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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 Gα proteins (agonist-stimulated [35S]GTPγS binding and immunoprecipitation of [35S]GTPγS-labelled Gα 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 Gαi/o to Gαz 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.

Cover letter

Dear Editor,

I am pleased to send you a revised version of the manuscript “Chronic treatment with the opioid antagonist naltrexone favours the coupling of spinal cord μ-opioid receptors to $G\alpha_z$ protein subunits” in which the new questions raised by the reviewer 1 have been incorporated, as outlined below in detail.

We hope that you will find the revised version suitable for publication in Neuropharmacology.

Thank you for your consideration.

Best regards,

María A Hurlé

Reviewer 1

-Since the authors performed an important positive control experiment (antagonism of delta opioid receptor agonist-mediated activation of G proteins by naltrindole) this figure should be accessible to the readers, maybe as a supplementary figure, rather than indicating "data not shown", p.16 line 8.

The figure was included in the manuscript as supplementary figure S4

-Evidence of downregulation of Galphaz following intracerebroventricular administration of ODN should be shown by western-blotting on spinal cord extracts (to support the behavioral result shown in the new figure 4).

We performed western blotting experiments that shown a down-regulation of Galphaz protein content in the spinal cord from animals treated with Galphaz-ONA (p15, lines 8-10). A new figure (Fig 5) showing these results was included.

***Response to Reviewers**

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***Title page**

Chronic treatment with the opioid antagonist naltrexone favours the coupling of spinal cord μ-opioid receptors to $G\alpha_z$ 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 Gα proteins (agonist-stimulated [^{35}S]GTPγS binding and immunoprecipitation of [^{35}S]GTPγS-labelled Gα subunits) as well as μ-opioid receptor-dependent inhibition of the adenylyl cyclase (AC) activity. Chronic naltrexone treatment augmented DAMGO-stimulated [^{35}S]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 $G\alpha_z$ subunits. Immunoprecipitation assays confirmed the transduction switch from $G\alpha_{i/o}$ to $G\alpha_z$ proteins. The consequence was an enhancement of the antinociceptive response to sufentanil that, in consonance with the neurochemical data, was prevented by $G\alpha_z$ 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

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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 Gα proteins (agonist-stimulated [^{35}S]GTPγS binding and immunoprecipitation of [^{35}S]GTPγS-labelled Gα subunits) as well as μ-opioid receptor-dependent inhibition of the adenylyl cyclase (AC) activity. Chronic naltrexone treatment augmented DAMGO-stimulated [^{35}S]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 $G\alpha_z$ subunits. Immunoprecipitation assays confirmed the transduction switch from $G\alpha_{i/o}$ to $G\alpha_z$ proteins. The consequence was an enhancement of the antinociceptive response to sufentanil that, in consonance with the neurochemical data, was prevented by $G\alpha_z$ 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.

Keywords: Opioid antagonist, Opioid receptors, G-protein, adenylyl cyclase, constitutive activity, inverse agonism

Abbreviations:

AC: Adenyly cyclase

DAMGO: Tyr-D-Ala-Gly-Me-Fe-Gly-ol-enkephalin

FK: Forskolin

ODN: oligodeoxyribonucleotides

PTX: Pertussis toxin

1. Introduction

Opioid drugs produce their pharmacological effects by interacting with specific G-protein–coupled receptors (namely μ-, δ- and κ-opioid receptors) (Snyder and Pasternak, 2003). The functional interaction of opioid receptors with the pertussis toxin (PTX)–sensitive $G\alpha_{i1,2,3}$ and $G\alpha_o$ transducers as well as PTX-resistant $G\alpha_z$ has been clearly demonstrated in heterologous expression systems, neural cell lines and in the CNS (Chan et al., 1995; Chalecka-Franaszek et al., 2000; Tso and Wong, 2000). In animal models, evidence that $G\alpha_{i1,2,3}$, $G\alpha_o$, $G\alpha_z$ and $G\alpha_q$ can contribute to μ-opioid signalling and antinociception was provided by studies using PTX, specific antisera or antisense oligodeoxyribonucleotides against specific G-protein subunits and null transgenic mouse strains (Sánchez-Blázquez et al., 1995; Standifer et al., 1996; Garzón et al., 1998; Sánchez-Blázquez et al., 2001; Hendry et al., 2000; Yang et al., 2000; Yoburn et al., 2003; Mostany et al., 2008; Lamberts et al., 2011). In addition to the specificity of receptor–G-protein coupling, different selective agonists for a specific-receptor can induce different modes of ligand-receptor interaction, and the particular activation pattern of G-protein subtypes determines the intrinsic activity of the agonist for the elicited biological response (Garzón et al., 1998; Sánchez-Blázquez et al., 2001; Valdizán et al., 2010).

