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RESEARCH PAPER

Sensitivity to μ-opioid receptor-mediated anti-nociception is determined by cross-regulation between μ- and δ-opioid receptors at supraspinal level

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BACKGROUND AND PURPOSE

The perception of pain and its inhibition varies considerably between individuals, and this variability is still unexplained. The aim of the present study is to determine whether functional interactions between opioid receptors are involved in the inter-individual variability in the sensitivity to μ-opioid receptor agonists.

EXPERIMENTAL APPROACH

Anti-nociceptive tests, radioligand binding, stimulation of [^{35}S]GTP–γ–S binding, inhibition of cAMP production and co-immunoprecipitation experiments were performed in two strains of rat (Sprague–Dawley bred at our university – SDU – and Wistar) that differ in their sensitivity to opioids.

KEY RESULTS

The increased anti-nociceptive potency of μ-opioid receptor agonists in SDU rats was reversed by the δ-opioid receptor antagonist, naltrindole. Inhibition of the binding of [^{3}H] naltrindole by μ-opioid receptor agonists was different in brain membranes from SDU and Wistar rats. Differences were also evident in the effect of δ-opioid receptor ligands on the binding of [^{35}S]GTP-γ–S stimulated by μ-opioid receptors agonists. No strain-related differences were detected in spinal cord membranes. The potency of morphine to inhibit cAMP production in brain membranes varied between the strains, in the presence of deltorphin II and naltrindole. Co-immunoprecipitation experiments demonstrated that δ-opioid receptors were associated with μ-opioid receptors to a higher extent in brain synaptosomal fractions from SDU than in those from Wistar rats.

CONCLUSIONS AND IMPLICATIONS

There was increased supraspinal cross-talk between μ and δ-opioid receptors in SDU, as compared with Wistar rats. This was related to an enhanced sensitivity to anti-nociception induced by μ-opioid receptor agonists.

Abbreviations

%MPE, percentage of the maximum possible effect; 3-d, *N*-[1-fluorophenyl)pyrazol-3-yI]-N-[1-(2-phenethyl)-4-piperidyl)]propanamide; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2; DADL, [D-Ala^2, D-Leu^5]-enkephalin; DAMGO, [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin; E_{max}, maximum effect; HINT1, histidine triad nucleotide-binding protein 1; K_d, dissociative constant; K_I, inhibitory constant; pCl-DPDPE, [D-Pen^2, pCl-Phe^4, D-Phen^5]-enkephalin; RGS, regulator of G-protein signalling; SDU, Sprague–Dawley rats bred at our university; SNC80 (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide; TIPPΨ, H-Tyr-TicΨ-[CH_2NH]Phe-Phe-OH

Introduction

Opioids are the most powerful analgesics available currently for patients, yet their use and efficacy remains limited by undesirable side effects and the inter-individual variability found in opioid responses. The perception of pain and its inhibition varies considerably between individuals, both in humans (Dionne *et al.*, 2005) and in other animals (Mogil *et al.*, 1996; Más *et al.*, 2000). This variability is still unexplained. From the clinical point of view, it is important to recognize that optimal pain control may be achievable through understanding the molecular-genetic mechanisms, yielding individualized analgesic medications and dose regimens based upon each person's genetic endowment (Kim and Dionne, 2009). Several genetic models have been identified or produced using rodents, in an attempt to investigate the mechanisms that underlie variation between individuals in their response to opioid drugs. Differences have been described in nociceptive sensitivity (Hoffman *et al.*, 1998) and in the anti-nociceptive effect of opioids (Hoffman *et al.*, 1998; Morgan *et al.*, 1999; Más *et al.*, 2000), as well as in other actions of opioids (Hoffman *et al.*, 1998; Más *et al.*, 2000).

The effects of opioid drugs and endogenous opioid peptides are mediated by μ-, δ-, and κ-opioid receptors found in the CNS and other tissues (receptor nomenclature follows Alexander *et al.*, 2011). Opioid receptors belong to the family of GPCRs and their multiplicity provides a basis for explaining the complex pharmacology of opioids.

At present, the existence of interactions between opioid receptors is widely assumed. There is indirect evidence that opioid receptors do not necessarily act independently from each other. The existence of opioid receptor complexes was reported more than 30 years ago from radioligand binding and anti-nociception experiments (Vaught and Takemori, 1979). The cross-talk between μ-opioid receptors and δ-opioid receptors is documented, mainly from the observation that δ receptor agonists modulate μ receptor–mediated analgesia (Vaught *et al.*, 1982). Allosteric coupling between μ and δ-opioid receptors has been also postulated (Rothman *et al.*, 1993). Furthermore, immunohistochemical studies have demonstrated co-localization of opioid receptors in some neurons (Ji *et al.*, 1995), and cross-linking and co-immunoprecipitation studies have suggested the existence of a receptor complex both at spinal and supraspinal levels (Schoffelmeer *et al.*, 1990; Gomes *et al.*, 2004; Garzón *et al.*, 2005), opening the possibility for physical interactions between the receptors. It has been also shown that μ- and δ-opioid receptors coexist in a complex with a series of signalling elements such as G-proteins and RGS proteins (Garzón *et al.*, 2005). Functional interactions between μ- and δ-opioid receptors have been demonstrated in knockout models. Mice lacking μ-opioid receptors show reduced anti-nociception and the absence of respiratory depression induced by δ-opioid receptor agonists (Matthes *et al.*, 1998). In mice lacking δ-opioid receptors, δ receptor agonists retain supraspinal analgesia, suggesting the existence of a second δ-receptor like analgesic system or μ receptor-dependent anti-nociception (Zhu *et al.*, 1999; Scherrer *et al.*, 2004). Also, δ-opioid receptor knockout mice do not develop analgesic tolerance to morphine (Zhu *et al.*, 1999). Co-expression of δ- and μ-opioid receptors enhances the signalling of both receptors, suggesting that there is functional interaction between them (Snook *et al.*, 2008). The putative coordinated action of the opioid receptors, which may have therapeutic implications, needs to be clarified.

The aim of the present work was to assess whether the extent of functional interaction between μ- and δ-opioid receptors could determine the sensitivity to anti-nociception induced by μ-opioid receptor agonists. We addressed this question using a number of *in vivo* and *in vitro* approaches in two strains of rats – Sprague–Dawley bred at our university (SDU) and Wistar – that differ in their sensitivity to morphine. Our findings demonstrate that the sensitivity to the anti-nociceptive effect of μ-opioid receptor agonists was related to the extent of the interaction between μ- and δ-opioid receptors at a supraspinal level.

Methods

Animals

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee and followed guidelines regarding ethical standards for the experimental investigation of pain in animals (Zimmermann, 1983). All experiments were carried out on adult male rats that were 12–15 weeks old. The strains used were SDU rats, derived from a line bred at our University, and Wistar rats that were purchased commercially (Harlam, Barcelona, Spain). The animals were housed in clear plastic cages, three to four rats per cage, and maintained on a 12 h light/dark cycle, with sawdust bedding. Food and water were provided *ad libitum*, and the humidity and temperature were stabilized (20–22°C). The experiments were carried out between 1000 and 1500 h.

Nociceptive tests

The tail flick test was performed as described by Más *et al.* (2000). The light intensity (55–60°) was adjusted such that the baseline latencies ranged between 3 and 5 s, and the individual cut-off was calculated using the formula: cut-off = baseline latency × 10/4.5 (Menendez *et al.*, 1993). The hot plate response was assessed as previously described (Le Bars *et al.*, 2001). The heated surface of the plate was kept at a temperature of 55 ± 0.5°C. The reaction times of the first evoked behaviour regardless of whether it was paw licking or jumping were measured, and the cut-off was 30 s. Dose–response curves for morphine, fentanyl, methadone and 3-d were obtained for each animal by using a cumulative-dosing procedure (Paronis and Holtzman, 1991). After habituation and baseline trials, each animal was injected s.c. or i.p. with a low dose of morphine, fentanyl, methadone or 3-d, such that the cumulative dose was increased by 0.15 log units. Anti-nociception was evaluated by hot plate and tail flick tests 30 min after each cumulative s.c. dose of morphine (3.3–27 $mg \cdot kg^{-1}$ for the hot plate response and 0.3–9.6 $mg \cdot kg^{-1}$ for the tail flick test) and by tail flick test alone 30 min after each cumulative dose of methadone (0.85–4.8 $mg \cdot kg^{-1}$), 15 min after each dose of fentanyl (2.5–57 $\mu g \cdot kg^{-1}$), and 5 min after each dose of 3-d (0.15–1.7 $mg \cdot kg^{-1}$). This procedure was continued until the rat showed no nociceptive response within

the cut-off period. Non-cumulative dose–response curves were obtained for SNC80 (6.8–13.5 mg·kg^{-1}). The effect of the selective δOR antagonist naltrindole on the morphine and fentanyl dose–response curves was determined by administration of naltrindole by s.c. injection 20 min before different doses of morphine or fentanyl were administered.

Results are expressed as means ± SEM. The anti-nociceptive responses were expressed as a percentage of the maximum possible effect (%MPE), which was calculated as: %MPE = 100 × [(test latency – baseline latency) / (cut-off time – baseline latency)]. The anti-nociceptive dose–response curves were fitted to the logit–log equation, and the ED_{50} was estimated with Prism (GraphPad Software Inc., La Jolla, CA). The dose–response curves of nociceptive tests were compared using a two-way ANOVA, with strain or co-administration of naltrindole as the between-groups factor and dose as the repeated-measures factor. This was followed by the Bonferroni test for multiple comparisons. The level of significance was set at $P < 0.05$.

Receptor binding

For each batch, pooled membranes from the whole brain (minus cerebellum) or spinal cord from six rats were prepared (Fang *et al.*, 1994). Protein concentrations were determined using the Bradford method (Bradford, 1976). The binding of [^{3}H]naltrindole (specific activity 60 Ci·mmol^{-1}) to the microsomal brain membranes was performed as described by Fang *et al.* (1994). In the inhibition experiments, 0.1 nM [^{3}H]naltrindole was used. The binding of [^{3}H]DAMGO (specific activity 60 Ci·mmol^{-1}) was performed essentially as described by Raynor *et al.* (1995). In the inhibition experiments, 1 nM [^{3}H]DAMGO was used. Non-specific binding of both radioligands was determined in the presence of 10 μM naloxone. Experimental data were expressed as specific binding (fmol·mg^{-1} protein). The data on saturation binding were fitted to a one binding-site saturation model with Prism. The results of the inhibition experiments were fitted to one- and two-binding sites models using the programme Prism, and *F*-tests were performed to determine whether the experimental data fitted significantly better to a model with one or two binding sites.

