.0 ± 0.2) g compared with the contralateral hindpaw (12.2 ± 0.2) g, indicating that mechanical allodynia/hyperalgesia had developed by 14 days post-CCI surgery. As expected, mean baseline PWTs after sham-CCI surgery (11.7 ± 0.4) g and for non-operated control rats (10.9 ± 0.3) g did not differ significantly between hindpaws (Table 1). PWT versus time curves for control CCI rats following i.t. administration of 0.9% saline are shown in Fig. 1(D).

Mean (±SEM) PWT versus time curves following i.t administration of single bolus doses of oxycodone or morphine to CCI-, sham-CCI, and non-operated SD rats are shown in Fig. 1(A–C). For the ipsilateral hindpaw of CCI-rats, ΔPWT AUC values produced by each of the i.t. bolus doses of oxycodone and morphine tested (Fig. 1C) did not differ significantly ($p < 0.05$). In the contralateral hindpaw of CCI-rats, the extent and duration of the antinociceptive response (ΔPWT AUC) produced by i.t. oxycodone (35 nmol) was significantly greater ($p < 0.05$) than that produced in the hindpaws of sham-operated or non-injured rats (Fig. 1A and Fig. 2A). Importantly, i.t. nor-BNI pre-treatment abolished the antinociceptive effects of i.t. oxycodone in both hindpaws of CCI-rats (Fig. 2C and E).

By contrast, the ΔPWT AUC values for the contralateral hindpaw of CCI-rats administered i.t. morphine (35 nmol) did not differ significantly ($p < 0.05$) from those produced in the hindpaws of sham-operated or non-injured rats (Figs. 1B and 2B). Although the antinociceptive effects of i.t. morphine in the hindpaws of CCI-rats were blocked by pre-treatment with i.t. naloxone, i.t. pre-treatment with nor-BNI did not significantly attenuate i.t. morphine antinociception in the hindpaws of these animals (Fig. 2D and F).

Table 1
Means (±SEM) baseline pre-dosing paw withdrawal threshold (PWT) values for CCI, sham-CCI, and non-injured SD rats, 14 days after CCI or sham-CCI surgery

	Ipsilateral (g)	Contralateral (g)
Oxycodone		
CCI ($n = 6$)	4.2 ± 0.5*	13.3 ± 0.3
Sham-CCI ($n = 4$)	11.5 ± 0.9	11.5 ± 1.0
Non-injured ($n = 4$)	10.2 ± 0.2	10.5 ± 0.3
Morphine		
CCI ($n = 6$)	5.0 ± 0.4*	11.9 ± 0.5
Sham-CCI ($n = 4$)	12.0 ± 0.0	12.0 ± 0.2
Non-injured ($n = 4$)	11.5 ± 0.3	11.3 ± 0.4

* $p < 0.05$ ipsilateral vs contralateral.

3.2. Behavioural studies in STZ-diabetic rats administered s.c. opioids

Elevated blood glucose concentrations (~22 mM), polyuria, polydypsia, and markedly increased food intake were present in STZ-diabetic DA rats, throughout the 24-week study period, consistent with the maintenance of diabetes over the duration of the study. Body weight was maintained over the 24-week study period. There was a significant ($p < 0.05$) temporal decrease in the mean (±SEM) baseline PWT values from 11.9 (±0.15) g just prior to the induction of STZ-diabetes to 8.0 (±0.3) g by 1 week post-STZ administration, with a further significant ($p < 0.05$) decrease to 5.2 (±0.3) g by 3 weeks post-STZ. Thereafter, the mean (±SEM) baseline PWTs were 5.0 (±0.1) and 4.7 (±0.1) g at 9 and 12 weeks post-STZ, respectively, with a further small but significant ($p < 0.05$) decrease after 12 weeks such that at 24 weeks post-STZ administration, the mean (±SEM) PWT was 3.3 (±0.1) g. Taken together, these data show that once mechanical allodynia/hyperalgesia (defining symptom of PDN) has developed in STZ-diabetic rats, it is maintained for at least 24 weeks. As expected, in control non-diabetic DA rats, mean (±SEM) PWTs did not differ significantly ($p > 0.05$) from pre-STZ baseline PWTs for the diabetic rat cohort.