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 $G\alpha_i$ 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 Dickenson, 2010; Chang et al., 2007; Dierssen et al., 1990; Gullapalli and Ramarao, 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 6β-naltrexol, is a well-known phenomenon in rodents (Sirohi et al., 2007). For example, we previously reported that the antinociceptive and respiratory depressant potencies of μ-agonists are enhanced following interruption of long-term treatment with naltrexone in rats (Díaz et al., 2002). In most of the studies in the literature, this increased responsiveness to agonists has been correlated with opioid receptor up-regulation (Díaz et al., 2002; Lesscher et al., 2003; Patel et al., 2003; Sirohi 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 $G\alpha_{i/o}$ to PTX-resistant $G\alpha_z$ 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 antinociceptive effect of the μ-opioid agonist sufentanil.

2. Material 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 #86/606/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 2001 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, [^{35}S]GTPγS binding and adenylyl cyclase studies) were carried out 24 h after withdrawal from a chronic saline or naltrexone treatment.

To interfere with the expression of $G\alpha_z$ 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′-CGTGATCTCACCCTTGCTCTCTGCCGGGCCAGT-3′. The ODN was phosphorothioate-modified at the two bases on each end. The sequence of the missense oligodeoxynucleotide was 5′-CCCTTATTTACTTTCGCC-3′, and it was phosphorothioate-modified at positions 5′-CC and GC-3′ (Sánchez-Blázquez et al., 1995; Serres et al., 2000). ODNs (5 μg/10 μl) were administered twice intracerebroventricularly (i.c.v) under light isofluorane-induced anaesthesia with a 24-h interval between administrations (figure S1 B). The $G\alpha_z$-antisense ODN injection was performed on days 5 and 7 for the rats receiving chronic naltrexone treatment (figure S1 C). 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 $G\alpha_z$.

To prevent the activation of $G\alpha_{i1,2,3}$ / $G\alpha_o$ proteins, PTX was administered (1 μg/10 μl, i.c.v.), and the antinociceptive response of sufentanil was tested 48 h later (figure S1

D). PTX was injected on day 6 to the rats receiving chronic naltrexone treatment (figure S1 E).

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 latency was within 3 to 5 sec. A cut-off time of 10 sec 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 (Díaz et al., 2002).

2.4. Autoradiography of μ-opioid agonist-stimulated [^{35}S]GTPγS binding

[^{35}S]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 EGTA and 100 mM NaCl; pH 7.4) for 15 min at 25ºC, followed by a second 15-min preincubation in the same assay buffer containing 2 mM GDP and 10 mU/ml adenosine deaminase. Sections were then incubated for 2 h at 25ºC in assay buffer containing 1 mM DTT and 0.04 nM [^{35}S]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) (Díaz et al., 2002). The μ-opioid selective agonist DAMGO was used at concentrations ranging from 10^{-10} to 10^{-4} 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 [^{35}S]GTPγS were exposed to autoradiographic films (Kodak-MR films, GE Healthcare, Spain) along with [^{14}C]-radioactive microscales (GE Healthcare, Spain). In order to generate the autoradiograms, films were developed following a 48-h ([^{35}S]GTPγS binding period. Autoradiographic densitometry was performed using Scion Image software (Scion Corporation, Maryland, USA). Autoradiographic values of net agonist-stimulated [^{35}S]GTPγS binding were calculated by subtracting basal binding from agonist-stimulated binding.

