Chronic treatment with the opioid antagonist naltrexone favours the coupling of spinal cord µ-opioid receptors to Gαz protein subunits

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ABSTRACT

Sustained administration of opioid antagonists to rodents results in an enhanced antinociceptive response to agonists. We investigated the changes in spinal µ-opioid receptor signalling underlying this phenomenon. Rats received naltrexone (120 µg/h; 7 days) via osmotic minipumps. The antinociceptive response to the µ-agonist sufentanil was tested 24 h after naltrexone withdrawal. In spinal cord samples, we determined the interaction of µ-receptors with Gαz proteins (agonist-stimulated [35S]GTPγS binding and immunoprecipitation of [35S]GTPγS-labelled Gαz subunits) as well as µ-opioid receptor-dependent inhibition of the adenylyl cyclase (AC) activity. Chronic naltrexone treatment augmented DAMGO-stimulated [35S]GTPγS binding, potentiated the inhibitory effect of DAMGO on the AC/cAMP pathway, and increased the inverse agonist effect of naltrexone on cAMP accumulation. In control rats, the inhibitory effect of DAMGO on cAMP production was antagonized by pertussis toxin (PTX) whereas, after chronic naltrexone, the effect became resistant to the toxin, suggesting a coupling of µ-receptors to PTX-insensitive Gαz subunits. Immunoprecipitation assays confirmed the transduction switch from Gαz to Gαq proteins. The consequence was an enhancement of the antinociceptive response to sufentanil that, in consonance with the neurochemical data, was prevented by Gαz-antisense oligodeoxyribonucleotides but not by PTX. Such changes in opioid receptor signalling can be a double-edged sword. On the one hand, they may have potential applicability to the optimisation of the analgesic effects of opioid drugs for the control of pain. On the other hand, they represent an important homeostatic dysregulation of the endogenous opioid system that might account for undesirable effects in patients chronically treated with opioid antagonists.

This article is part of a Special Issue entitled ‘Post-Traumatic Stress Disorder’.

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One of the best-characterised effector systems linked to the opioid receptor signalling cascade is the adenyl cyclase (AC)/cAMP pathway (Law et al., 2000). Following receptor activation, opioid drugs exert an inhibitory effect on AC activity through Gi subunits, resulting in reduced cAMP production (George et al., 2000; Laugwitz et al., 1993; Mostany et al., 2008). The AC/cAMP pathway has long been known to play a crucial role in the processing of painful stimuli, and studies have demonstrated an important role of several AC isoforms in inflammatory and neuropathic pain models, as well as in opioid-induced analgesia (see Pierre et al., 2009).

Chronic treatment with opioid ligands (agonists and antagonists) as well as other non-opioid drugs (for example, calcium channel blockers) critically modifies, quantitatively and qualitatively, opioid receptor signalling. This modification results in important changes in the pharmacological potency, efficacy and intrinsic activity of opioid drugs as well as in the quality of the elicited response (Bannister and Dickinson, 2010; Chang et al., 2007; Diaz et al., 2000; Dierssen et al., 1990; Gullapalli and Ramaraoo, 2002; Hurlé et al., 2000; Mostany et al., 2008; Santillán et al., 1998; Vanderah et al., 2001). In this context, the development of functional super-sensitivity to opioid agonists after long-term exposure to opioid receptor antagonists, such as naloxone, naltrexone and 6-fluoromethylxoxol, is a well-known phenomenon in rodents (Singh et al., 2007). For example, we previously reported that the antinoceptive and respiratory depressant potencies of μ-agonists are enhanced following interruption of long-term treatment with naltrexone in rats (Diaz et al., 2002). In most of the studies in the literature, this increased responsiveness to agonists has been correlated with opioid receptor up-regulation (Diaz et al., 2002; Lesscher et al., 2003; Patel et al., 2003; Siroti et al., 2007; Unterwald et al., 1995; Yoburn et al., 1986).

Sustained opioid receptor blockade by naltrexone is among the currently available treatments for substance abuse and dependence disorders, and the recently introduced long-acting, sustained-release formulations of naltrexone are considered to be promising strategies for the treatment of heroin (Krupitsky and Blokhina, 2010), alcohol (Anton, 2008; Ray et al., 2010) and nicotine (David et al., 2006) dependence. However, the advantages and disadvantages of these new therapies have not been systematically analysed.

The neurochemical adaptations produced by continued opioid antagonist treatment have scarcely been studied. Here, we further analyse the molecular mechanisms underlying the increased functional responsiveness to opioid agonists produced by sustained administration of antagonists in rats. We demonstrate that following long-term treatment with naltrexone, spinal μ-opioid receptors undergo a transductional shift from PTX-sensitive G_{i0} to PTX-resistant G_{i2} transducer proteins. Consequently, the inhibitory effect of agonists on the AC/cAMP effector pathway is enhanced. In addition, the population of constitutively active μ-receptors in the spinal cord appears to be increased. These neurochemical changes correlate with the pharmacological super-sensitivity to the antinoceptive effect of the μ-opioid agonist sufentanil.