Following s.c. administration of bolus doses of oxycodone and morphine to STZ-diabetic DA rats, the

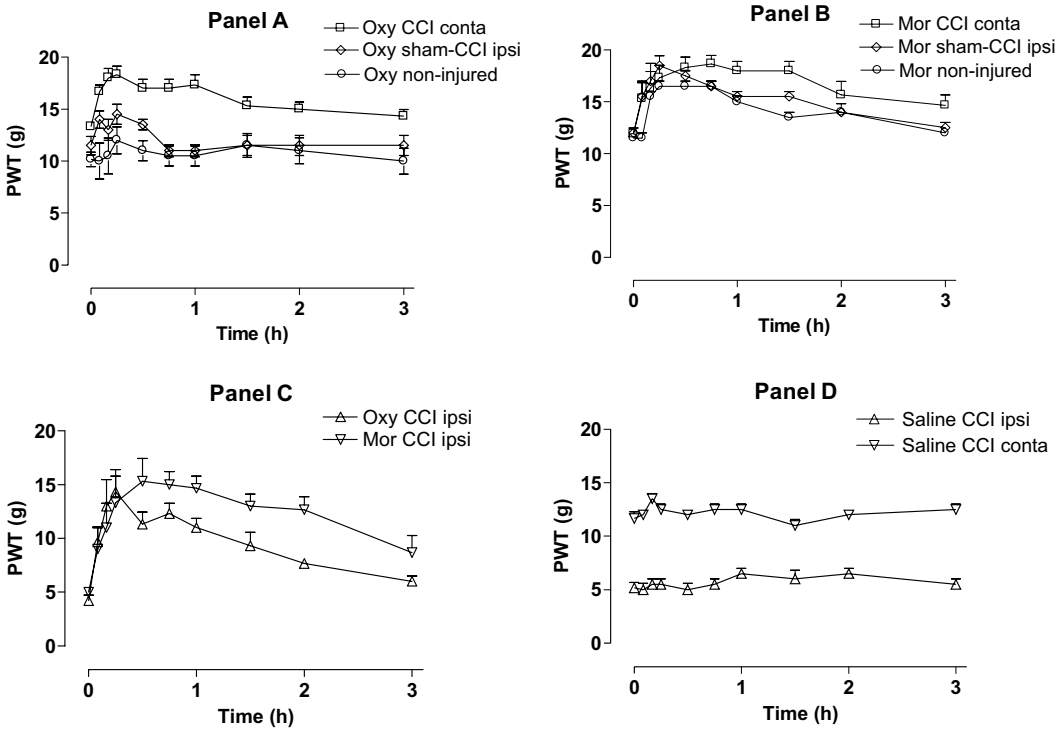

Fig. 1. Mean (±SEM) paw withdrawal threshold (PWT) versus time curves for CCI, sham-CCI, and non-operated SD rats, following i.t. administration of a single 35 nmol dose of oxycodone (Oxy), morphine (Mor) or 0.9% saline, 14 days after CCI or sham-CCI surgery.

peak antinociceptive responses were produced within 15 and 30 min, respectively. Thereafter, responses decreased in a mono-exponential manner. ED_{50} values for oxycodone and morphine determined from dose–response curves at 3 and 9 weeks post-STZ administration were not significantly different ($p < 0.05$) (Fig. 3; Table 2). However, oxycodone was ~3 times more potent than morphine for the alleviation of mechanical allodynia/hyperalgesia at 3 and 9 weeks after the induction of diabetes with STZ (Table 2). Comparison with the ED_{50} values for oxycodone and morphine from control non-diabetic DA rats indicates that, by 3 weeks post-STZ administration, there was a ~2.5-fold decrease in the antinociceptive potency of morphine ($p < 0.05$), and a ~1.7-fold decrease ($p < 0.05$) in the antinociceptive potency of oxycodone (Table 2).