2.5. Immunoprecipitation of [^{35}S]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 x g, 5 min, 4°C), and the resulting supernatants were centrifuged again (14,000 rpm, 15 min, 4°C). Resuspended pellets (500 μg protein/ml/assay) were incubated with 20 nM [^{35}S]GTPγS and 10 μM DAMGO in a final volume of 100 μl for 30 min at 30ºC. Non-specific binding was determined in the presence of 10 μM of GTPγS. Membrane suspensions were then solubilised on ice with in a solution containing 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 2.5 mM CHAPS, 0.1 mM phenylmethylsulfonylfluoride, 0.01 M aprotinin, 1 μg/ml leupeptin, 1 μg/ml pestatin, 1 μl/ml antipain, and 10 μg/ml chymostatin for 30 min. Solubilised membranes were incubated for 3 h at room temperature with 15 μl of specific rabbit anti-Gα_o, anti-Gα_{i1-2-3}, and anti-Gα_z antibodies immobilised to superparamagnetic Dynabeads® Protein A (overnight, 4ºC). 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., 2009). The amount of coupling of μ-opioid receptors to the diverse G protein subunits induced by DAMGO (10^{-4} 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 (1:60 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 EGTA, 5 mM EDTA, 5 mM DTT, 25 μM leupeptin and 300 mM sucrose; pH 7.4). The homogenates were centrifuged at 1,500 x g (5 min at 4ºC), and the resulting supernatants were centrifuged at 13,000 x g (15 min at 4ºC). The pellets were resuspended (120 μg protein/ml) in assay buffer (80 mM Tris-HCl, 0.2 mM EGTA, 1 mM EDTA, 2 mM $MgCl_2$, 100 mM NaCl, 60 mM sucrose, 1 mM DTT, 10 μM GTP, 0.5 mM IBMX, 5 mM phosphocreatine, 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^{-9} to 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 μM NAD, and 10 mM thymidine; pH 7.4, 30ºC). The inverse agonism of naltrexone (10^{-7} to 10^{-3} M) was analysed by measuring cAMP accumulation in the absence of NaCl and FK (Mato et al., 2002). The effects of selective opioid antagonists (μ, β-funaltrexamine; δ, naltrindole; and κ, *nor*-binaltorphimine) added to the media at a concentration of 10^{-4} M were evaluated.

Membranes under the different experimental protocols were preincubated for 5 min at 37ºC, then ATP was added to a final concentration of 200 μM and the mixture was incubated for 10 min at 37ºC. 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 [^{3}H] 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

Sufentanil was kindly provided by Janssen Cylag, S.A. (Madrid, Spain). DAMGO, naltrexone and FK were purchased from Sigma (Madrid, Spain). The selective antagonists of μ-opioid receptors (β-funaltrexamine), δ-opioid receptors (naltrindole) and κ-opioid receptors (*nor*-binaltorphimine) were obtained from Tocris Bioscience (Biogen S.L., Madrid, Spain). [^{35}S]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 $G\alpha_o$, $G\alpha_{i1,2,3}$, and $G\alpha_z$ 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 [^{35}S]GTPγS binding and AC assays were fitted to sigmoidal concentration-response curves to determine potency (EC_{50} and IC_{50}, respectively) and theoretical maximal effect (E_{max} and I_{max}, respectively). The EC_{50} and IC_{50} values were normalised as -logEC_{50} (pEC_{50}) and -

$\log IC_{50}$ (pIC_{50}) for statistical comparison. Data represent the mean ± standard error of the mean (S.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 [^{35}S]GTPγS binding

Basal and agonist-induced μ-opioid receptor activation of G-proteins was determined by [^{35}S]GTPγS binding using spinal cord sections. The basal level of [^{35}S]GTPγS binding was not different between groups. However, animals chronically treated with naltrexone exhibited a significant increase in μ-opioid receptor-mediated stimulation of [^{35}S]GTPγS binding. The maximal stimulatory effect induced by the selective μ-opioid agonist DAMGO on spinal cord [^{35}S]GTPγS binding was significantly enhanced in comparison with saline-treated rats, as indicated by the E_{max} values determined from the concentration-response curves. In contrast, the potency between groups was not different (table 1; figure 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 ^{3}H-DAMGO to μ-opioid receptors (Díaz 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} to 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 DAMGO to inhibit FK-induced cAMP accumulation was significantly enhanced with no change in potency (table 1). PTX causes the ADP-ribosylation and inactivation of $G\alpha_{i/o}$ proteins, with the exception of $G\alpha_z$ (Casey et al., 1990). The presence of PTX in the medium did not modify either basal or FK-stimulated cAMP accumulation (table 1, figure 2). However, in saline-treated animals, the maximal inhibitory effect of DAMGO was almost completely prevented by PTX pretreatment, suggesting the involvement of $G\alpha_{i/o}$ 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-induced cAMP accumulation, suggesting the involvement of $G\alpha_z$ proteins (figure 2).

3.3. Chronic naltrexone treatment increases μ-opioid receptor coupling to $G\alpha_z$ protein subunits

To further assess the existence of specific changes in the coupling of μ-opioid receptors to the PTX-resistant $G\alpha_z$ subunits, we performed immunoprecipitation of DAMGO-activated [^{35}S]GTPγS-labelled Gα protein subunits.