[^{35}S]GTP-γ-S binding

The binding of [^{35}S]GTP-γ-S (specific activity 1000 Ci·mmol^{-1}) to pooled membranes from brain or spinal cord from both strains, prepared as above, was investigated as previously described (Sim *et al.*, 1995), with minor modifications. The basal level of binding was determined by incubation of samples in the absence of agonists. Non-specific binding was determined in the presence of 100 μM unlabelled GTP-γ-S. To study interactions between the μ- and δ-opioid receptor ligands, increasing concentrations of δ receptor ligands were used in the presence or absence of a fixed dose of the μ receptor agonists, DAMGO (30 nM for deltorphin II and TIPPΨ and 3 μM for naltrindole) or morphine (30 nM for deltorphin II and TIPPΨ and 0.5 μM for naltrindole).

Experimental data on [^{35}S]GTP-γ-S were expressed as the percentage of the basal binding. Results were fitted to appropriate binding models with the programme Prism. When necessary, *F*-tests were performed to determine whether the experimental data fitted significantly better to one- or two-binding sites model.

cAMP assay

For the cAMP assay, membranes were prepared from the whole brain (minus cerebellum), and cAMP was extracted from the tissue homogenate essentially as described by Izenwasser *et al.* (1993). The ability of morphine or deltorphin II to inhibit the adenylyl cyclase activity stimulated by 50 μM forskolin was evaluated by incubation of aliquots of the brain homogenate (protein concentration of 40 μg/60 μL final volume) with increasing concentrations of morphine or deltorphin II (0.01–100 μM, in both cases), for 5 min at 30°C. The basal activity of adenylyl cyclase was determined by incubation of the membranes in the absence of forskolin. To study the interactions of δ-opioid receptor ligands with morphine, we used the same protocol to evaluate the ability of increasing concentrations of morphine to inhibit stimulated adenylyl cyclase activity in the presence of either 0.5 μM deltorphin II or 0.1 μM naltrindole. Ethanol extraction and analysis of samples by radioimmunoassay were performed following the manufacturer's instructions.

The activity of adenylyl cyclase was expressed as pmol of cAMP produced per mg protein in 5 min. The results were expressed as means ± SEM or as a percentage of the adenylyl cyclase activity stimulated by 50 μM forskolin. The concentration–response curves for cAMP were fitted to the logit–log equation. The morphine concentration–response curves in the absence and presence of δ-opioid receptor ligands were compared using two-way ANOVA, with treatment as the between-groups factor and concentration as the repeated-measures factor. The level of significance was set at $P < 0.05$.

Co-immunoprecipitation experiments

Co-immunoprecipitation experiments were performed as described by Garzón *et al.* (2005). Briefly, synaptosomal membranes from the whole brain (minus cerebellum) were prepared from both strains. To obtain synaptosomal extract, synaptosomes (600 μg of protein) were sonicated (two cycles of 5 s each) in 1 mL solubilization buffer containing 50 mM Tris–HCl (pH 7.7), 50 mM Na Cl, 1% Nonidet P-40, 50 μL of protease and phosphatase inhibitor mixtures (Sigma-Aldrich), and solubilized overnight at 4°C. The target proteins were immunoprecipitated from the synaptosomal extract with selected affinity purified biotinylated IgGs. The immunocomplexes were recovered with streptavidin agarose and the agarose pellets were solubilized in 2× Laemmli buffer. Biotinylated antibodies used were raised against: residues 208–216 of the second external loop of μ-opioid receptors (2EL; Garzón *et al.*, 2005; Rodríguez-Muñoz *et al.*, 2008); residues 110–120 of the first external loop of δ-opioid receptors (D1EL; Schulz *et al.*, 1998; Garzón *et al.*, 2005).

In some experiments, synaptosomal extracts were immunodepleted of μ receptors or δ receptors before the immunoprecipitation with biotinylated IgGs directed against the target proteins. To immunodeplete μ receptors, the synaptosomal extracts were subjected to an overnight incubation with the biotinylated antibody 2EL. Then, μ receptors were

removed by incubating the extract with streptavidin agarose. The sample was centrifuged, and the supernatant was collected. Next, the supernatant was incubated overnight with the biotinylated antibody D1EL. The immunocomplexes were recovered with streptavidin agarose and the agarose pellets were solubilized in 2× Laemmli buffer. To immunodeplete δ receptors, the synaptosomal extracts were subjected to an overnight incubation with biotinylated antibody D1EL. Then, δ receptors were removed by incubating the extract with streptavidin agarose. The sample was centrifuged and the supernatant was collected. Next, the supernatant was incubated overnight with the biotinylated antibody 2EL. The immunocomplexes were recovered with streptavidin agarose, and the agarose pellets were solubilized in 2× Laemmli buffer.

Detection of immunoprecipitated proteins by immunoblotting

The recovered immunocomplexes were resolved on 10% SDS-PAGE and subsequently transferred onto 0.2 μm PVDF membranes (Bio-Rad 162–0176, Madrid, Spain) and probed with previously characterized antibodies directed towards: residues 2–16 of the extracellular N-terminal of the μ-opioid receptor (NT; Garzón *et al.*, 2005); residues 2–16 of the extracellular N-terminal of the δ- opioid receptor (DNT; Garzón *et al.*, 2005). The primary antibodies were detected using the corresponding secondary antibodies conjugated to horseradish peroxidase (diluted 1:10 000). Antibody binding was visualized with ECL Plus Western Blotting detection system (GE #RPN2132), and the chemiluminescence will be recorded with a ChemiImager IS-5500 (Alpha Innotech, San Leandro, CA) equipped with a Peltier-cooled CCD camera that provides a real-time readout of 30 frames per second. Data analysis was performed using the Image Gauge 4.0 software (Fujifilm Co., Barcelona, Spain).

Materials

All compounds for s.c. and i.p. injection were dissolved in normal saline, and injection volumes ranged from 0.2 to 0.6 mL. Morphine HCl was obtained from Alkaliber Laboratory (Madrid, Spain), methadone HCl from Dr Esteve Laboratories (Barcelona, Spain), fentanyl citrate from Roche Laboratories (Madrid, Spain), SNC80 and deltorphin II from Tocris (Bristol, UK), naltrindole HCl from Sigma-Aldrich (Madrid, Spain), and 3-d was provided by Dr Martín-Fontelles (University Juan Carlos I, Madrid, Spain). All other chemicals were of reagent grade and were obtained from Sigma-Aldrich. Radiochemicals and the cAMP assay kit were purchased from Tocris (Madrid, Spain), PerkinElmer (Madrid, Spain) or GE Healthcare (Barcelona, Spain).

Results

Anti-nociceptive response to μ-opioid receptor agonists

The baseline responses before the administration of drugs were similar in both strains (tail flick test: Wistar = 3.8 ± 0.1 s, n = 51; SDU = 3.9 ± 0.1, n = 68; hot plate test: Wistar = 7.9 ± 0.3 s, n = 29; SDU = 7.2 ± 0.3 s, n = 28). The potency of morphine was 1.5–2-fold higher in SDU than in Wistar rats in both the tail flick and the hotplate tests (Figure 1A, Table 1). Statistical analyses of the dose–response curves obtained with the tail flick and hot plate tests indicated that strain had a significant effect (Table 1).

We examined whether the higher sensitivity of SDU rats to morphine was specific to this opioid or was attributable to μ-opioid receptor-induced anti-nociception. Dose–response curves for the μ receptor agonists, fentanyl and methadone, and the highly selective μ receptor agonist, 3-d (Jagerovic *et al.*, 2002) were obtained in the tail flick test. As before, the baseline response latency before drug administration was similar in SDU and Wistar rats. For each drug tested, the ED_{50} value was significantly lower in SDU than in Wistar rats (Table 1).

Anti-nociceptive response to the δ-opioid receptor agonist, SNC80

We studied whether the higher sensitivity of the SDU strain to μ receptor-induced anti-nociception was also observed with δ receptor-induced anti-nociception. Dose–response curves for the non-peptidic δ receptor agonist SNC80 in the tail flick test were obtained (Figure 1B), but we could not use

Table 1

Estimated ED_{50} values of μ-opioid receptor agonists in Wistar and SDU rats

Drug	Test	Wistar	SDU	F (d.f.)[a]
Morphine	Hot plate	8.70 ± 0.69	5.74 ± 0.38	7.64 (1,124)**
	Tail flick	1.45 ± 0.06	0.75 ± 0.04	23.92 (1,165)***
+Naltrindole[b]	Tail flick	1.70 ± 0.03	1.40 ± 0.12	0.075 (1,30)[n.s.]
Fentanyl	Tail flick	17.20 ± 2.00	4.20 ± 0.10	46.22 (1,123)***
Methadone	Tail flick	1.31 ± 0.19	0.82 ± 0.01	29.96 (1,143)*
3-d	Tail flick	0.38 ± 0.050	0.19 ± 0.003	9.75 (1,94)**

Values shown represent mean ± SEM (n = 10–16 animals per group). ED_{50} values are expressed in mg·kg^{-1}, except for those for fentanyl, which are expressed in μg·kg^{-1}.

[a]F, strain factor; (d.f.), degrees of freedom; two-way ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001; [n.s.]non-significant, differences between strains.

[b]ED_{50} of morphine in the presence of naltrindole.