The antinociceptive efficacy of s.c. morphine was abolished by 12 weeks post-STZ administration, and it remained abolished at 24 weeks post-STZ administration (Fig. 3 and Table 2). By contrast, the antinociceptive efficacy of s.c. bolus doses of oxycodone was maintained over the 24-week period that followed the induction of STZ-diabetes in DA rats, although by 12 weeks post-STZ there was a further ~2-fold decrease in the potency of oxycodone (Fig. 3; Table 2). At 24 weeks post-STZ administration, the antinociceptive potency of oxycodone was not significantly different ($p > 0.05$) from that determined at 12 weeks post-STZ (Fig. 3 and Table 2).

3.3. Radioligand binding studies in untreated rodent brain membrane preparations

While morphine displaced [^{3}H]morphine with high affinity as expected, oxycodone demonstrated a substantially lower affinity for the displacement of [^{3}H]morphine (Table 3). Displacement of [^{3}H]DAMGO by unlabelled DAMGO in rat brain membrane preparations was best fit by a one-site binding model (Table 3). The observed K_i value (Table 3) was similar to previously reported values (Albrecht et al., 1997). When oxycodone was used to displace [^{3}H]DAMGO from rat brain membranes, the data were best fit by a two-site binding model with one relatively high affinity and one relatively low-affinity binding site (Table 3). Thus, oxycodone was found to interact with two populations of opioid binding sites, with one population being high affinity and the other being relatively low affinity.

Oxycodone did not significantly displace [^{3}H]DPDPE from δ-opioid binding sites in rat brain membranes, whereas unlabelled DPDPE displaced [^{3}H]DPDPE with high affinity (Table 3), in agreement with other studies (Vaughn et al., 1989). Similarly, [^{3}H]U69,593 demonstrated a high affinity for κ_1-opioid binding sites in guinea-pig brain membrane preparations with an observed K_i in close agreement with published values (Tiberi and Magnan, 1989), but oxycodone did not significantly displace [^{3}H]U69,593 binding (Table 3). Thus, oxycodone does not interact