In spinal cord homogenates from animals treated with chronic naltrexone, the coupling of μ-opioid receptors to Gα proteins was 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 figure 3, the DAMGO-induced activation of $G\alpha_z$ 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 $G\alpha_o$ (194.4 ± 7.7% *vs.* 172.6 ± 5.8%) and $G\alpha_{i1\text{-}2\text{-}3}$ (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 $G\alpha_z$ proteins

The functional relevance of the transduction switch from $G\alpha_{i/o}$ to $G\alpha_z$ proteins was assessed by analysing the consequences of PTX or $G\alpha_z$-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 $G\alpha_z$-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 $G\alpha_{i/o}$ transducer proteins rather than PTX-insensitive $G\alpha_z$ subunits.

In the rats that received the chronic naltrexone treatment (figure 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, $G\alpha_z$-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 $G\alpha_z$–proteins in the dorsal horn of the spinal cord induced by $G\alpha_z$-antisense ODN treatment was confirmed by western blotting experiments (Figure 5). Overall, these results indicate that following withdrawal from chronic naltrexone, the antinociceptive response mediated by µ-opioid receptor activation involved $G\alpha_z$-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_{max} = 29.1 ± 0.7 pmol/min/mg; pEC_{50} = 4.2 ± 0.3). This inverse-agonist action of naltrexone was potentiated after chronic administration of naltrexone because the maximal cAMP production appeared significantly enhanced (E_{max}= 35.3 ± 0.8, $p < 0.01$ *vs.* saline-treated group; pEC_{50} = 4.1 ± 0.2; p= NS), indicating the existence of constitutively active opioid receptors that uncovered the inverse-agonist effect of naltrexone (figure 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- (β-funaltrexamine), δ- (naltrindole) and κ- (*nor*-binaltorphimine)

receptors at a concentration of 10^{-4} M. The effect of each antagonist alone was examined in parallel, and only n*or*-binaltrophimine increased cAMP levels (data not shown), confirming its reported inverse agonism (Wang et al., 2007). As shown in figure 6B, β-funaltrexamine antagonised the naltrexone-induced cAMP increase both in the saline-treated group (109.1 ± 2.9% *vs.* 121.1 ± 5.4% in the absence of β-funaltrexamine; $p<0.05$) and in the chronic naltrexone-treated group (114.4 ± 0.4% *vs.* 147.2 ± 2.2% in the absence of β-funaltrexamine; $p<0.01$). Naltrindole (10^{-4}M) did not modify the naltrexone-induced cAMP increase in any group. The same concentration of naltrindole antagonised the binding of [^{35}S]GTPγS 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 $G\alpha_s$ (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 (Díaz 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 per cent 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}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 $G\alpha_i$ subunits were the preferential transducers linking μ-opioid receptor activation to the AC/cAMP pathway in naive 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 Gα proteins. A likely transducer candidate is $G\alpha_z$, which is the only $G\alpha$ 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 $G\alpha_z$ subunits was augmented following withdrawal from chronic naltrexone, whereas the coupling to $G\alpha_o$ and $G\alpha_{i1\text{-}2\text{-}3}$, 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 $G\alpha_i$ to $G\alpha_z$ 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 $G\alpha_z$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 $G\alpha$ subunits differ in their specificity for individual AC isoforms. For example, it has been suggested that the relatively high affinity of $G\alpha_z$ for AC type V, together with its slow GTPase activity, might account for its capacity to induce strong AC inhibition in cultured cells (Ammer and Christ, 2002). Finally, $G\alpha_z$ may be difficult to switch off after receptor activation unless external factors, such as RGS-Rz proteins, accelerate the rate of $G\alpha_z$GTP hydrolysis. In particular, RGSZ2 plays an important role in controlling μ-opioid signalling induced by $G\alpha_z$ transducer proteins (Garzón et al., 2005). Thus, it may be feasible that, following chronic naltrexone treatment, an inadequate control of $G\alpha_z$ 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 naive rats was prevented by PTX but not by $G\alpha_z$ antisense ODN, indicating the involvement of $G\alpha_{i/o}$ transducer proteins. In contrast, following withdrawal from chronic naltrexone treatment, the switch from $G\alpha_{i/o}$ to $G\alpha_z$ proteins appeared to be responsible for the enhanced antinociceptive

response to μ-opioid agonists because sufentanil-induced antinociception was prevented by $G\alpha_z$ antisense ODN but not by PTX pretreatment.

Aside from Gα 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 $G\alpha_z$ has been described in several reports (see Ho and Wong, 2001). Moreover, $G_{\beta\gamma}$-subunits broadly regulate Kir3 channels, voltage-gated Ca^{2+} channels, phospholipase Cβ, and several isoforms of AC, among other effectors (Dupré et al., 2008).