### 2. Materials and methods

#### 2.1. Subjects

The experiments were carried out using male Sprague–Dawley rats weighing 250–300 g (Charles River, Harlan, Barcelona, Spain). The animals were housed in sawdust-lined cages in an environmentally controlled animal facility at 22 °C with a 12:12 h light–dark cycle and food/water provided ad libitum. This study was approved by the Cantabria University Institutional Laboratory Animal Care and Use Committee and performed in strict accordance with the “European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes” (European Union Directive #86/609/EEC).

#### 2.2. Pharmacological treatments

Diagrams showing the pharmacological treatment schedules are depicted in Supplementary figure S1. Chronic saline (1 μl/h) or naltrexone (120 μg/h) infusion was administered using Alzet 0110 osmotic minipumps (Alza Corporation, Palo Alto, CA, USA) that were implanted subcutaneously under light ether-induced anaesthesia (Figure S1A). These pumps delivered the solutions at a constant rate of 1 μl/h for 7 days. On day 7, the minipumps were removed, and the in vivo (antinociceptive response to the μ-opioid receptor agonist sufentanil) or in vitro assays (autoradiographic, [35S]GTPγS binding and adenyl cyclase studies) were carried out 24 h after withdrawal from a chronic saline or naltrexone treatment.

To interfere with the expression of G_{i2} proteins, we used a synthetic antisense oligodeoxynucleotide (ODN) that has previously been characterised (Sanchez-Blazquez et al., 1995; Serres et al., 2000). The sequence was 5′-CGATCATC-CAATCCCTGTCCTGGCCGGCGCAGT-3′. The ODN was phosphorothioate-modified at the two bases on each end. The sequence of the missense oligodeoxynucleotide was 5′-CCCTATTACCGCGCCGAC-3′, and it was phosphorothioate-modified at positions 5′-CC and GC-3′ (Sanchez-Blazquez et al., 1995; Serres et al., 2000). ODNs (5 μg/ml/μl) were administered twice intracerebroventricularly (i.c.v.) under light isoflurane-induced anaesthesia with a 24-h interval between administrations (Figure S1B).

The G_{i2}-antisense ODN injection was performed on days 5 and 7 for the rats receiving chronic naltrexone treatment (Figure S1C). The rats were challenged with sufentanil 24 h later to test whether the antinociceptive response elicited by activation of μ-opioid receptors was mediated by G_{i2}.

To prevent the activation of G_{i4},G_{i0} proteins, PTX was administered (1 μg/10 μl i.c.v.), and the antinociceptive response of sufentanil was tested 48 h later (Figure S1D). PTX injection was performed on day 6 to the rats receiving chronic naltrexone treatment (Figure S1E).

#### 2.3. Evaluation of nociception

The tail-flick test was used to assess the nociceptive threshold. A tail-flick response was elicited by applying radiant heat to the surface of the tail. The intensity of the stimulus was adjusted so that control latencies were within 3–5 s. A cut-off time of 10 s was established to avoid permanent injury. Tail-flick latencies were measured before the drug injection and 30 min after subcutaneous administration of sufentanil (0.1 or 1 μg/kg; Figure S1). This drug administration schedule was based on dose-response curves obtained in previous studies (Diaz et al., 2002).

#### 2.4. Autoradiography of μ-opioid agonist-stimulated [35S]GTPγS binding

[35S]GTPγS binding using tissue sections was performed as described previously (Sim et al., 1996; Mostany et al., 2008). Sections were first preincubated in assay buffer (50 mM Tris–HCl, 3 mM MgCl₂, 0.2 mM EDTA and 100 mM NaCl; pH 7.4) for 15 min at 25 °C, followed by a second 15-min preincubation in the same assay buffer containing 2 mM GDP and 10 μM/ml adenosine deaminase. Sections were then incubated for 2 h at 25 °C in assay buffer containing 1 mM DTT and 0.04 mM [35S]GTPγS. Consecutive sections were used to define basal binding (in the absence of the opioid agonist), stimulated binding (in the presence of agonist) and non-specific binding (without agonist and in the presence of 10 μM GTPγS) (Diaz et al., 2002). The μ-opioid selective agonist DAMGO was used at concentrations ranging from 10⁻⁴ to 10⁻⁶ M. After this incubation, slides were rinsed twice in cold Tris buffer (50 mM Tris–HCl; pH 7.4) for 15 min, dipped in distilled water and dried under an ice-cold air stream.

Tissue sections incubated with [35S]GTPγS were exposed to autoradiographic films (Kodak-MR films, GE Healthcare, Spain) along with [³⁵S]-radioactive microscales (GE Healthcare, Spain). In order to generate the autoradiograms, films were developed following a 48-h [³⁵S]GTPγS binding period. Autoradiographic densitometry was performed using Scion Image software (Scion Corporation, Maryland, USA). Autoradiographic values of net agonist-stimulated [³⁵S]GTPγS binding were calculated by subtracting basal binding from agonist-stimulated binding.