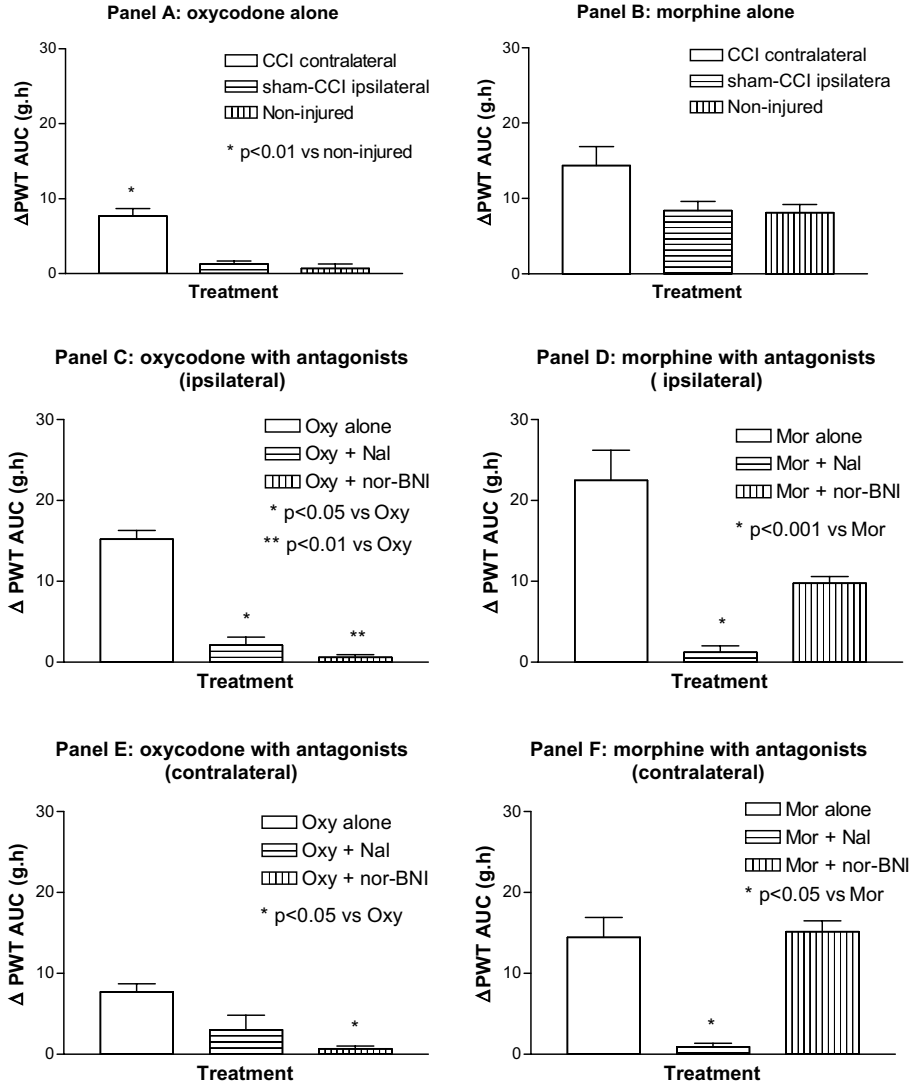

Fig. 2. Mean (±SEM) area under the delta paw withdrawal threshold versus time curves (ΔPWT AUC) values for CCI, sham-CCI, and non-operated SD rats following i.t administration of a single 35 nmol dose of oxycodone (Oxy) or morphine (Mor), 14 days after CCI or sham-CCI surgery (upper panels), and to similar CCI-rats that were pretreated with naloxone (Nal) or nor-binaltorphimine (nor-BNI) (middle and lower panels).

with either δ- or κ_1-opioid binding sites in rodent brain membranes, respectively.

3.4. Radioligand binding in μ- and δ-opioid receptor depleted rat brain membranes

Using rat brain membrane preparations that had been depleted of μ- and δ-opioid binding sites by the selective acylating agents, BIT and FIT, respectively, the displacement of [^{3}H]bremazocine by oxycodone relative to that of other unlabelled opioid ligands provided evidence that oxycodone binds to putative κ_{2b} sites (Table 4). Specifically, in 'depleted' brain membrane preparations, [^{3}H]bremazocine demonstrated a selective affinity for pharmacologically defined κ_2-opioid receptors comprising putative κ_{2a}- and κ_{2b}-opioid subtypes (Ni et al., 1993, 1995). The observations herein that the μ- selective opioid ligands, CTOP and morphine together with the δ-selective opioid ligand, DPDPE, failed to significantly displace [^{3}H]bremazocine from 'depleted' rat brain membranes (Table 4), confirmed that BIT and FIT treatment had effectively depleted μ- and δ-opioid binding site populations in these brain membrane preparations.

Unlabelled bremazocine displaced [^{3}H]bremazocine in 'depleted' rat brain membranes with high affinity (Table 4), and the observed $K_i$values were in agreement with published values for depleted rat ($K_d = 1.1$–1.3 nM) and guinea pig ($K_d = 0.6$–2.5 nM) brain membrane preparations (Tiberi and Magnan, 1989; Rothman et al., 1990b; Rothman et al., 1992; Ni et al., 1995).