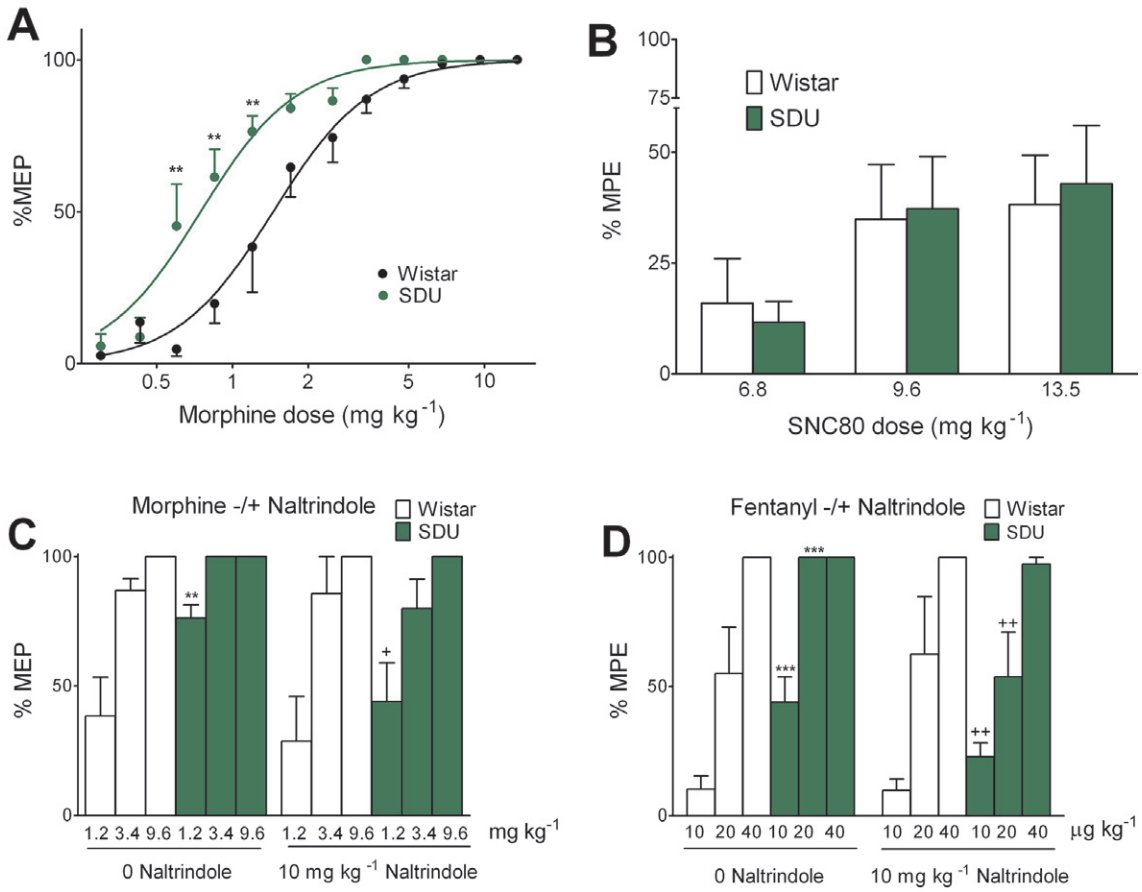

Figure 1

(A) Dose–response relationship for the anti-nociceptive effect of s.c. morphine evaluated by tail flick in Wistar and SDU rats. (B) Dose–response relationship for the anti-nociceptive effect of s.c. SNC80 evaluated by tail flick in Wistar and SDU rats. (C) Effect of s.c. naltrindole (10 mg·kg^{-1}) on the anti-nociceptive response to increasing doses of s.c. morphine and (D) fentanyl in Wistar and SDU rats, evaluated by the tail flick test. The results are expressed as the means ± SEM of %MPE. $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from Wistar rats ($n = 6$–26 animals per group); $^{+}P < 0.05$, $^{++}P < 0.01$, significant effect of naltrindole, Bonferroni's test ($n = 5$–11 animals per group). Estimated values of ED_{50} are given in Table 1.

doses of this agonist higher than 13.5 mg·kg^{-1} (%MPE: 40%, in both strains) because they induced restlessness. At the doses tested, no significant difference in the anti-nociceptive effect was apparent between strains.

Interaction of δ receptor antagonist naltrindole with the μ receptor agonists morphine and fentanyl

To test if interactions between δ- and μ-opioid receptors could account for the higher sensitivity of SDU rats to μ receptor agonists, dose–response curves for the μ receptor agonists morphine and fentanyl in the presence of the δ receptor antagonist naltrindole were obtained. Figure 1C shows that, in the tail flick test, subcutaneous administration of 10 mg·kg^{-1} naltrindole abolished the increased significant anti-nociceptive effect of 1.2 mg·kg^{-1} of morphine in SDU rats but not in Wistar rats ($P < 0.05$). This resulted in suppression of the significant differences in the estimates of ED_{50} of morphine and in the dose–response curves (Table 1). Naltrindole (10 mg·kg^{-1}, s.c.) also reversed the increased effect of 10 and 20 μg·kg^{-1} of the highly selective μ receptor agonist fentanyl in SDU rats, compared with Wistar rats ($P < 0.01$) (Figure 1D).

Binding of [^{3}H]naltrindole and [^{3}H]DAMGO to the brain and spinal cord microsomal fraction

To determine the density of brain μ- and δ-opioid receptors in SDU and Wistar rats, saturation binding of [^{3}H]naltrindole and [^{3}H] DAMGO to the brain and spinal cord microsomal fraction was determined The apparent K_d values of the μ receptor agonist [^{3}H]DAMGO and the δ receptor antagonist [^{3}H]naltrindole in brain microsomal fractions were similar in SDU and Wistar rats, as was the number of μ and δ receptor binding sites (Figure 2A.B; Table 2). Inhibition of the binding of [^{3}H]DAMGO and [^{3}H]naltrindole demonstrated that the μ/δ receptor selectivity of CTAP was similar in brain membranes from either strain (>700-fold). The δ/μ receptor selectivity of unlabelled naltrindole was also similar in brain membranes from either strain (~60-fold) (Figure 2C,D, Table 2). The inhibition of the binding of [^{3}H] DAMGO by increasing concentrations of morphine and DAMGO revealed

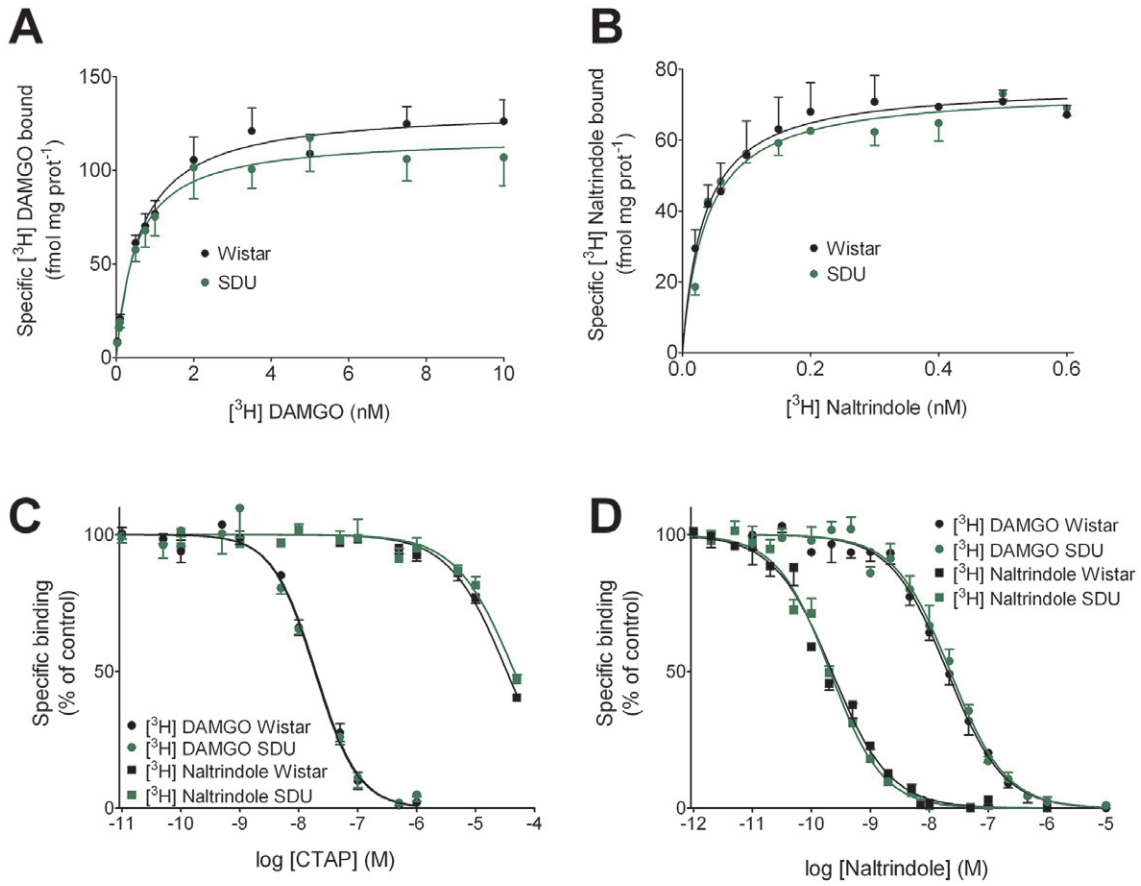

Figure 2

Saturation curves of [^{3}H] DAMGO (A) and [^{3}H] naltrindole (B) to brain membranes from Wistar and SDU rats. Inhibition of the specific binding of [^{3}H] DAMGO and [^{3}H]naltrindole to whole brain membranes from Wistar and SDU rats by CTAP (C) and naltrindole (D). The results are expressed as the means ± SEM from three experiments performed in triplicate. K_d, B_{max} and K_I values are given in Table 2.

that both μ receptor agonists inhibited the binding of [^{3}H] DAMGO with high affinity in rat brain membranes from both strains, according to a one-binding site model (Figure 3A,B, Table 2). In spinal cord, DAMGO inhibited the binding of [^{3}H] DAMGO also with high affinity in both strains according to a one-binding site model (Wistar: K_i = 0.94 ± nM; SDU: K_i = 1.18 ± nM) (Figure 3C). Experiments on the inhibition of the binding of [^{3}H]naltrindole to brain membranes by the μ receptor agonists morphine and DAMGO disclosed a clear difference between SDU and Wistar rats (Figure 3A,B). In the brain membranes of SDU rats, both μ receptor agonists inhibited the binding of [^{3}H]naltrindole according to a two-binding site model ($P < 0.01$ in both cases). The percentage of high-affinity binding sites for morphine and DAMGO showed reasonable agreement. In contrast, for the brain membranes of Wistar rats, the results fitted significantly to a one binding-site model. The K_I of morphine and DAMGO in the brain membranes of Wistar rats were similar to those for the low affinity sites in SDU rats (Table 2). In spinal cord membranes of both strains, DAMGO inhibited the binding of [^{3}H]naltrindole according to a one binding-site model. The affinity of DAMGO was similar in both strains (Wistar: K_I = 870 ± 127 nM; SDU: K_I = 588 ± 74 nM) (Figure 3C).

Binding of [^{35}S]GTP-γ–S stimulated by μ- and δ-opioid receptor agonists

To determine whether the strains display differences in the effects of δ and μ receptor ligands on the activation of G-proteins, the binding of [^{35}S]GTP-γ–S induced by μ and δ receptor agonists and the interactions between ligands for these receptors were assayed. As shown in Figure 4 and Table 3, no significant differences between strains were observed in the concentration–response curves of the μ or δ receptor agonists in brain and spinal cord membranes. However, for brain membranes, differences became obvious when concentration–response curves of δ receptor ligands were performed in the presence of a fixed concentration of μ receptor agonists. In brain membranes of Wistar rats, the presence of either 30 nM DAMGO or 30 nM morphine did not alter the binding of [^{35}S]GTP-γ–S stimulated by increasing concentrations of the δ receptor agonist deltorphin II (Figure 5A, Tables 3 and 4), but in SDU brain membranes, the presence of either 30 nM DAMGO or 30 nM morphine increased the potency of deltorphin II by 5- to 10-fold (Figure 5B, Tables 3 and 4). In presence of morphine, the E_{max} also increased. In brain membranes, naltrindole had no sig-

Table 2

Affinities and number of binding sites for [^{3}H]DAMGO and [^{3}H]naltrindole binding to brain membranes from Wistar and SDU rats