In addition to chronic naltrexone treatment, a number of pharmacological interventions induce analogous signalling plasticity on μ-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 (Dierssen et al., 1990; Hurlé et al., 2000; Mostany et al., 2008). The underlying mechanism involved efficient inhibition of cAMP production associated with a change in μ-opioid receptor–preferred G-protein coupling from PTX-sensitive $G\alpha_i$ to PTX-resistant $G\alpha_z$ 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 δ-selective antagonists enhance μ-opioid receptor signalling through a mechanism involving the formation of μ–δ hetero-oligomeric signalling units and a subsequent switch in opioid-receptor preference for $G\alpha_z$ over $G\alpha_i$ subunits, which are preferentially activated by individually expressed μ-

and δ-receptors (Fan et al., 2005; George et al., 2000; Hasbi et al., 2007). Experiments in vivo demonstrate that this change in opioid-receptor transduction leads to increased μ-receptor binding and signalling activity and to an enhancement of morphine antinociceptive potency in mice (Abul-Husn et al., 2007; Gomes et al., 2004). Taken together, these findings suggest that conditions favouring the coupling of μ-opioid receptors to $G\alpha_z$-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 (Sadée 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; Wang et al., 2007). On the other hand, the *in vitro* inverse agonist activity of naltrexone and other putative μ-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 μ-opioid receptors exhibit an enhanced cAMP accumulation not linked to the formation of constitutively active μ-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 μ-opioid receptors. In addition, immunoprecipitation assays indicated the lack of involvement of $G\alpha_s$ subunits in this effect, demonstrating that naltrexone could not induce the coupling of μ-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 μ-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 κ-opioid antagonist *nor*-binaltorphimine. In this regard, Wang et al. (2007) demonstrated that naltrexone has inverse agonist properties at μ- but not at δ- and κ-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 κ-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 μ- and κ-receptors.

5. Conclusions

Following long-term treatment with naltrexone, μ-receptors in the spinal cord experienced a transduction shift from PTX-sensitive $G\alpha_o$ and $G\alpha_{i1\text{-}2\text{-}3}$ proteins to PTX-resistant $G\alpha_z$ proteins. As a result, the inhibitory effect of the μ-agonist DAMGO on the AC/cAMP effector pathway was enhanced. In addition, constitutively active μ-opioid receptor expression, and possibly κ-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 μ-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.

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Figure legends

Figure 1. Effect of chronic naltrexone treatment on DAMGO-induced [^{35}S]GTPγS binding. **Left:** Concentration–response curves of DAMGO-stimulated [^{35}S]GTPγS binding using spinal cord sections from animals chronically treated for 7 days with saline and naltrexone (120 µg/h). Values (mean ± S.E.M.) represent specific [^{35}S]GTPγS binding in nCi/mg tissue. **Right:** Representative autoradiographic illustrations showing basal (A and B) and 10 µM DAMGO-stimulated (C and D) [^{35}S]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.

Figure 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).

Figure 3. Selective [^{35}S]GTPγS labelling of $G\alpha_o$, $G\alpha_{1,2,3}$ and $G\alpha_z$ 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 per cent 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 [^{35}S]GTPγS labelling of $G\alpha_z$ following chronic administration of naltrexone.

Figure 4. Antinociceptive effect of sufentanil in the tail-flick test. Naive rats (**A**) and rats chronically treated with naltrexone (**B**) were challenged with sufentanil after i.c.v pretreatment with saline, pertussis toxin (PTX; 1 µg/10 µl), $G\alpha_z$ antisense oligodeoxynucleotide (ODN; 2 x 5 µg/10 µl) or missense ODN (2 x 5 µg/10 µl). Data represent the mean ± S.E.M. of the per cent antinociception relative to the maximal possible effect (100%) (**$p < 0.01$ vs. saline; ***$p < 0.001$ vs. baseline; $^{\#\#}p < 0.001$ vs. saline (Newman-Keuls post-ANOVA).

Figure 5. Effect of $G\alpha_z$ antisense oligodeoxynucleotide on the levels of $G\alpha_z$ proteins in the dorsal horn of the spinal cord. Representative immunoblots and integrated optical density (OD) of the bands show a down-regulation of $G\alpha_z$ proteins following antisense ODN, both in saline and naltrexone (NTX) treated rats. Data represent the mean ± S.E.M. from four animals per group of the per cent OD relative to missense ODN. (*$p < 0.05$ vs missense ODN; Newman-Keuls post-ANOVA).