#### 2.5. Immunoprecipitation of [³⁵S]GTPγS-labelled Gα subunits

Spinal cord samples were homogenised [1:30 (w/v)] in ice-cold buffer (50 mM Tris–HCl 250 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, and 1 mM DTT; pH 7.4) using a motor-driven glass Teflon tissue potter (10 strokes, 1500 rpm). The homogenates were then centrifuged (1500 g, 5 min, 4 °C), and the resulting supernatants were centrifuged again (14000 rpm, 15 min, 4 °C). Resuspended pellets (500 μg protein/ml/assay) were incubated with 20 nM [³⁵S]GTPγS and 10 μM DAMGO in a final volume of 100 μl for 30 min at 30 °C. Non-specific binding was determined in the presence of 10 μM of GTPγS. Membrane suspensions were then solubilised on ice with a solution containing 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 2.5 mM CHAPS, 0.1 mM phenylmethylsulfonyl fluoride, 0.01 M aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml chymostatin and 10 μg/ml aprotinin (10 μg/ml). These membranes were incubated for 3 h at room temperature with 15 μl of specific rabbit anti-Gα_{i2}, anti-Gα_{i1,2} and anti-Gα_{i} antibodies immobilised to superparamagnetic Dynabeads® Protein A (overnight, 4 °C). After three washes with 1 ml of PBS, the beads were pelleted, and the bound radioactivity was counted in 4 ml of Ecolite scintillation cocktail. Antibody specificity was confirmed in our experimental
conditions by western blot analysis, as previously described (Mato et al., 2010). The amount of coupling of μ-opioid receptors to the diverse G-protein subunits induced by DAMGO (10−8 M) was expressed as percentage over the basal values in the absence of the agonist (100%).

2.6. Cyclic AMP assays

Ac assays were performed using spinal cord samples as described previously (Mostany et al., 2008). Samples were homogenised (1:50 weight/volume dilution) with a Teflon/glass grinder (10 strokes, 800 r.p.m.) in an ice-cold homogenisation buffer (20 mM Tris–HCl, 1 mM EDTA, 5 mM EDTA, 5 mM DTG, 25 mM leupeptin and 300 mM sucrose; pH 7.4). The homogenates were centrifuged at 1500 x g (5 min at 4 °C), and the resulting supernatants were centrifuged at 13,000 x g (15 min at 4 °C). The pellets were resuspended (120 μg protein/ml) in assay buffer (30 mM Tris–HCl, 0.2 mM EGTA, 1 mM EDTA, 2 mM MgCl2, 100 mM NaCl, 60 mM MgCl2 sucrose, 1 mM EDTA, 10 μM GTP, 0.5 mM IBMX, 5 mM phosphatase, 50 U/ml creatine phosphokinase, and 5 U/ml myokinase; pH 7.4) without (basal AC activity) or with 10 μM forskolin (FK) (FK-stimulated CAMP accumulation). Opioid receptor-mediated inhibition of FK-stimulated CAMP accumulation was determined using different concentrations of the agonist DAMGO (10−8−10−4 M). To test the effect of PTX on DAMGO-induced inhibition of FK-stimulated CAMP accumulation, samples were preincubated for 30 min with or without PTX (1 μg/ml) in buffer (25 mM Tris–HCl buffer containing 0.05% SDS, 10 mM DTT, 1 mM EDTA, 2.5 mM NAD, and 10 mM thymidine; pH 7.4, 30 °C). The inverse agonism of naltrexone (10−7−10−5 M) was analysed by measuring CAMP accumulation in the absence of NaCl and FK (Mato et al., 2002). The effects of selective opioid antagonists (β, β-funaltrexamine; δ, naltrindole; and κ, nornaltrexamine) added to the media at a concentration of 10−8 M were evaluated. Membranes under the different experimental protocols were preincubated for 5 min at 37 °C, then ATP was added to a final concentration of 200 μM and the mixture was incubated for 10 min at 37 °C. The reaction was stopped by boiling for 5 min, and the CAMP concentration was determined in a 50 μl sample of the supernatant using a commercial kit (Cyclic AMP [3H] assay system, Amersham Biosciences, Barcelona, Spain). Each CAMP assay was performed in triplicate, and the results are expressed as pmol CAMP/min/mg protein.

2.7. Drugs and chemicals

Subentanil was kindly provided by Janssen Cych, S.A. (Madrid, Spain), DAMGO, naltrindone and FK were purchased from Sigma (Madrid, Spain). The selective antagonists of μ-opioid receptors (β-funaltrexamine), δ- and κ-opioid receptors (naltrexone and nornaltrexone) were obtained from Tocris Bioscience (Biogen S.L., Madrid, Spain). [35S]GTPγS (1250 Ci/mmol) was purchased from Perkin Elmer (Madrid, Spain), PTX was purchased from Calbiochem (Roche Diagnostics, Barcelona, Spain). The ODNs were synthesised by Sigma-Genosys Ltd. (Cambridge, UK). Selective rabbit polyclonal antibodies against Gκ4, Gα12, and Gα13 subunits were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Representative immunoblots showing the specificity of the antibodies are shown in Supplementary figure S2.