Radioligand	Wistar	SDU
[^{3}H]DAMGO		
Saturation: K_d	0.58 ± 0.07	0.65 ± 0.06
B_{max}	133 ± 4	138 ± 3
Displacements		
Morphine: K_i	2.09 ± 0.12	2.92 ± 0.37
DAMGO: K_i	0.84 ± 0.03	0.92 ± 0.03
CTAP: K_i	7.10 ± 0.48	7.31 ± 0.92
Naltrindole: K_i	8.95 ± 1.06	7.46 ± 0.73
[^{3}H]naltrindole		
Saturation: K_d	0.031 ± 0.007	0.034 ± 0.008
B_{max}	75 ± 4	73 ± 3
Displacements		
Morphine: K_{i1}	–	19.94 ± 5.98
$\%_1$	–	34.3 ± 0.13
K_{i2}	238.5 ± 22.84	558.2 ± 101.5
DAMGO: K_{i1}	–	19.64 ± 5.96
$\%_1$	–	19.1 ± 0.3
K_{i2}	946.5 ± 70.99	1466 ± 101.5
CTAP: K_i	>5000	>5000
Naltrindole: K_i	0.15 ± 0.02	0.12 ± 0.01

Binding was performed as described in Methods. Values shown represent mean ± SEM of three experiments conducted in duplicate. K_d and K_i values are expressed in nM. B_{max} values are expressed in fmol·mg^{-1} protein. $\%_1$ represents the percentage of high-affinity binding sites.

nificant effect on the binding of [^{35}S]GTP-γ–S alone or in the presence of either 30 nM DAMGO or 30 nM morphine (data not shown). However, naltrindole inhibited the binding of [^{35}S]GTP-γ–S stimulated by 3 μM DAMGO or 5 μM morphine in a concentration-dependent manner. In the case of Wistar rats, the concentration–response data fitted significantly to a one binding-site model. In SDU rats, experimental data fitted significantly to a two binding sites model. High-affinity phase represented approximately 45% of the binding (Figure 6A,B, Table 4). In the brain membranes of Wistar rats, the ID_{50} values for naltrindole were fairly similar to the ID_{50} of low affinity sites in SDU rats. The δ receptor antagonist TIPPΨ, did not affect the binding of [^{35}S]GTP-γ–S to the brain membranes of either strain (Figure 7A,B). However, both 30 nM DAMGO or 30 nM morphine enhanced the binding of [^{35}S]GTP-γ–S to SDU brain membranes in a concentration-dependent manner (Figure 7B). This reached a maximum at concentrations of approximately 10^{-7} M. At higher concentrations, a second inhibitory phase was also apparent. For the brain membranes of Wistar rats, in the presence of either 30 nM DAMGO or 30 nM morphine, TIPPΨ did not enhance the binding of [^{35}S]GTP-γ–S (Figure 7A).

In spinal cord membranes from both strains, 30 nM DAMGO did not have any significant effect on the ED_{50} of the

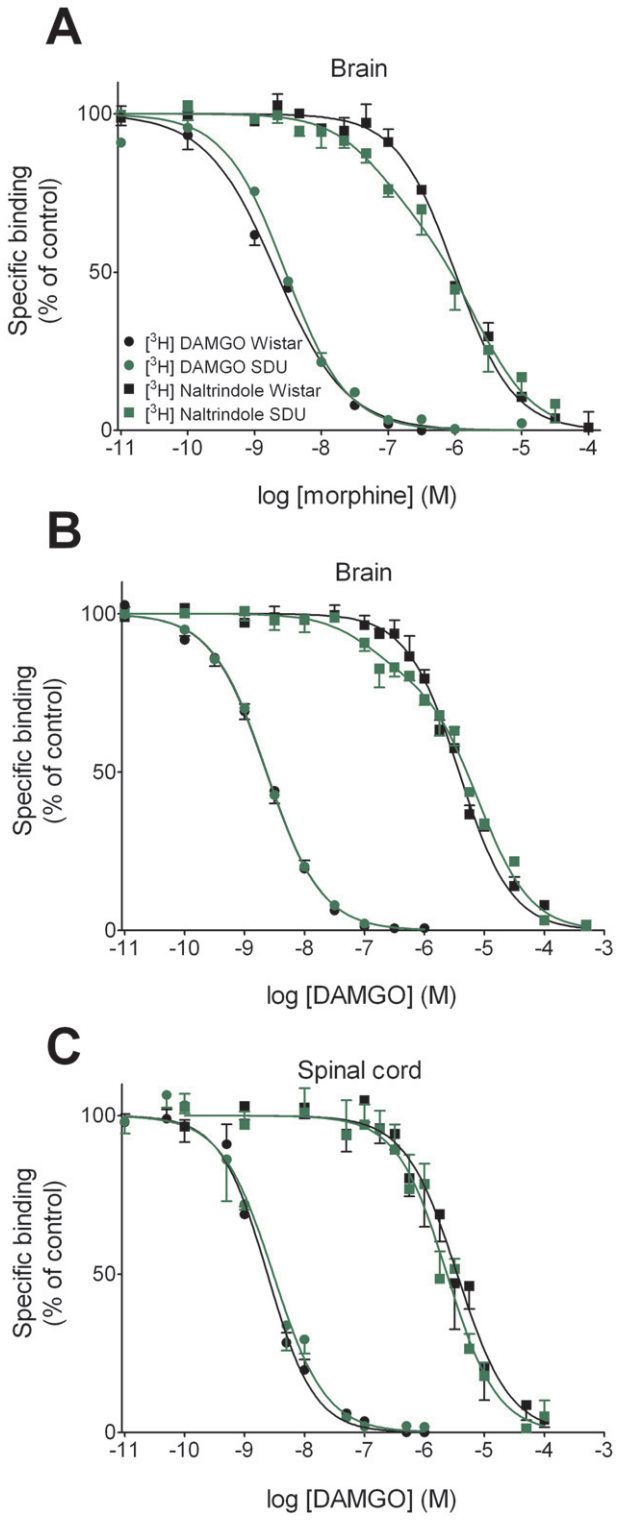

Figure 3

Inhibition of the specific binding of [^{3}H] DAMGO and [^{3}H]naltrindole to whole brain membranes from Wistar and SDU rats by (A) morphine and (B) DAMGO. In (C) inhibition of the specific binding of [^{3}H] DAMGO and [^{3}H]naltrindole to spinal cord membranes from Wistar and SDU rats by DAMGO. The results are expressed as the means ± SEM from three experiments performed in triplicate. K_i values are given in the text and in Table 2.

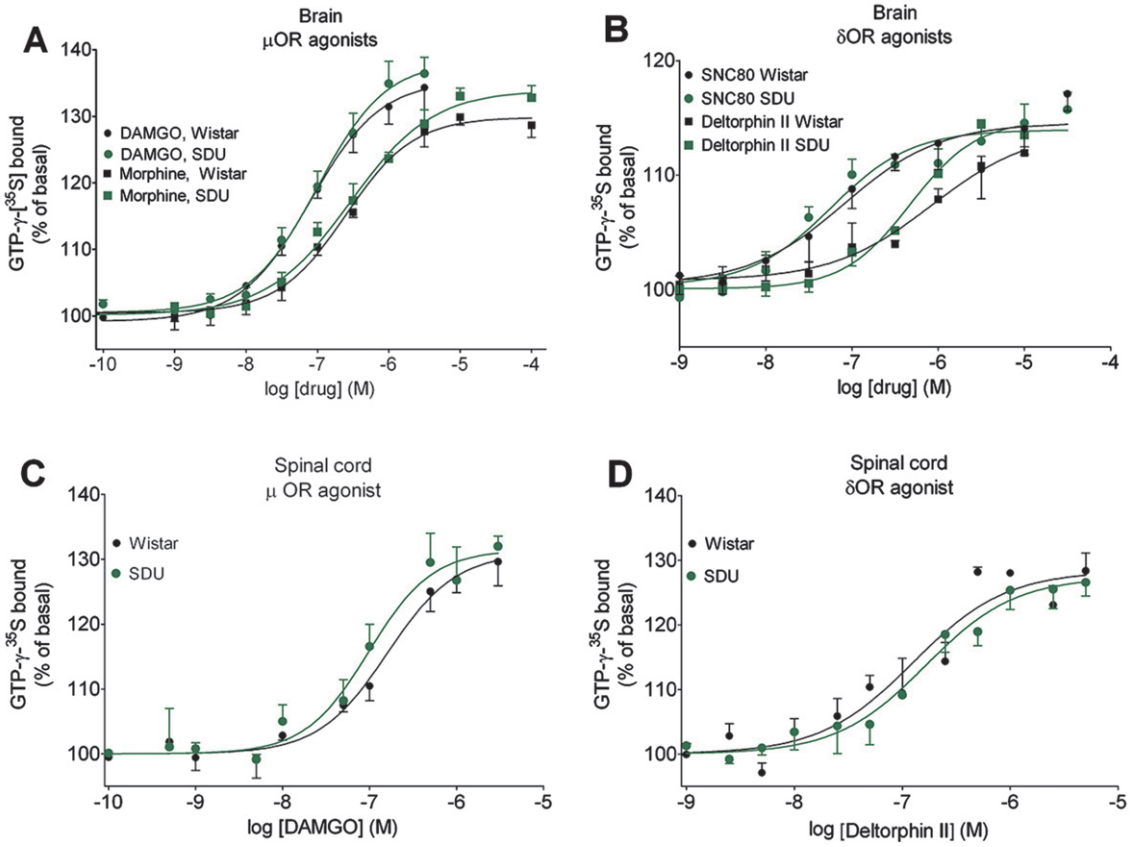

Figure 4

Binding of [^{35}S]GTP-γ-S stimulated by the μ opioid receptor (μOR) agonists, DAMGO and morphine (A), and (B) the δ-opioid receptor (δOR) agonists, SNC80 and deltorphin II in brain membranes from Wistar and SDU rats. In (C), binding of [^{35}S]GTP-γ-S stimulated by the μ-opioid receptor agonist DAMGO and in (D) the δ-opioid receptor agonist deltorphin II in spinal cord membranes from Wistar and SDU rats. Results are expressed as the means ± SEM of the percentage of basal binding from three to six experiments performed in triplicate. Corresponding ED_{50} and E_{max} values are shown in Table 3.