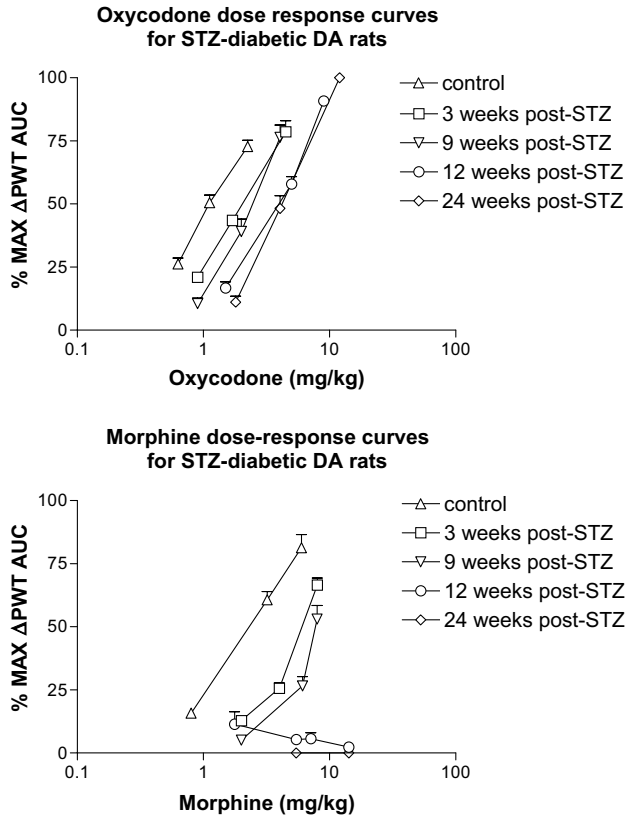

Fig. 3. Mean (±SEM) dose–response curves based on the percent maximum area under the ΔPWT versus time curve (% MAX ΔPWT AUC) values for s.c. bolus doses of oxycodone (upper) and morphine (lower) administered to DA rats with streptozotocin (STZ)-induced diabetes at 3, 9, 12 and 24 weeks post-STZ administration, and after administration of oxycodone and morphine to control non-diabetic DA rats.

Additionally, the displacement of [^{3}H]bremazocine by oxycodone in 'depleted' rat brain membrane preparations was best fit by a two-site binding model (Table 4). Leu-enkephalin (LE) has been shown to have a high affinity ($K_i = 11.7 \pm 2.1$ nM) for putative κ_{2b}-opioid binding sites (Rothman et al., 1992). In the presence of LE (500 nM), which acted to block putative κ_{2b}-opioid binding sites in rat brain membranes, the displacement of [^{3}H]bremazocine by oxycodone at concentrations below 1 μM was insignificant (Table 4). These data support the notion that oxycodone has a relatively high affinity for putative κ_{2b} opioid binding sites.

4. Discussion

Using behavioural methods in rat models of neuropathic pain and radioligand binding, the present study confirmed and extended our previous *in vivo* work in rats (Ross and Smith, 1997), showing that the intrinsic antinociceptive effects of oxycodone appear to be mediated by κ-opioid receptors, in contrast to morphine, the prototypic μ-opioid agonist (Pasternak, 2001; Gaveriaux-Ruff and Kieffer, 2002).

4.1. Behavioural studies in rat models of neuropathic pain

In the present work, i.t. administration of oxycodone and morphine to CCI-, sham-CCI and non-operated rats confirmed that these opioids have distinctly different antinociceptive profiles. Specifically, i.t. oxycodone (35 nmol) produced significant antinociception in both hindpaws of CCI-rats (Fig. 1) that was abolished by pre-treatment with nor-BNI (Fig. 2), whereas in sham-CCI and non-operated rats, oxycodone produced insignificant antinociception (Fig. 1). These latter findings are consistent with the low antinociceptive potency of i.t. oxycodone (~2–7%) relative to morphine in non-injured rats (Plummer et al., 1990; Pöyhiä and Kalso, 1992), and with the low epidural potency of oxycodone (~11%) relative to morphine in patients with post-operative pain (Backlund et al., 1997).

By contrast, i.t. morphine (35 nmol) not only produced significant antinociception in both hindpaws of CCI-rats, it also produced potent antinociception in sham-CCI and non-operated rats, such that antinociception was approximately equipotent in the contralateral hindpaw of CCI-rats and in the hindpaws of sham-CCI and non-operated rats (Fig. 1). In CCI-rats, pre-treatment with nor-BNI did not significantly attenuate i.t. morphine antinociception whereas pre-treatment with the non-selective opioid antagonist, naloxone, abolished morphine antinocieption (Fig. 2). Collectively, these findings in CCI-rats confirm the notion that oxy-

Table 2
Mean (±SEM) ED_{50} values for the relief of hindpaw mechanical allodynia/hyperalgesia in STZ-diabetic rats and for producing antinociception in the hindpaws of control non-diabetic DA rats, following administration of single s.c. bolus doses of oxycodone or morphine

Treatment group	Oxycodone (mg/kg)	Morphine (mg/kg)
Control non-diabetic	1.2 ± 0.1	2.4 ± 0.3
Three-week STZ-diabetic	$2.0 \pm 0.15^*$	$6.1 \pm 0.3^*$
Nine-week STZ-diabetic	$2.1 \pm 0.4^*$	$6.1 \pm 0.4^*$
Twelve-week STZ-diabetic	$4.1 \pm 0.3^*$	Efficacy abolished
Twenty-four week STZ-diabetic	$4.2 \pm 0.3^*$	Efficacy abolished

* ED_{50} significantly different ($p < 0.05$) from ED_{50} in respective control non-diabetic rats.