2.8. Data analysis

Data analysis was performed using the GraphPad Prism statistical software package (GraphPad Software, Inc, San Diego, CA, USA). Data from the [35S]GTPγS binding and AC assays were fitted to sigmoidal concentration-response curves to determine potency (EC50 and IC50, respectively) and theoretical maximal effect (Emax) and its changes in the data (μ.E.M.). Statistical analysis was performed using Student’s t-tests and one-way ANOVA followed by the Newman–Keuls post-hoc test when appropriate. A p < 0.05 was considered to be statistically significant.

3. Results

3.1. Chronic naltrexone treatment increases DAMGO-induced [35S]GTPγS binding

Basal and agonist-induced μ-opioid receptor activation of G-proteins was determined by [35S]GTPγS binding using spinal cord sections. The basal level of [35S]GTPγS binding was not different between groups. However, animals chronically treated with naltrexone exhibited a significant increase in μ-opioid receptor-mediated stimulation of [35S]GTPγS binding. The maximal stimulatory effect induced by the selective μ-opioid agonist DAMGO on spinal cord [35S]GTPγS binding was significantly enhanced in comparison with saline-treated rats, as indicated by the Emax values determined from the concentration-response curves. In contrast, the potency between groups was not different (Table 1; Fig. 1). These data suggest that the μ-opioid receptor coupling to G-proteins was increased following chronic naltrexone treatment.

We also confirmed that chronic naltrexone treatment increased the specific binding of [3H]-DAMGO to μ-opioid receptors (Diaz et al., 2002), consistent with the reported up-regulation of μ-opioid receptors induced by chronic naltrexone treatment (Yoburn et al., 1986, 1995; Unterwald et al., 1995; Lesscher et al., 2003; Patel et al., 2003; Sirohi et al., 2007).

3.2. Chronic naltrexone treatment causes potentiation of μ-opioid agonist-induced inhibition of CAMP accumulation through a mechanism involving PTX-resistant G-proteins

Basal AC activity and the ability of the AC activator FK (10 μM) to increase CAMP levels were not altered following long-term administration of naltrexone in comparison with saline-treated animals (Table 1). In control rats, incubation of the spinal cord membranes with increasing concentrations (10−9−10−4 M) of the selective μ-opioid agonist DAMGO produced a concentration-dependent inhibition of FK-stimulated CAMP accumulation. Following long-term treatment with naltrexone, the maximal ability of FK to inhibit FK-stimulated CAMP accumulation was significantly enhanced with no change in potency (Table 1). PTX causes the ADP-ribosylation and inactivation of Gαi4 proteins, with the exception of Gαi2 (Casey et al., 1990). The presence of PTX in the medium did not modify either basal or FK-stimulated CAMP accumulation (Table 1, Fig. 2). However, in saline-treated animals, the maximal inhibitory effect of DAMGO was almost completely prevented by PTX pretreatment, suggesting the involvement of Gαi4 proteins. On the contrary, in the group of animals chronically treated with naltrexone, PTX did not antagonise the maximal inhibitory effect of DAMGO on FK-stimulated CAMP accumulation, suggesting the involvement of Gαi2 proteins (Fig. 2).

3.3. Chronic naltrexone treatment increases μ-opioid receptor coupling to Gα2 protein subunits

To further assess the existence of specific changes in the coupling of μ-opioid receptors to the PTX-resistant Gα2 subunits, we performed immunoprecipitation of DAMGO-activated [35S]GTPγS-labelled Gz protein subunits.

In spinal cord homogenates from animals treated with chronic naltrexone, the coupling of μ-opioid receptors to Gz proteins was increased (Table 1), suggesting the involvement of Gα2 proteins in the mechanism of action of naltrexone.