Figure 6. Naltrexone-induced cAMP accumulation in spinal cord homogenates from animals chronically treated for 7 days with saline or naltrexone (120 µg/h) and selective opioid antagonists. (**A**): Concentration–response curves of naltrexone-induced cAMP accumulation (pmol/min/mg protein); (**B**) Antagonism of naltrexone-induced cAMP accumulation by the selective antagonists of μ- (β-funaltrexamine), δ- (naltrindole) and κ- (*nor*-binaltrophimine) receptors (percentage with respect to the

basal value, 100%). Data represent the mean ± S.E.M. Note the increase in the naltrexone inverse agonist effect following chronic administration of the opioid antagonist, which is completely abolished by treatment with the selective μ-opioid antagonist β-funaltrexamine, supporting the interaction of naltrexone with constitutively active μ-opioid receptors (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ *vs.* basal value of cAMP level (Newman-Keuls post-ANOVA); $^{+}p < 0.05$ and $^{++}p < 0.01$ *vs.* naltrexone (Newman-Keuls post-ANOVA).

Table 1

Effect of chronic administration of naltrexone (120 μg/hr, 7 days) in μ-opioid receptor density and functionality, and on adenylyl cyclase (AC) activity

Type of assay	**Saline**	**Chronic naltrexone**
Autoradiographic density of [^{3}H]-DAMGO binding (fmol/mg tissue)	42.42±4.15	75.42±7.52**
[^{35}S]GTPγS binding autoradiography		
Basal binding (nCi/mg tissue)	250. 22±40.12	278. 23±24.12
DAMGO-stimulated binding		
Emax (nCi/mg tissue)	538.20±48.01	736.5±39.75**
pEC_{50} (-log EC_{50})	5.89±0.35	5.86±0.14
Adenylyl cyclase (AC) activity		
Basal AC activity (pmol/min/mg protein)	21.30±3.90	27.50±3.79
Forskolin-induced cAMP accumulation (pmol/min/mg protein)	599.61±25.34	553.98±43.79
DAMGO inhibition	462,42±14.28	389.44±29,36*
Imax (pmol/min/mg protein)	6.48±0.47	6.88±0.16
pIC_{50} (-log IC_{50})		

Emax represents DAMGO-induced maximal stimulation of [^{35}S]GTPγS binding; Imax represents DAMGO-induced maximal inhibition of forskolin-induced cAMP-accumulation. Values are given as means±S.E.M. of data from 6-7 animals/group. *p< 0.05 and **p< 0.01 vs saline-treated group (un-paired *t* test).

Figure 1

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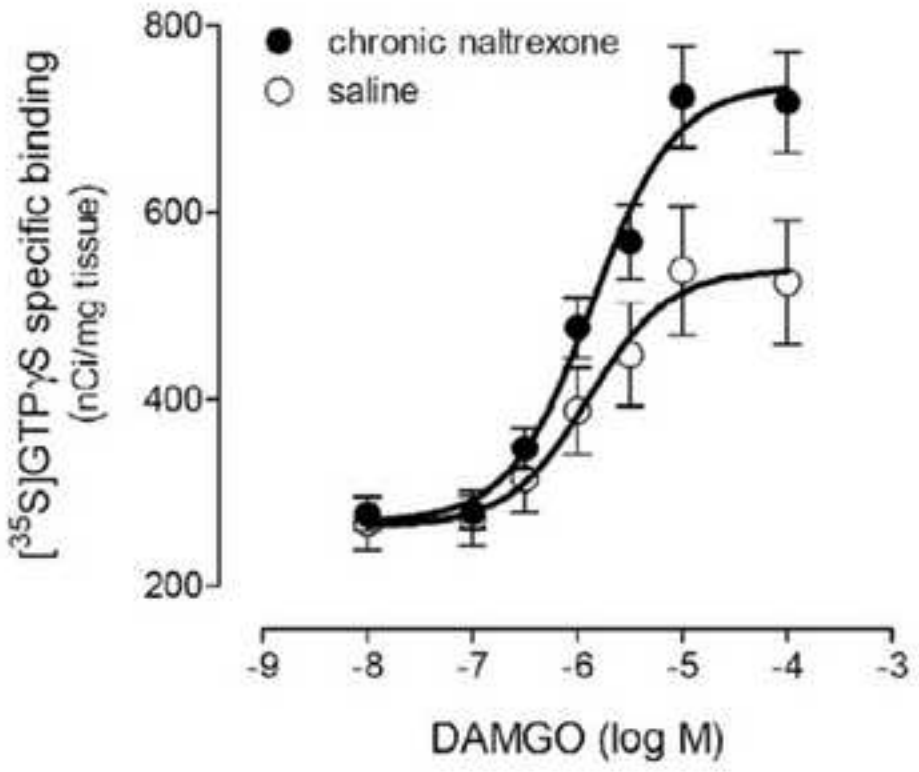