Table 3

Affinities and E_{max} of the stimulation of [^{35}S]GTP-g-S binding to brain and spinal cord membranes from Wistar and SDU rats by μ– or δ-opioid receptor agonists

	Brain				Spinal Cord			
	ED_{50}		E_{max}		ED_{50}		E_{max}	
Drug	Wistar	SDU	Wistar	SDU	Wistar	SDU	Wistar	SDU
DAMGO	0.08 ± 0.01	0.10 ± 0.01	135.0 ± 2.5	138.6 ± 2.7	0.15 ± 0.03	0.16 ± 0.05	130.1 ± 1.7	131.5 ± 2.6
Morphine	0.25 ± 0.03	0.29 ± 0.03	129.9 ± 1.1	133.8 ± 1.4	–	–	–	–
SNC80	0.07 ± 0.01	0.04 ± 0.01	114.5 ± 1.4	113.9 ± 0.9	–	–	–	–
Deltorphin II	0.83 ± 0.18	0.47 ± 0.05	114.0 ± 0.6	114.8 ± 0.3	0.14 ± 0.04	0.16 ± 0.04	127.9 ± 2.2	127.4 ± 1.8

Binding was performed as described in Methods. Values shown represent mean ± SEM of three experiments conducted in triplicate. ED_{50} values are expressed in μM. E_{max} values are expressed as the percentage of basal binding.

binding of [^{35}S]GTP-γ–S stimulated by increasing concentrations of deltorphin II (Wistar: ED_{50} = 0.17 ± 0.05 μM; SDU: ED_{50} = 0.17 ± 0.06 μM) (Figure 5C,D; Table 3). In spinal cord membranes, naltrindole had no significant effect on the binding of [^{35}S]GTP-γ–S alone or in the presence of 30 nM DAMGO (data not shown). However, naltrindole inhibited the binding of [^{35}S]GTP-γ–S stimulated by 3 μM DAMGO in a concentration-dependent manner (Figure 6C). For both strains, the concentration-response data fitted significantly to a one binding-site model. The ID_{50} values were fairly similar

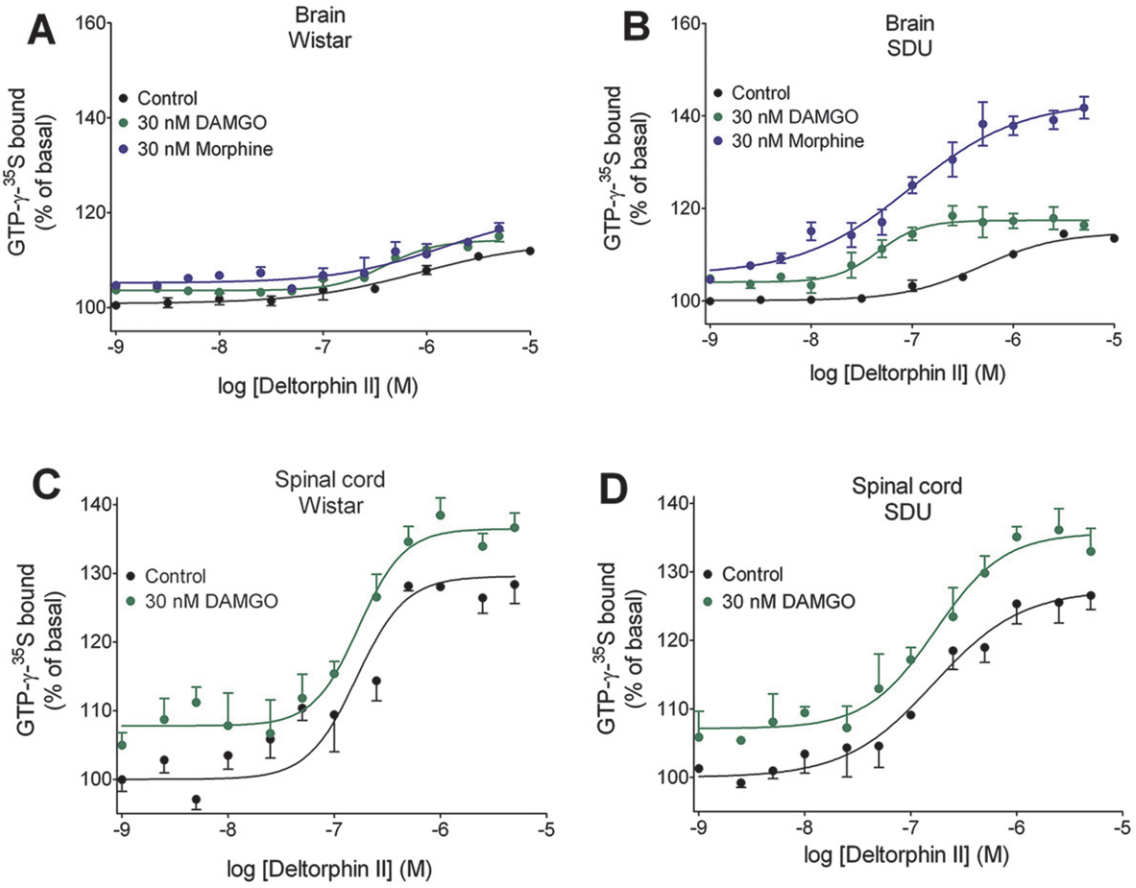

Figure 5

Binding of [^{35}S]GTP-γ-S induced by increasing concentrations of the δ-opioid receptor agonist, deltorphin II in the absence or presence of 30 nM DAMGO and 30 nM morphine in brain membranes from Wistar (A) and SDU (B) rats. In (C), binding of [^{35}S]GTP-γ-S induced by increasing concentrations of deltorphin II in the absence or presence of 30 nM DAMGO in spinal cord membranes from Wistar and in (D) SDU rats. The results are expressed as the means ± SEM of the percentage of the basal binding from three to six experiments performed in triplicate. ED_{50} and E_{max} values are given in Tables 3 and 4, and in the text.

Table 4

Affinities and E_{max} of the concentration-response curves of the [^{35}S]GTP-g-S binding to brain membranes from Wistar and SDU rats by δ-opioid receptor agonists and antagonists in the presence of fixed concentrations of μ–opioid receptor agonists

	High-affinity phase				Low-affinity phase			
	ED_{50}		E_{max}		ID_{50}		I_{max}	
Drug	**Wistar**	**SDU**	**Wistar**	**SDU**	**Wistar**	**SDU**	**Wistar**	**SDU**
DAMGO								
+Deltorphin II	0.68 ± 0.07	0.05 ± 0.001	110.3 ± 0.3	112.9 ± 0.9	–	–	–	–
+Naltrindole	–	0.003 ± 0.0007[a]	–	44.0 ± 5.7[b]	0.06 ± 0.01	0.31 ± 0.06	90.7 ± 2.9	56.0 ± 7.3
Morphine								
+Deltorphin II	1.41 ± 0.31	0.10 ± 0.01	110.1 ± 0.7	137.4 ± 2.9	–	–	–	–
+Naltrindole	–	0.003 ± 0.0004[a]	–	47.0 ± 3.3[b]	0.06 ± 0.009	0.49 ± 0.06	97.4 ± 2.3	53.0 ± 3.7

[a]ID_{50}. [b]I_{max}. Binding was performed as described in Methods. Values shown represent mean ± SEM of three experiments conducted in triplicate. ED_{50} values are expressed in μM. E_{max} and I_{max} values are expressed as the percentage of binding in the absence of δ receptor ligand.

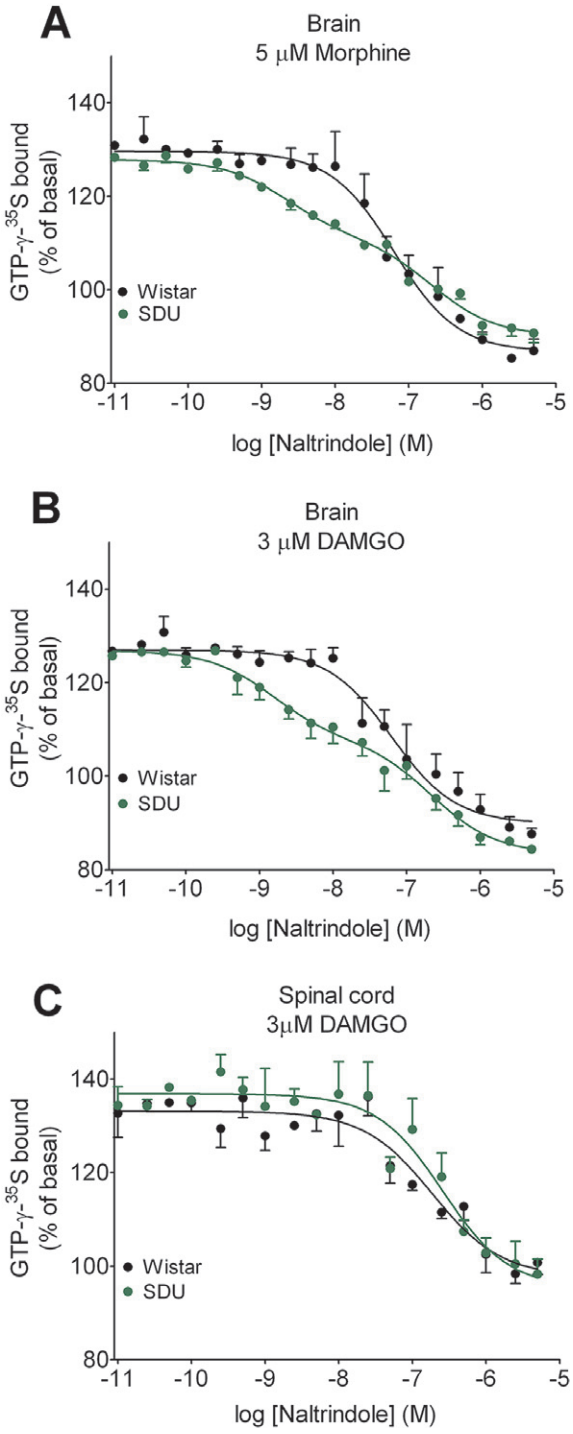

Figure 6

Inhibition of the binding of [^{35}S]GTP-γ-S induced by 5 μM morphine (A) and 3 μM DAMGO (B) by increasing concentrations of naltrindole in brain membranes from Wistar and SDU rats. In (C), inhibition of the binding of [^{35}S]GTP-γ-S induced by 3 μM DAMGO by increasing concentrations of naltrindole in spinal cord membranes from Wistar and SDU rats. The results are expressed as the means ± SEM of percentage of the basal binding from three to six experiments performed in triplicate. IC_{50} and E_{max} values are given in Table 4, and in the text.

(Wistar: 0.19 ± 0.06 μM; SDU: 0.26 ± 0.09 μM). The δ receptor antagonist TIPPΨ, did not change binding of [^{35}S]GTP-γ-S to spinal cord membranes of either strain, alone or in the presence of 30 nM DAMGO (Figure 7C,D).

Inhibition of adenylyl cyclase activity stimulated by forskolin

To determine whether there were differences between the strains in the inhibition of adenylyl cyclase activity, concentration–response curves of μ and δ receptor agonists and the interactions between μ receptor agonists and δ receptor ligands were carried out. The basal level of cAMP formation in the brain membranes of both strains (37.5 ± 5.2 and 28.3 ± 3.1 pmol·mg^{-1} protein·min^{-1}; Wistar and SDU respectively) or that stimulated by 50 μM forskolin (234 ± 17 and 270 ± 30% stimulation; Wistar and SDU, respectively, n = 8) were not different. The parameters of the inhibition by either morphine or deltorphin II, of cAMP production stimulated by forskolin were the same in brain membranes from Wistar and SDU rats (Figure 8A,B, Table 5). However, the interaction of fixed concentrations of either deltorphin II or naltrindole with increasing concentrations of morphine was clearly strain–dependent. In the brain membranes from Wistar rats, both the potency and the E_{max} of morphine were not affected by the presence of 0.5 μM deltorphin II. In the presence of 0.1 μM naltrindole, the E_{max} of morphine was lower, whereas its potency was unchanged in Wistar rats (Figure 8C, Table 5). Analysis of the morphine concentration–response curves indicated a main effect of naltrindole ($F_{1,24}$ = 7.97; P < 0.01). In the brain membranes of SDU rats, deltorphin II and naltrindole shifted the concentration–response curves of morphine significantly to the left and right respectively (Figure 8D). In the presence of 0.5 μM deltorphin II (a concentration that by itself did not inhibit significantly the adenylyl cyclase activity stimulated by forskolin), the potency of morphine in inhibiting the adenylyl cyclase activity stimulated by forskolin was increased by approximately fivefold, with no change in the E_{max} (Table 5). In the presence of 0.1 μM naltrindole, morphine, at concentrations of up to 10^{-4} M, did not significantly inhibit the adenylyl cyclase activity stimulated by forskolin. Morphine only induced inhibition, of about 50%, at a concentration of 10^{-3} M. Analysis of the morphine concentration–response curves indicated a main effect of deltorphin II ($F_{1,24}$ = 4.32; P < 0.05) and naltrindole ($F_{1,28}$ = 19.11; P < 0.001) in SDU rats.