<table>
<thead>
<tr>
<th>Type of assay</th>
<th>Saline</th>
<th>Chronic naltrexone</th>
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<tbody>
<tr>
<td>Autoregulatory density of [3H]-DAMGO binding (fmol/mg tissue)</td>
<td>42.42 ± 4.15</td>
<td>75.42 ± 7.52**</td>
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<tr>
<td>[35S]GTPγS binding autoradiography</td>
<td></td>
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<tr>
<td>Basal binding (nci/mg tissue)</td>
<td>250.22 ± 40.12</td>
<td>278.23 ± 24.12</td>
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<tr>
<td>DAMGO-stimulated binding</td>
<td></td>
<td></td>
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<tr>
<td>Emax (nci/mg tissue)</td>
<td>538.20 ± 48.01</td>
<td>736.5 ± 39.75**</td>
</tr>
<tr>
<td>pEC50 (–log EC50)</td>
<td>5.89 ± 0.35</td>
<td>5.86 ± 0.14</td>
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<tr>
<td>Adenylyl cyclase (AC) activity</td>
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<tr>
<td>Basal AC activity (pmol/min/mg protein)</td>
<td>21.30 ± 3.90</td>
<td>27.50 ± 3.79</td>
</tr>
<tr>
<td>Forskolin-induced CAMP accumulation (pmol/min/mg protein)</td>
<td>599.61 ± 25.34</td>
<td>553.98 ± 43.79</td>
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<tr>
<td>DAMGO inhibition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I50 (pmol/min/mg protein)</td>
<td>462.42 ± 14.28</td>
<td>389.44 ± 29.36*</td>
</tr>
<tr>
<td>pEC50 (–log EC50)</td>
<td>6.48 ± 0.47</td>
<td>6.88 ± 0.16</td>
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</table>

Emax represents DAMGO-induced maximal stimulation of [35S]GTPγS binding; I50 represents DAMGO-induced maximal inhibition of forskolin-induced CAMP accumulation. Values are given as means ± S.E.M. of data from 6 to 7 animals/group. *p < 0.05 and **p < 0.01 vs. saline-treated group (un-paired t test).
significantly increased in comparison with saline-treated animals (183.0 ± 13.3% vs. 140.1 ± 9.0% of basal binding; p < 0.05). Western blot analysis of spinal cord samples revealed no change in the expression of any Gα subunit after chronic naltrexone treatment (see methods and Figure S3 in the supplementary information). However, as shown in Fig. 3, the DAMGO-induced activation of Gα subunits was significantly increased in chronic naltrexone-treated animals in comparison with the saline group (301.6 ± 39.9% vs. 170.7 ± 10.2% of basal binding; p < 0.05). Significant differences in the coupling with Gαo (194.4 ± 7.7% vs. 172.6 ± 5.8%) and Gα1,2,3 (178.9 ± 7.3% vs. 160.6 ± 9.6%) were not observed between naltrexone- and saline-treated animals.

3.4. Potentiation of μ-opioid antinociception following withdrawal from chronic naltrexone involves Gαz proteins

The functional relevance of the transduction switch from Gα1,2,3 to Gαz proteins was assessed by analysing the consequences of PTX or Gαz-antisense ODN pretreatment on the antinociceptive response to sufentanil. Under baseline conditions (Fig. 4A), sufentanil, acutely administered at the dose of 1 μg/kg (n = 10), produced an antinociceptive response that almost reached the MPE; this effect was prevented by PTX (1 μg/10 μl, i.c.v.) administered 48 h beforehand (n = 5). In contrast, pretreatment with Gαz-antisense ODN (5 μg/10 μl, two i.c.v. injections on alternate days; n = 5) did not significantly modify the effect of sufentanil. Neither saline nor missense ODN administered i.c.v. modified the tail-flick basal response or sufentanil-induced antinociception. These results indicate that sufentanil-induced antinociception in naïve animals is dependent on the interaction of μ-opioid receptors with PTX-sensitive Gα1,2,3 transducer proteins rather than PTX-insensitive Gαz subunits.

In the rats that received the chronic naltrexone treatment (Fig. 4B), the antinociceptive response of sufentanil (0.1 μg/kg; n = 5) was significantly potentiated, confirming “in vivo” the development of functional super-sensitivity to the antinociceptive effect of sufentanil. PTX injected on the 6th day of chronic naltrexone infusion did not prevent the development of opioid super-sensitivity. On the other hand, Gαz-antisense ODN injected i.c.v. on days 5 and 7 of the chronic naltrexone infusion completely prevented the development of super-sensitivity to the antinociceptive response elicited by sufentanil. The reduction in the expression levels of Gαz proteins in the dorsal horn of the spinal cord

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**Fig. 1.** Effect of chronic naltrexone treatment on DAMGO-induced [35S]GTPγS binding. Left: Concentration-response curves of DAMGO-stimulated [35S]GTPγS binding using spinal cord sections from animals chronically treated for 7 days with saline and naltrexone (120 μg/10 μl). Values (mean ± S.E.M.) represent specific [35S]GTPγS binding in nCi/mg tissue. Right: Representative autoradiographic illustrations showing basal (A and B) and 10 μM DAMGO-stimulated (C and D) [35S]GTPγS binding in spinal cord sections from animals chronically treated for 7 days with saline (A and C) and naltrexone (B and D). Note the enhanced response to DAMGO following withdrawal from chronic naltrexone in the outer layers of the dorsal horn (laminae I and II). Abbreviations: DH, dorsal horn; VH, ventral horn.