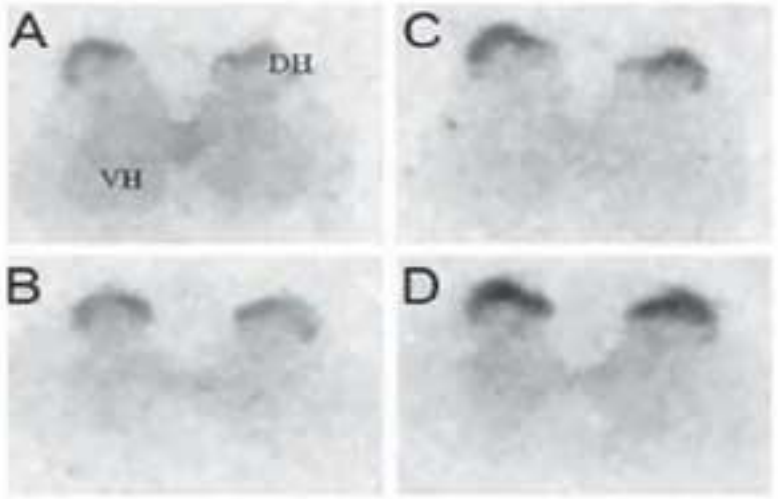

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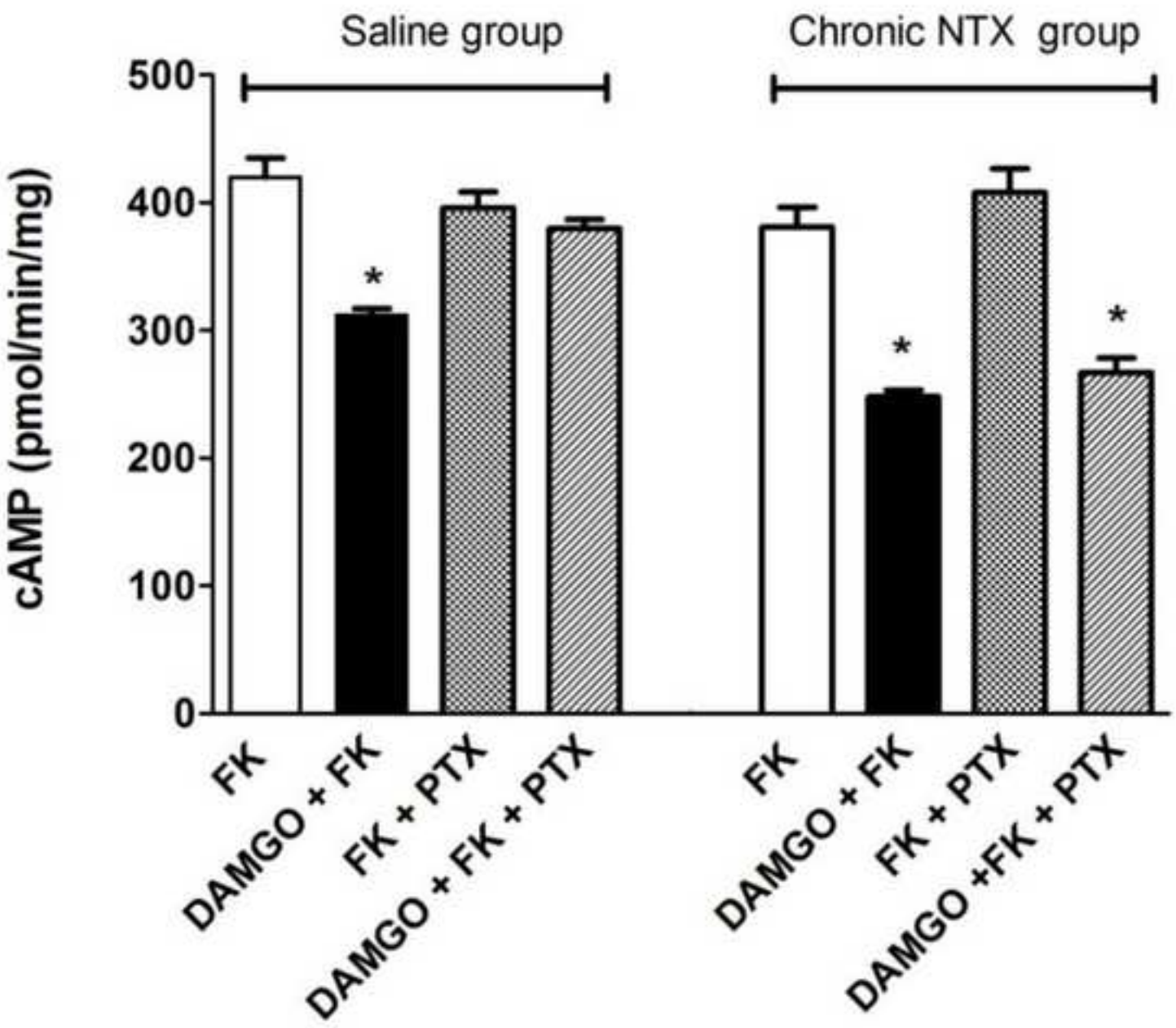