Table 3
Mean K_i (±SEM) values for the displacement of [^{3}H]DAMGO, [^{3}H]DPDPE, [^{3}H]morphine, and [^{3}H]U69,593 in untreated rodent brain membrane preparations

Opioid ligands	Tissue source	1 or 2 site fit	K_i
Morphine vs [^{3}H]morphine	SD rat	1	1.2 ± 0.4 nM
Oxycodone vs [^{3}H]morphine	SD rat	1	357 ± 121 nM
DAMGO vs [^{3}H]DAMGO	SD rat	1	0.7 ± 0.1 nM
DAMGO vs [^{3}H]DAMGO	DA rat	1	0.5 ± 0.5 nM
Oxycodone vs [^{3}H]DAMGO	DA rat	2*	1.9 ± 0.7 nM
			74.6 ± 0.9 nM
DPDPE vs [^{3}H]DPDPE	SD rat	1	1.4 ± 0.3 nM
Oxycodone vs [^{3}H]DPDPE	SD rat	1 or 2	>100 μM
U69,593 vs [^{3}H]U69,593	Guinea pig	1	2.6 ± 0.2 nM
Oxycodone vs [^{3}H]U69,593	Guinea pig	1 or 2	>100 μM

Data are means of three independent experiments each performed in duplicate.
* Statistical significance of 2-site *vs.* 1-site fit for pooled data: $F = 6.30$, $p < 0.001$.

codone is a putative κ-opioid agonist, consistent with our earlier work showing that nor-BNI pre-treatment of rats abolished i.c.v. oxycodone, but not morphine, antinociception (Ross and Smith, 1997), and with more recent findings in STZ-diabetic mice where s.c. oxycodone antinociception was abolished by nor-BNI pre-treatment (Nozaki et al., 2005).

In the 24-week longitudinal study in STZ-diabetic rats reported herein, there were striking differential temporal changes in the efficacy and potency of morphine and oxycodone for the relief of mechanical allodynia/hyperalgesia. Our long-term data in STZ-diabetic rats are the first to show the progressive development of morphine hyposensitivity, such that s.c. morphine efficacy was abolished by 12 weeks after STZ-diabetes induction, and it remained abolished at 24 weeks post-STZ administration. These observations are consistent with anecdotal clinical reports that morphine lacks efficacy for the symptomatic relief of painful diabetic neuropathy (PDN) in patients (Attal, 2000). Additionally, the present observation that morphine potency was reduced by ~2.5-fold as early as 3 weeks after STZ-induced diabetes is in agreement with previous reports (Kamei et al., 1992; Suh et al., 1996; Courteix et al., 1998; Malcangio and Tomlinson, 1998; Nozaki et al., 2005).

However, in contrast to the abolition of morphine efficacy by 12 weeks after the induction of STZ-diabetes, oxycodone retained full efficacy for the relief of mechanical allodynia/hyperalgesia in the same rats over the 24 weeks post-STZ study period, albeit with a ~3- to 4-fold decrease in potency. These findings extend those of a recent short-term study in mice which showed that at 2 weeks post-STZ administration, there was a large drop in the antinociceptive potency of s.c. morphine whereas s.c. oxycodone potency was not significantly affected (Nozaki et al., 2005). Together, these findings in STZ-diabetic rodents support recent clinical trials showing that oral oxycodone provided significant relief of moderate to severe pain in patients with PDN (Gimbel et al., 2003; Watson et al., 2003).