**Fig. 2.** Effect of pertussis toxin (PTX) on DAMGO-induced inhibition of FK-stimulated cAMP accumulation in spinal cord homogenates from rats chronically treated with saline or naltrexone. Data represent the mean ± S.E.M. PTX prevented opioid-induced inhibition of FK-stimulated cAMP accumulation in spinal cord homogenates from saline- but not naltrexone-treated rats (***p < 0.01 and ****p < 0.01 vs. FK).

**Fig. 3.** Selective [35S]GTPγS labelling of Gα0, Gα1,2,3 and Gαz protein subunits activated by the μ-opioid agonist DAMGO in the spinal cord homogenates from rats chronically treated with saline or naltrexone. G-protein subunits were isolated using antibodies against each subtype immobilised to superparamagnetic Dynabeads. Data represent the mean ± S.E.M. of the percent bound relative to basal binding (100%) for each specific G-protein subunit (*p < 0.05 vs. saline (Newman–Keuls post-ANOVA)). Note the selective increase of [35S]GTPγS labelling of Gαz following chronic administration of naltrexone.
induced by Gαz-antisense ODN treatment was confirmed by western blotting experiments (Fig. 5). Overall, these results indicate that following withdrawal from chronic naltrexone, the antinociceptive response mediated by μ-opioid receptor activation involved Gαz-transducer proteins.

3.5. Chronic naltrexone treatment increases the constitutive activity of μ-opioid receptors

Incubation of spinal cord membranes from saline-treated rats with increasing concentrations of naltrexone induced a concentration-dependent increase in the levels of cAMP ($E_{\text{max}} = 29.1 \pm 0.7 \text{ pmol/min/mg}$; $pEC_{50} = 4.2 \pm 0.3$). This inverse agonist action of naltrexone was potentiated after chronic administration of naltrexone because the maximal cAMP production appeared significantly enhanced ($E_{\text{max}} = 35.3 \pm 0.8$, $p < 0.01$ vs. saline-treated group; $pEC_{50} = 4.1 \pm 0.2$, $p = \text{NS}$), indicating the existence of constitutively active opioid receptors that uncovered the inverse agonist effect of naltrexone (Fig. 6A).

To determine the subtype of opioid receptor that exhibited constitutive activity, the effect of naltrexone on cAMP levels was evaluated in the presence of selective antagonists to μ- (b-funaltrexamine), δ- (naltrindole) and κ- (nor-binaltorphimine) receptors at a concentration of $10^{-4} \text{ M}$. The effect of each antagonist alone was examined in parallel, and only nor-binaltrophimine increased cAMP levels (data not shown), confirming its reported inverse agonism (Wang et al., 2007). As shown in Fig. 6B, b-funaltrexamine antagonised the naltrexone-induced cAMP increase both in the saline-treated group (109.1 ± 2.9% vs. the effect of naltrexone alone; $p < 0.05$) and in the chronic naltrexone group (114.4 ± 0.4% vs. 147.2 ± 2.2% in the absence of b-funaltrexamine; $p < 0.05$). Naltrindole ($10^{-4} \text{ M}$) did not modify the naltrexone-induced cAMP increase in any group. The same concentration of naltrindole antagonised the binding of $[35S]GTP\gamma S$ induced by the δ-specific agonist DSLET (Figure S4). Nor-binaltrophimine not only was unable to antagonise but also increased naltrexone-induced cAMP accumulation in both the saline group (142.2 ± 2.1%; $p < 0.01$ vs. the effect of naltrexone alone) and the chronic naltrexone group (165.3 ± 10.1%; $p < 0.05$ vs. the effect of naltrexone alone). Furthermore, immunoprecipitation assays that were carried out using spinal cord samples from saline and chronic naltrexone-treated animals demonstrated the absence of naltrexone-induced coupling with Gαz (104 ± 8.1%). Overall, our data support the interaction of naltrexone with constitutively active μ-opioid receptors.
4. Discussion

Functional super-sensitivity to opioid agonists induced by sustained exposure to antagonists is a well-known phenomenon in rodents. Most studies addressing the underlying mechanisms have focused on the up-regulation of opioid receptors in the CNS subsequent to blockade (Diaz et al., 2002; Lesscher et al., 2003; Patel et al., 2003; Sirohi et al., 2007; Unterwald et al., 1995; Yoburn et al., 1986, 1995). Treatment with naltrexone clearly induces the up-regulation of μ- and, to a lesser extent, δ- and κ-opioid receptors throughout the brain, with differences in the percent change across various brain regions (Lesscher et al., 2003; Yoburn et al., 1995). Furthermore, the increase in the maximal stimulatory effect of DAMGO on the spinal cord binding of \[^{35}\text{S}\]GTPγS indicates the existence of enhanced coupling between μ-opioid receptors and their cognate G-proteins.