Co-immunoprecipitation experiments

Synaptosomal μ-opioid receptors were immunoprecipitated with a specific biotinylated anti-μ-opioid receptor antibody. When the immunoprecipitate was probed with a different anti-μ receptor antibody, a major band of ~60 kDa was apparent. Minor bands of MWs ranging from 47 to 250 kDa were also evident. The 47 kDa corresponds to non-glycosylated μ receptors (Rodríguez-Muñoz *et al.*, 2011). The higher MW bands represent different degrees of glycosylation (Garzón *et al.*, 1995; 2005). Only the major band was analysed. No differences in the amount of this band between strains were observed (Figure 9A,B). The antibody used precipitated almost all μ receptors, as a second immunoprecipitation of the unbound synaptosomal extract revealed practically no μ

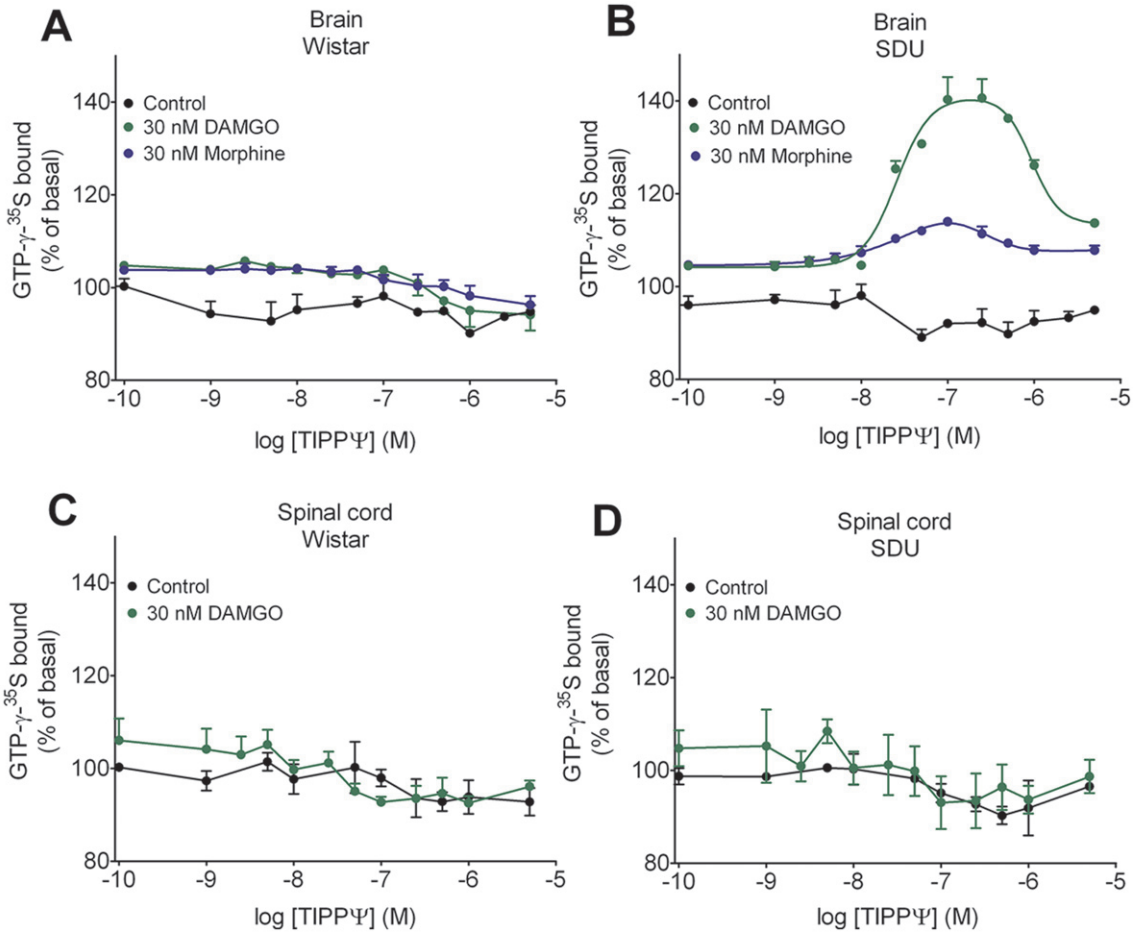

Figure 7

Effect of increasing concentrations of the δ-opioid receptor antagonist TIPPΨ on the binding of [^{35}S]GTP-γ-S in brain membranes from Wistar (A) and SDU rats (B) in the absence or presence of 30 nM DAMGO and 30 nM morphine. Effect of increasing concentrations of TIPPΨ on the binding of [^{35}S]GTP-γ-S in spinal cord membranes from Wistar (C) and SDU rats (D) in the absence or presence of 30 nM DAMGO. The results are expressed as the means ± SEM of percentage of the basal binding from three to six experiments performed in triplicate. ED_{50} and E_{max} values are given in Table 4 and in the text.

Table 5

Parameters of the concentration–response curves for morphine and deltorphin II, and morphine in the presence of deltorphin II or naltrindole in the inhibition of cAMP production in brain membranes from Wistar and SDU rats

Drug	ID_{50}		E_{max}	
	Wistar	SDU	Wistar	SDU
Morphine	498 ± 82	493 ± 98	60.3 ± 7.5	55.9 ± 5.0
Deltorphin II	847 ± 189	1920 ± 392	48.6 ± 4.2	52.9 ± 5.6
Morphine + Deltorphin II	983 ± 247	94.2 ± 15	51.3 ± 4.5	52.1 ± 2.8
Morphine + Naltrindole	382 ± 50	*n.a.*	31.6 ± 1.2	*n.a.*

n.a., not applicable. The activity of adenylyl cyclase was determined as described in Methods. Values shown represent mean ± SEM of three experiments conducted in triplicate. ID_{50} values are expressed in nM. E_{max} values are expressed as the percentage of the adenylyl cyclase activity stimulated by 50 μM forskolin.

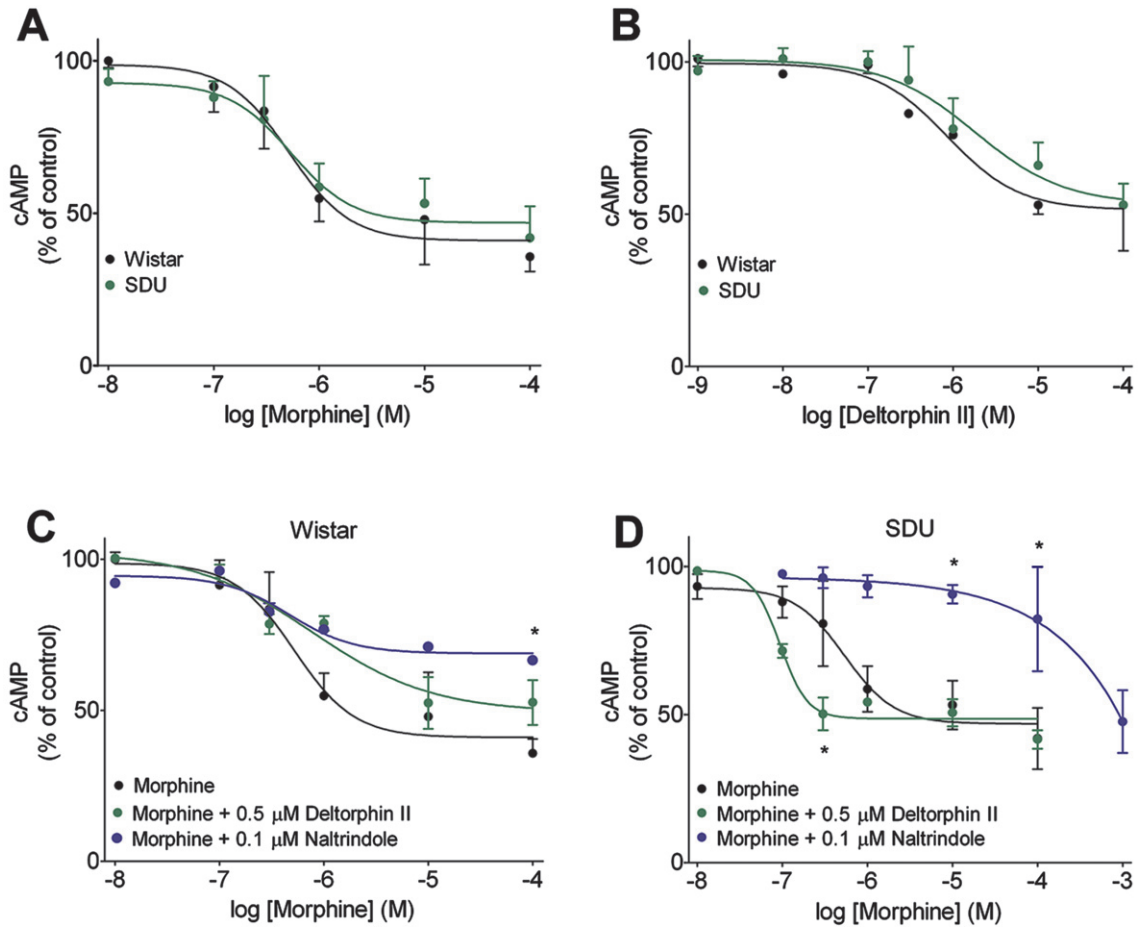

Figure 8

Inhibition of forskolin-stimulated cAMP production induced by increasing concentrations of morphine (A) and deltorphin II (B) in brain membranes from Wistar and SDU rats. Inhibition of forskolin-stimulated cAMP production induced by increasing concentrations of morphine in the absence or presence of 0.5 μM deltorphin II or 0.1 μM naltrindole in homogenates of brain from Wistar (C) and SDU (D) rats. The results are expressed as means ± SEM of the percentage of forskolin-stimulated adenylyl cyclase activity in the absence of drugs from three experiments performed in triplicate. *$P < 0.05$, significantly different from morphine alone, Bonferroni's test. ED_{50} and E_{max} values are given in Table 5.

receptors (Figure 9C,D). When immunoprecipitated synaptosomal μ receptors were probed with an anti-δ receptor antibody, a major band of 58 kDa was observed. Minor bands ranging from 50 to 165 kDa were also evident. Given that it has been demonstrated that the high MW bands represent different degrees of glycosylation (Garzón *et al.*, 2005), only the major band was analysed. The amount of the δ receptor 58 kDa band associated with immunoprecipitated μ receptor was significantly higher in SDU than in Wistar rats (Figure 9E,F). Immunodepletion of δ receptors before the immunoprecipitation of μ receptors reduced the intensity of the major band of μ receptors in both strains. A larger reduction was evident in SDU compared with Wistar, but this difference was not statistically significant (Figure 9G,H).