The long-term results presented herein together with those of short-term studies (Kamei et al., 1992, 1994; Zurek et al., 2001) show that STZ-diabetic rodents are

Table 4
Mean K_i (±SEM) values for the displacement of [^{3}H]bremazocine in rat brain membrane preparations depleted of μ- and δ-opioid receptors by pre-treatment with BIT and FIT, respectively

Opioid ligands	Tissue source	1 or 2 site fit	K_i
Bremazocine	SD rat	1	1.4 ± 0.1 nM
Bremazocine	DA rat	1	2.5 ± 0.1 nM
Oxycodone	DA rat	2*	25.1 ± 0.2 nM
			>4 μM
Oxycodone + LE (500 nM)	DA rat	1 or 2	>100 μM
Oxycodone + LE (500 nM)	SD rat	1	>3 μM
CTOP	DA rat	1	>100 μM
Morphine	DA rat	1	>0.2 μM
Morphine	SD rat	1	> 1.5 μM
DPDPE	DA rat	1	>100 μM

Data are means of three independent experiments each performed in duplicate.
* Statistical significance of 2-site *vs.* 1-site fit for pooled data: $F = 12.44$, $p < 0.0001$.

selectively hyporesponsive to μ-opioid agonists, while maintaining normal responses to δ- and κ-opioid agonists (Kamei et al., 1992, 1994, 1997). While the mechanistic basis through which diabetes selectively decreases μ-opioid agonist potency is unclear, it is likely that multiple pharmacodynamic and pharmacokinetic factors contribute.

In short-term studies (4 weeks post-STZ administration) in diabetic rodents, enhanced protein kinase C (PKC) activity (Narita et al., 1996; Ohsawa et al., 1997) has been implicated in the development of μ-opioid agonist hyposensitivity (Kamei et al., 1992, 1994; Zurek et al., 2001), as PKC-mediated activation of the NMDA receptor is involved in both the development and maintenance of persistent neuropathic pain and morphine tolerance (Mao et al., 1995). Increased neuronal Ca^{2+} concentrations following NMDA receptor activation appear to have a central role in the development of morphine hyposensitivity in diabetes, as i.c.v. administration of Ca^{2+} chelators to STZ-diabetic mice restored μ-opioid agonist potency to that of non-diabetic mice (Hoffmeister and Tettenborn, 1986; Ohsawa et al., 1998). Although changes in μ-opioid agonist binding affinity or density were not seen in the brain or spinal cord of rats 4 weeks after STZ-induced diabetes (Courteix et al., 1998; Chen and Pan, 2003), impaired G-protein coupling to μ-opioid receptors has been documented (Chen et al., 2002). Finally, pharmacokinetic factors such as an increase in the clearance and volume of distribution of systemically administered morphine in STZ-diabetic rats may also contribute by reducing the amount of morphine available to enter the CNS (Courteix et al., 1998).

4.2. Radioligand binding in rat brain membranes

Radioligand binding studies of oxycodone's opioid receptor binding profile in rat brain membrane preparations showed that it did not displace the δ-selective opioid radioligand, [^{3}H]DPDPE, ($K_i > 100$ μM) or the κ_1-selective ligand, [^{3}H]U69,593 ($K_i > 100$ μM), in a manner similar to that reported recently for membranes expressing cloned mouse δ (mDOR1)- or human κ_1 (hKOR1)-opioid receptors, respectively (Lalovic et al., 2006). As i.c.v. and i.t. oxycodone antinociception in rats are abolished by pretreatment with nor-BNI, an opioid antagonist that does not discriminate between κ_1 and κ_2-opioid receptor subtypes (Takemori et al., 1988; Horan et al., 1991), these findings collectively suggest that oxycodone may act as a κ_2-opioid agonist.

In displacement studies with [^{3}H]DAMGO, a radioligand that reportedly labels both μ- and κ_{2b}-opioid receptor subtypes (Rothman et al., 1992; Ni et al., 1993), oxycodone displaced [^{3}H]DAMGO from both high-affinity and low-affinity binding sites in rat brain membranes (Table 1). This finding suggests the possibility that oxycodone may bind with relatively high affinity to putative κ_{2b}-opioid receptors, and with lower affinity to μ-opioid receptors, as reported by Chen et al. (1991) for [^{3}H]DAMGO in rat brain homogenate and more recently by Lalovic et al. (2006) for [^{3}H]diprenorphine in membranes from cells expressing cloned human μ (hMOR1)-opioid receptors.