Regarding intracellular effectors, one of the best-characterised signalling cascades linked to opioid receptor activation is the AC/cAMP pathway (Law et al., 2000). This pathway has long been known to play a crucial role in processing nociception. In addition to opioids, other pharmacological agents with analgesic properties exert an inhibitory influence on this pathway (Pierre et al., 2009). In agreement with previous reports (George et al., 2000; Laugwitz et al., 1993; Mostany et al., 2008), we observed that PTX-sensitive Ga subunits were the preferential transducers linking μ-opioid receptor activation to the AC/cAMP pathway in naïve animals. Following chronic naltrexone treatment, the inhibitory effect of DAMGO on the AC/cAMP pathway was significantly potentiated. However, under these experimental conditions, the effect was not prevented by PTX, in contrast to the saline-treated group. Thus, our data indicate that following chronic treatment with antagonists, μ-opioid receptors underwent a shift in the transduction of their signal, showing a higher efficiency of interaction with PTX-resistant over PTX-sensitive Ga proteins. A likely transducer candidate is Ga₂ζ, which is the only Ga subunit resistant to PTX (Casey et al., 1990) that inhibits AC (Kozasa and Gilman, 1995; Mostany et al., 2008). Consistent with this assumption, the immunoprecipitation data indicated that the coupling of μ-opioid receptors to Ga₂ζ subunits was augmented following withdrawal from chronic naltrexone, whereas the coupling to Ga₃ζ and Ga₅ζ subunits remained similar to that observed in saline-treated rats.

Although the present study provides no information about the mechanisms that could explain why the switch from Ga₃ζ to Ga₂ζ transducer proteins resulted in an enhancement of the opioid inhibitory effect on the AC/cAMP effector pathway, several observations led us to propose some putative mechanisms for such a phenomenon. First, the rate of Ga₂ζ-GTP hydrolysis is as much as 200-fold slower than that determined for other G-protein α subunits. This extremely slow rate of GTP hydrolysis would then result in a long-lasting signal (Casey et al., 1990; Jeong and Ikeda, 1998). Second, the inhibitory Gaζ subunits differ in their specificity for individual AC isoforms. For example, it has been suggested that the relatively high affinity of Ga₂ζ for AC type V, together with its slow GTase activity, might account for its capacity to induce strong AC inhibition in cultured cells (Ammers and Christ, 2002). Finally, Ga₂ζ may be difficult to switch off after receptor activation unless external factors, such as RGS-Rζ proteins, accelerate the rate of Ga₂ζ-GTP hydrolysis. In particular, RGS22 plays an important role in controlling μ-opioid signalling induced by Ga₂ζ transducer proteins (Garzón et al., 2005). Thus, it may be feasible that, following chronic naltrexone treatment, an inadequate control of Ga₂ζ activity may lead to strong inhibition of the AC/cAMP pathway.

The functional relevance of the particular transducer protein linking μ-opioid receptor activation to the AC/cAMP signalling pathway is strengthened by our data that demonstrate the close relationship between agonist-activated signalling “in vitro” and agonist-induced pharmacological effects “in vivo”. Thus, as observed in the AC assay, sufentanil-induced antinociception in naïve rats was prevented by PTX but not by Ga₂ζ-antisense ODN, indicating the involvement of Ga₂ζζ transducer proteins. In contrast, following withdrawal from chronic naltrexone treatment, the switch from Ga₂ζζ to Gaζ proteins appeared to be responsible for the enhanced antinociceptive response to μ-opioid agonists because sufentanil-induced antinociception was prevented by Ga₂ζζ-antisense ODN but not by PTX pretreatment.

Aside from Gaζ transducer proteins and the AC/CAMP pathway, chronic treatment with naltrexone could have additional consequences on other elements linked to μ-opioid receptor signalling that were not analysed in this study. In this regard, voltage-gated Ca\(^{2+}\) channels and G-protein-coupled inwardly rectifying K\(^+\) channels are fundamental determinants of opioid-induced antinociception.
(Law et al., 2000; Heinke et al., 2011), whose modulation by G_{z} has been described in several reports (see Ho and Wong, 2001). Moreover, G_{z}-subunits broadly regulate Kir3 channels, voltage-gated Ca^{2+} channels, phospholipase C{\beta}, and several isoforms of AC, among other effectors (Dupr{\acute{e}} et al., 2009).

In addition to chronic naltrexone treatment, a number of pharmacological interventions induce analogous signalling plasticity on {\mu}-opioid receptors with similar functional consequences. In this regard, we previously reported that 7 days of combined treatment with nimodipine (L-type calcium channel blocker) and sufentanil prevents the development of tolerance and strongly enhances the antinociception in rats (Diaz et al., 2000; Dierssen et al., 1990; Hurl{\acute{e}} et al., 2000; Mostany et al., 2008). The underlying mechanism involved efficient inhibition of CAMP production associated with a change in {\mu}-opioid receptor-preferred G-protein coupling from PTX-sensitive G_{z} to PTX-resistant G_{z} subunits (Mostany et al., 2008). Changes in sensitivity to agonists have also been reported to occur upon heterodimerisation of opioid receptors. Studies using cultured cells provide evidence that {\delta}-selective antagonists enhance {\mu}-opioid receptor signalling through a mechanism involving the formation of {\mu}-{\delta}-hetero-oligomeric signalling units and a subsequent switch in opioid receptor preference for G_{z} over G_{z} subunits, which are preferentially activated by individually expressed {\mu}- and {\delta}-receptors (Fan et al., 2005; George et al., 2000; Hashi et al., 2007). Experiments in vivo demonstrate that this change in opioid receptor transduction leads to increased {\mu}-receptor binding and signalling activity and to an enhancement of morphine antinociceptive potency in mice (Abul-Husn et al., 2007; Comes et al., 2004). Taken together, these findings suggest that conditions favouring the coupling of {\mu}-opioid receptors to G_{z}-protein subunits would increase agonist-induced AC/cAMP signalling pathways, leading to an enhancement of the pharmacological responses.