To confirm the results of immunoprecipitation with the μ receptor antibody, a parallel set of experiments immunoprecipitating δ receptors with a specific biotinylated δ receptor antibody was performed. When the immunoprecipitated δ receptors were probed with a different anti-δ receptor antibody, a major band of 58 kDa and minor bands ranging from 37 to 165 kDa were observed. As before, only the major 58 kDa band was analyzed and there were no differences between strains in the extent of this δ- receptor band (Figure 10A,B). The antibody used precipitated almost all δ receptors, as a second immunoprecipitation of the unbound synaptosomal extract revealed practically no δ receptors (Figure 10C,D). In agreenment with the earlier experiments, the extent of the μ receptor 60 kDa band associated with immunoprecipitated δ receptors was significantly higher in SDU than in Wistar rats (Figure 10E,F). Immunodepletion of μ receptors before the immunoprecipitation of δ receptors resulted in a significantly higher reduction of the 58 kDa band δ receptors in SDU than in Wistar rats (Figure 10G,H).

Discussion

The most significant finding presented here is that δ-opioid receptors determine the sensitivity to the anti-nociceptive effects of μ-opioid receptor agonists. Using different

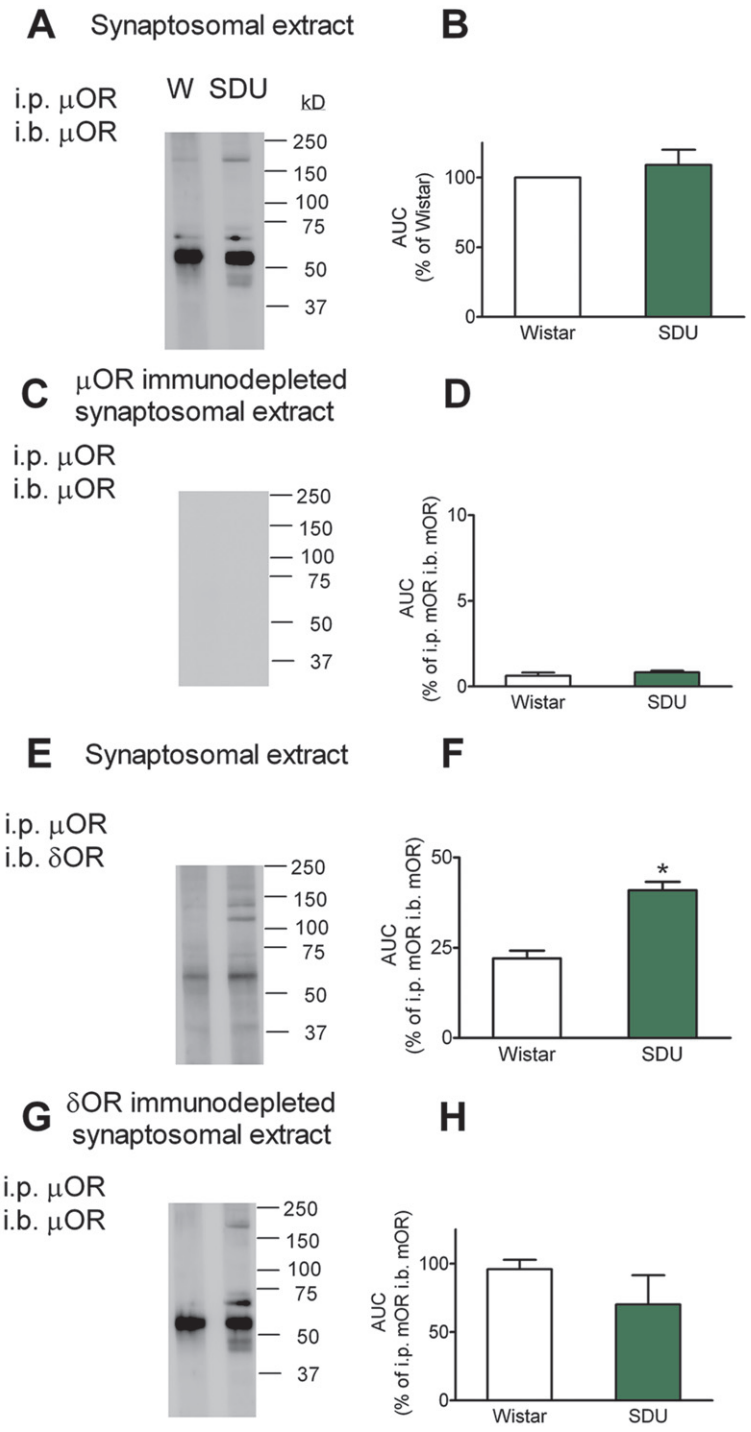

Figure 9

Western blots of immunoprecipitated μ-opioid receptors (μOR) from solubilized synaptosomal fraction of Wistar and SDU rats. (A) μ -opioid receptors were immunoprecipitated (i.p.) with a biotinylated anti-μ receptor antibody and probed (i.b.) with a different anti-μ receptor antibody. (B) The levels of μ-opioid receptors in SDU were normalized to the levels in Wistar rats. (C) The μ receptor-immunodepleted synaptosomal fraction was immunoprecipitated with a biotinylated anti-μ receptor antibody and probed with a different anti-μ receptor antibody. (D) For each strain, the levels of μ receptor in the μ receptor-immunodepleted synaptosomal fraction were normalized to total levels of μ receptors. (E) The μ receptors were immunoprecipitated with a biotinylated anti-μ receptor antibody, and probed with an anti-δ receptor antibody. (F) For each strain, the levels of δ receptors were normalized to μ receptors. (G) The δ receptor-immunodepleted synaptosomal fraction was immunoprecipitated with a biotinylated anti-μ receptor antibody and probed with a different anti-μ receptor antibody. (H) For each strain, the levels of μ receptors in the δ receptor-immunodepleted synaptosomal fraction were normalized to total levels of μ receptors. Experiments ($n = 3$) were performed and analyzed as described in Methods. Results are expressed as the means ± SEM. A representative experiment is shown in panels A, C, E and G. *$P < 0.05$, significantly different from Wistar rats, Student's *t*-test.

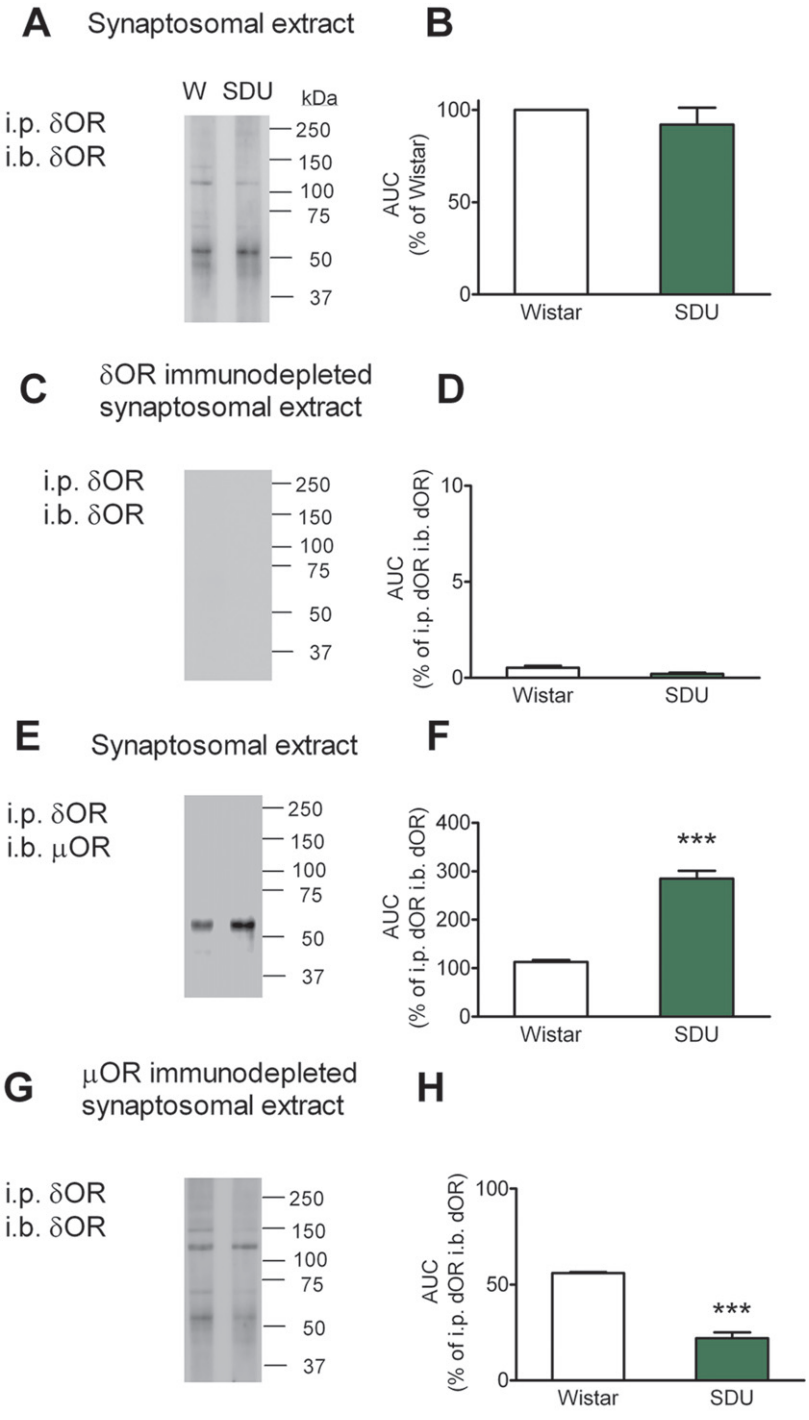

Figure 10

Western blots of immunoprecipitated δ-opioid receptors (δOR) from solubilized synaptosomal fraction of Wistar and SDU rats. (A) The δ receptors were immunoprecipitated (i.p.) with a biotinylated anti-δ receptor antibody and probed (i.b.) with a different anti-δ receptor antibody. (B) The levels of δ receptors in SDU were normalized to Wistar. (C) The δ receptor-immunodepleted synaptosomal fraction was immunoprecipitated with a biotinylated anti-δ receptor antibody and probed with a different anti-δ receptor antibody. (D) For each strain, the levels of δ receptor in the δ receptor-immunodepleted synaptosomal fraction were normalized to total levels of δ receptor. (E) The δ receptors were immunoprecipitated with a biotinylated anti-μ receptor antibody, and probed with an anti-μ receptor antibody. (F) For each strain, the levels of μ receptors were normalized to δ receptors. (G) The μ receptor-immunodepleted synaptosomal fraction was immunoprecipitated with a biotinylated anti-δ receptor antibody and probed with a different anti-δ receptor antibody. (H) For each strain, the levels of δ receptors in the μ receptor-immunodepleted synaptosomal fraction were normalized to total levels of δ receptors. Experiments (n = 3) were performed and analyzed as described in Methods. Results are expressed as the means ± SEM. A representative experiment is shown in panels A, C, E and G. ***P < 0.001, significantly different from Wistar rats, Student's t test.

experimental approaches, we have provided evidence that the extent of the interactions between μ and δ-opioid receptors varies between two strains of rats that show different sensitivities to the anti-nociceptive effect of μ receptor agonists.