Further investigation of the binding of oxycodone at putative κ_{2b}-opioid receptors using rat brain membranes depleted of their μ- and δ-opioid receptor populations by irreversible acylation with the reagents, BIT and FIT, respectively, showed that oxycodone displaced the κ_2-ligand, [^{3}H]bremazocine with relatively high affinity, demonstrating that oxycodone is a κ_2-opioid ligand. Although previous radioligand binding studies suggest that κ_2-opioid receptors may be subdivided into κ_{2a} and κ_{2b} subtypes (Rothman et al., 1992; Rothman et al., 1993), there are no radioligands that differentially label κ_{2a} and κ_{2b} sites. Hence, indirect methods employing a saturating concentration of leu-enkephalin (500 nM) were used, as this ligand reportedly has high selectivity for κ_{2b} sites ($K_i \sim 11$ nM), but very low affinity for putative κ_{2a} ($K_i \sim 5$ μM) sites in the CNS (Rothman et al., 1992; Rothman et al., 1993). In the presence of leu-enkephalin (500 nM), high affinity binding of oxycodone was eliminated, indicating that oxycodone appears to be a relatively high affinity ligand for κ_{2b}-opioid binding sites in the rat brain.

Studies in opioid receptor 'knockout' mice suggest that κ_2-opioid receptors may arise from heterodimerization of opioid receptors produced by the 3 known genes encoding opioid receptors μ-(MOR), δ-(DOR), and κ-(KOR), respectively (Simonin et al., 2001). This notion is consistent with that proposed by Jordan and Devi (1999) who showed that the *in vitro* pharmacological characteristics of δ-κ-opioid receptor heterodimers are similar to those of the previously pharmacologically defined κ_2-opioid receptor subtype. Thus, it is plausible that oxycodone may act as an agonist at heterodimeric δ–κ-opioid receptor complexes in the CNS. Furthermore, the present observation that the potency of i.t. oxycodone was markedly increased in CCI-rats relative to sham-CCI and non-operated animals suggests the possibility that nerve injury may augment the expression of the δ–κ-opioid heterodimer; however, this remains to be assessed.

In conclusion, the present study confirms that the antinociceptive profiles of oxycodone and morphine are distinctly different when assessed in rat models of neuropathic pain. Specifically, the antinociceptive efficacy of morphine was abolished in diabetic rats by 12 wks after STZ administration, whereas the efficacy of oxycodone was maintained over a 24-week post-STZ study period. In CCI-rats, nor-BNI pre-treatment abolished the antinociceptive effects of i.t. oxycodone but not i.t. morphine, in agreement with a recent study

that showed nor-BNI pre-treatment of STZ-diabetic mice abolished s.c. oxycodone antinociception, and with our previous findings showing that i.c.v. oxycodone's antinociceptive effects were completely attenuated in nor-BNI pre-treated rats (Ross and Smith, 1997). Complementary radioligand binding data in the present study support the notion that oxycodone acts as a κ_{2b}-opioid receptor agonist, and has a relatively low affinity for μ-opioid receptors in agreement with others.

5. Competing interests statement

The University of Queensland has licensed intellectual property to QRx Pharma Pty Ltd of which MS and FR are inventors. Under University policy, MS and FR will receive a portion of any income received by UQ from QRx in the event of successful commercialization. In the past, MS's laboratory has undertaken contract research for QRx Pharma and MS has acted as an occasional consultant to QRx Pharma.

Acknowledgements

The authors thank Mr Andrew Wright, School of Pharmacy, The University of Queensland, for assistance in the initial stages of this project.

CN was supported financially by an Australian Postgraduate Award. SL was supported financially by the Australian–American Fulbright Commission. This research was supported financially by the Queensland Pharmacy Research Trust and the Brain Research Fund of The University of Queensland.

Some of our data were presented in preliminary form at the Australian Society of Clinical and Experimental Pharmacologists and Toxicologists Conference held in Dunedin, New Zealand, in December 2001, at the 2nd Asia Pacific Conference on Pain Control held in Sydney, Australia, in January 2001 (Smith et al., 2001), and at the 10th World Congress on Pain held in San Diego, USA, in August 2002.

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