Another relevant adaptive response prompted by sustained opioid receptor blockade arises from the observation that the inverse agonist effect of naltrexone on the AC activity was significantly potentiated. Opioid receptors, similar to other G-protein-coupled receptors, may exhibit spontaneous constitutive activity even in the absence of agonists (Sadee et al., 2005). It has also been reported that antagonists, such as naloxone and naltrexone, display inverse agonist activity when the population of constitutively active opioid receptors increases, which is typically more prominent following chronic treatment with opioid agonists (Liu and Prather, 2001; Wang et al., 2001, 2007). On the other hand, the in vitro inverse agonist activity of naltrexone and other putative {\mu}-inverse agonists has been questioned by Divin et al., 2009. These authors observed that, under chronic treatment and the subsequent rapid removal of opioid agonist, cells expressing {\mu}-opioid receptors exhibit an enhanced CAMP accumulation not linked to the formation of constitutively active {\mu}-opioid receptors.

Our present results demonstrate for the first time in native tissue that the inverse agonism of naltrexone, reflected by CAMP accumulation, occurs after sustained treatment with opioid antagonists. Furthermore, our findings support the fact that the stimulatory effect of naltrexone on CAMP accumulation was mediated by {\mu}-opioid receptors. In addition, immunoprecipitation assays indicated the lack of involvement of G_{z} subunits in this effect, demonstrating that naltrexone could not induce the coupling of {\mu}-opioid receptors to these stimulatory subunits. Considering that receptor over-expression leads to a proportional increase in the number of spontaneously active receptors (see Leurs et al., 1998), constitutive signalling may be enhanced after withdrawal from chronic naltrexone treatment as a consequence of {\mu}-opioid receptor up-regulation. However, sensitisation of the receptor to the effects of inverse agonists cannot be ruled out (Divin et al., 2008; Liu and Prather, 2001; Wang et al., 2007).

Interestingly, we observed a potentiation of naltrexone inverse agonism by the {\kappa}-opioid antagonist nor-binaltorphimine. In this regard, Wang et al. (2007) demonstrated that naltrexone has inverse agonist properties at {\mu}- but not at {\delta}- and {\kappa}-opioid receptors in cultured cells over-expressing opioid receptors. In this study and in our study (data not shown), nor-binaltorphimine exhibited inverse agonist activity at {\kappa}-receptors. Thus, such an increase in cAMP accumulation induced by naltrexone in the presence of nor-binaltorphimine may be explained by the sum of their respective inverse agonist effects on {\mu}- and {\kappa}-receptors.

5. Conclusions

Following long-term treatment with naltrexone, {\mu}-receptors in the spinal cord experienced a transduction shift from PTX-sensitive G_{z} and G_{z}3,4,2,3,6 proteins to PTX-resistant G_{z} proteins. As a result, the inhibitory effect of the {\mu}-agonist DAMGO on the AC/cAMP effector pathway was enhanced. In addition, constitutively active {\mu}-opioid receptor expression, and possibly {\kappa}-opioid receptor expression, in the spinal cord appeared to be increased. The functional consequence of these neurochemical changes is the development of pharmacological super-sensitivity to the antinociceptive effect of {\mu}-receptor agonists, such as sufentanil. Such changes in opioid receptor signalling activity can be a double-edged sword. On the one hand, they may have potential applicability to the optimisation of the analgesic effects of opioid drugs for the control of pain. On the other hand, they represent an important homeostatic dysregulation of the endogenous opioid system that might account for undesirable paradoxical pharmacological effects in patients chronically treated with certain opioid antagonists.

Conflicts of interest

None.

Acknowledgements

This work was supported by grants from: Instituto de Salud Carlos III (RTICs: RD06/001/1016 and RD06/001/1006) and Ministerio de Ciencia e Innovaci{\acute{o}}n (SAF2007/65451, SAF2007/61862 and SAF2010/16894). We wish to thank Ms Beatriz Romero, Ms Rebeca Madureira and Ms Susana Dawalibi for their technical assistance.

Appendix. Supplementary material

Supplementary material related to this article can be found at doi:10.1016/j.neuropharm.2011.08.029.

References