We have demonstrated an enhanced anti-nociceptive potency of agonists of μ receptors, but not δ receptors, in SDU rats when compared with Wistar rats, in nociceptive tests. It could be argued that the excitatory effects of SNC80 (Jutkiewicz *et al.*, 2004) could bias the apparent anti-nociception. Nonetheless, the fact that, at all the doses of SNC80 tested, similar results were obtained in both strains strongly suggests that the anti-nociceptive effect induced by δ receptor agonists did not differ between SDU and Wistar rats. Differences in the anti-nociceptive effect induced by morphine or fentanyl were already evident at low doses (0.6 $mg \cdot kg^{-1}$ and 10 $\mu g \cdot kg^{-1}$ respectively). Hence, SDU rats displayed enhanced anti-nociception that was mediated by μ receptors. The higher sensitivity to morphine and the highly selective μ receptor agonist fentanyl, shown by SDU rats depends on δ receptor modulation of the μ receptor-mediated anti-nociception because, in the presence of the selective δ receptor antagonist naltrindole, the anti-nociception induced by morphine or fentanyl in SDU rats was restored to values that resembled those in Wistar rats. Naltrindole did not alter the anti-nociception induced by morphine and fentanyl in Wistar rats. The dose of naltrindole used here is selective for δ-opioid receptor antagonism (Portoghese *et al.*, 1988; Ossipov *et al.*, 1994), although at higher doses, naltrindole has agonist effects at δ- and κ-opioid receptors (Stapelfeld *et al.*, 1992; Takemori *et al.*, 1992; Stewart and Hammond, 1994). Therefore, our data suggest that a mechanism that involves activation of δ receptors underlies the enhanced sensitivity of SDU rats to anti-nociception induced by μ receptor agonists. Several reports have suggested that anti-nociceptive activity mediated by μ receptors can be modulated by δ receptors. In the present study, the modulation observed is positive. This has also been described by others (Lee *et al.*, 1980; Vaught *et al.*, 1982; Ward and Takemori, 1983; Heyman *et al.*, 1989; Negri *et al.*, 1995; Gomes *et al.*, 2004). Other reports suggest a negative regulation (Lee *et al.*, 1980; Heyman *et al.*, 1989; Schiller *et al.*, 1999; Gomes *et al.*, 2004; Guan *et al.*, 2005; He *et al.*, 2011). Many factors could account for these apparently conflicting results, such as the ligands, route of administration, level of the interaction, animal species or experimental approach.

Interactions between δ- and μ-opioid receptors were evident in radioligand-binding, stimulation of [^{35}S]GTP-γ–S binding and inhibition of adenylyl cyclase activity in brain membranes of SDU but not Wistar rats. Labelled antagonists are useful to detect changes in the binding properties of agonists. They have the same affinity towards different states of receptors and allow the detection of any change in affinity of competing agonists (Georgoussi *et al.*, 1993; Garzón *et al.*, 1998). In Wistar brain membranes, μ receptor agonists inhibited the specific binding of the δ receptor antagonist [^{3}H] naltrindole with low affinity. In SDU, an additional high-affinity binding site was apparent. In spinal cord, only one low affinity binding site was detected in both strains. The δ/μ receptor selectivity of naltrindole and the μ/δ receptor selectivity of CTAP are nearly identical in the brain membranes of both strains. Thus, the possible binding of [^{3}H] naltrindole to μ receptors, inhibited by μ receptor agonists in the brain of SDU rats, but not Wistar, can be ruled out.

In the brains of SDU, but not Wistar, rats the binding of [^{35}S]GTP-γ-S induced by the δ receptor agonist deltorphin II and the δ receptor antagonist TIPPΨ was enhanced in the presence of low concentrations of μ receptor agonists, suggesting a cross-regulation between μ and δ receptors in SDU rats. Naltrindole inhibited the binding of [^{35}S]GTP-γ-S induced by high concentrations of μ receptor agonists with low affinity in Wistar brain membranes. In SDU brain membranes, an additional high-affinity binding site was apparent. In SDU brains, the ratio of the IC_{50}s of the low and high-affinity phases was ~170. This ratio is similar to the δ/μ receptor selectivity ratio of naltrindole for the inhibition of GTP-γ–^{35}S binding to δ and μ receptors cloned in COS cells (143-fold) (Xu *et al.*, 2001). In contrast to a previous study, the results obtained in spinal cord membranes did not show any significant interaction in the binding of [^{35}S]GTP-γ–S induced by δ receptor ligands in the presence of a μ receptor agonist (Gomes *et al.*, 2004). The use of different animal species may explain this discrepancy.

We also examined the interaction of δ and μ receptors on the inhibition of forskolin-stimulated adenylyl cyclase activity by morphine. The δ receptor agonist deltorphin II increased the potency of morphine only in SDU rats. The δ receptor antagonist naltrindole decreased the E_{max} of morphine in Wistar rats. In contrast, in SDU rats in the presence of 100 nM naltrindole morphine, at concentrations up to 100 μM, did not have any effect on forskolin-stimulated cAMP production. It has been reported that 100 nM naltrindole attenuates the inhibition of forskolin-stimulated cAMP production induced by 1 μM DAMGO only in some brain areas (Izenwasser *et al.*, 1993). Thus, the effect of 100 nM naltrindole in Wistar rats was probably due to its binding to μ receptors. In SDU rats, additional mechanisms, probably through δ receptors, could be involved. These results indicate that interactions between δ and μ receptors *in vivo* modifies signal transduction in our model. Taken together, these experiments, as well as the radioligand and [^{35}S]GTP-γ-S binding experiments, provide a molecular basis for the greater anti-nociceptive effect of μ receptor agonists in SDU rats and its reversal by a δ receptor antagonist.

Classically, it was thought that a particular effect mediated by a drug or other ligand resulted from its interaction with a single type of receptor. However, there is increasing evidence that interactions between receptors play a major role in the actions of opioids (Smith and Lee, 2003). Since the late 1970s, compelling data from functional and radioligand binding studies have suggested that μ and δ receptors can interact (Vaught and Takemori, 1979). There is evidence for modulation of μ receptor-mediated analgesia by δ receptor agonists (Vaught *et al.*, 1982), allosteric coupling between μ and δ receptors (Rothman *et al.*, 1993), co-localization of opioid receptors in some neurons (Ji *et al.*, 1995) and the possible existence of a μ-δ receptor complex (Schoffelmeer *et al.*, 1990; Gomes *et al.*, 2004; Garzón *et al.*, 2005). The functional role of these interactions upon co-expression of both opioid receptors is also evident *in vitro* (Snook *et al.*, 2008), as well as in mice lacking the μ receptor (Matthes *et al.*,

1998) or the δ receptor (Zhu *et al.*, 1999; Scherrer *et al.*, 2004). In mice lacking δ-opioid receptors, δ receptor agonists still produce supraspinal analgesia, suggesting the existence of a second δ receptor-like analgesic system (Zhu *et al.*, 1999) or μ receptor-dependent anti-nociception (Scherrer *et al.*, 2004). In our study, the different effect of naltrindole on anti-nociception induced by μ receptor agonists in SDU and Wistar rats cannot be attributed to the binding of naltrindole to μ receptors in the SDU strain. The μ and δ receptors can coexist in a complex with a series of signalling elements such as G-proteins and RGS proteins (Garzón *et al.*, 2005). We found, in co-immunoprecipitation experiments, that the μ receptors were associated with δ receptors to a greater extent in SDU than in Wistar rats. Whether such association results from direct specific interactions, is promoted indirectly by scaffolding proteins or results from the high density of receptors within given lipid microdomains remain to be established (Snook *et al.*, 2006).

It has been reported that μ-δ-opioid receptor heteromers generate distinct pharmacological properties, such as the enhancement of the μ receptor binding and signalling by δ receptor antagonists (Gomes *et al.*, 2004). We detected some association of μ receptors with δ receptors in brains of Wistar rats, yet no functional or pharmacological evidence was observed in anti-nociception, radioligand binding or signal transduction. Thus, the differences between Wistar and SDU rats cannot be explained simply by the absence of μ-δ-receptor heteromers in Wistar and their presence in SDU rats. In the CNS, opioid receptors exist in a complex with G-proteins, RGS proteins, the HINT1 protein and scaffolding proteins. The affinity of agonists to μ-opioid receptors depends on the nature of G-protein coupled to the μ receptor (Garzón *et al.*, 1998). RGS proteins regulate the effect of opioid receptor agonists. For instance, knockdown of the RGS7 protein, a member of the R7 subfamily of RGS proteins, enhances the anti-nociceptive potency of μ- and δ-opioid receptor agonists (Garzón *et al.*, 2003). HINT1 forms homodimers in cells and these dimers act in the cross-regulation between NMDA receptors and μ-opioid receptors (Rodríguez-Muñoz *et al.*, 2011). The possible interaction of different signalling proteins with μ receptors could underlie the increased sensitivity to μ receptor agonists in SDU rats. This interaction and its regulation by δ receptors should be further explored.

In conclusion, we report here an *in vivo* animal model in which the degree of μ- and δ-opioid receptor cross-regulation at supraspinal level was correlated with the sensitivity to the anti-nociceptive effect of μ-opioid receptor agonists. *In vivo* interactions between exogenous μ-opioid receptor agonists and endogenous δ-opioid receptor peptides may produce the differential pharmacological effects reported here in the nociceptive tests. The lack of correlation between the *in vivo* sensitivity to opioids and baseline results of the *in vitro* assays could be explained by the absence of endogenous peptides in the *in vitro* assays. Provided that the results presented here are relevant to the clinical setting, one future challenge is to determine which factors influence the degree of μ- and δ-opioid receptor cross-regulation. This strategy, together with the identification of human biomarkers for opioid sensitivity, could represent the next step in pain relief.

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Conflicts of interest

There are no conflicts of interest in this work.

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