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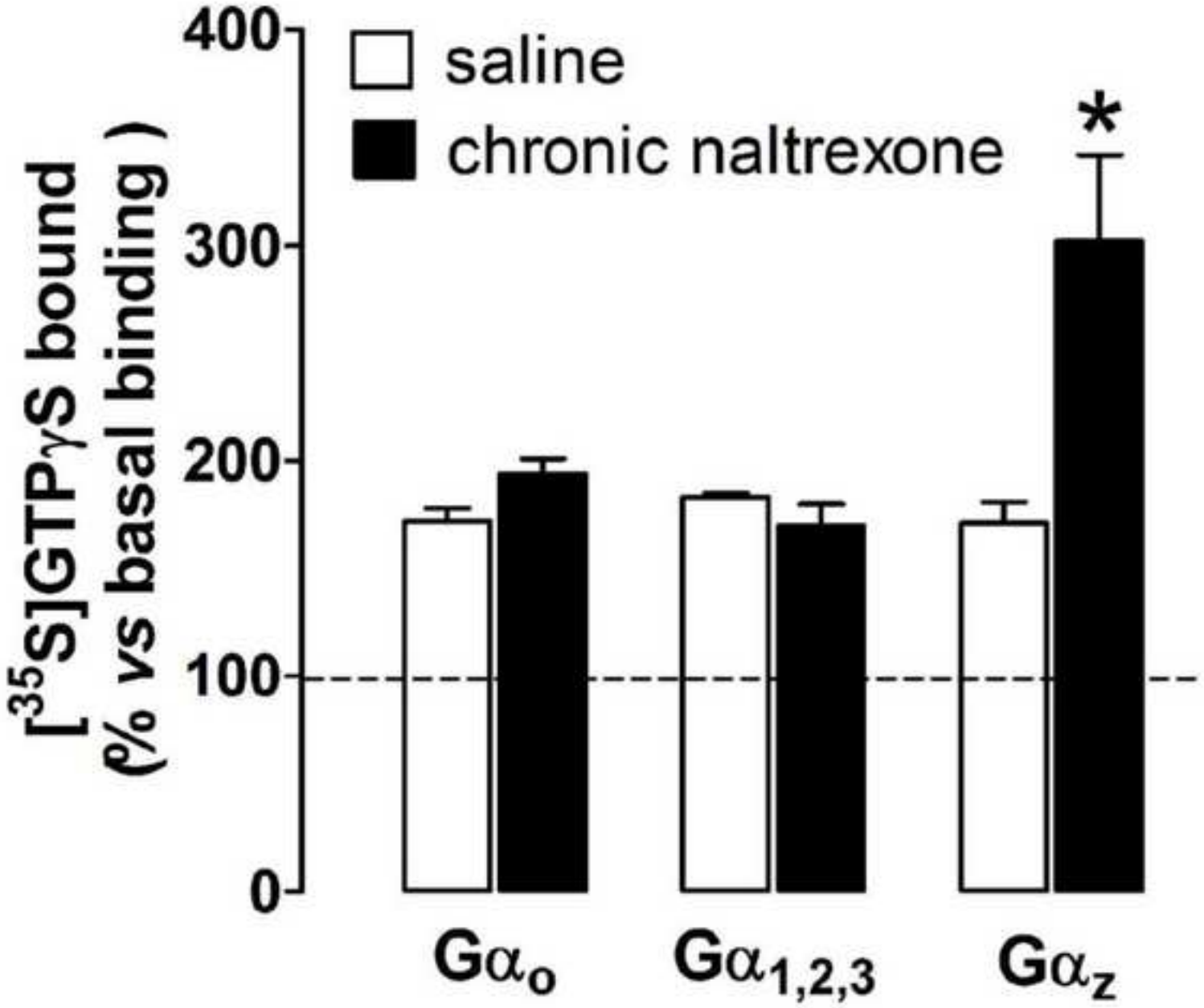

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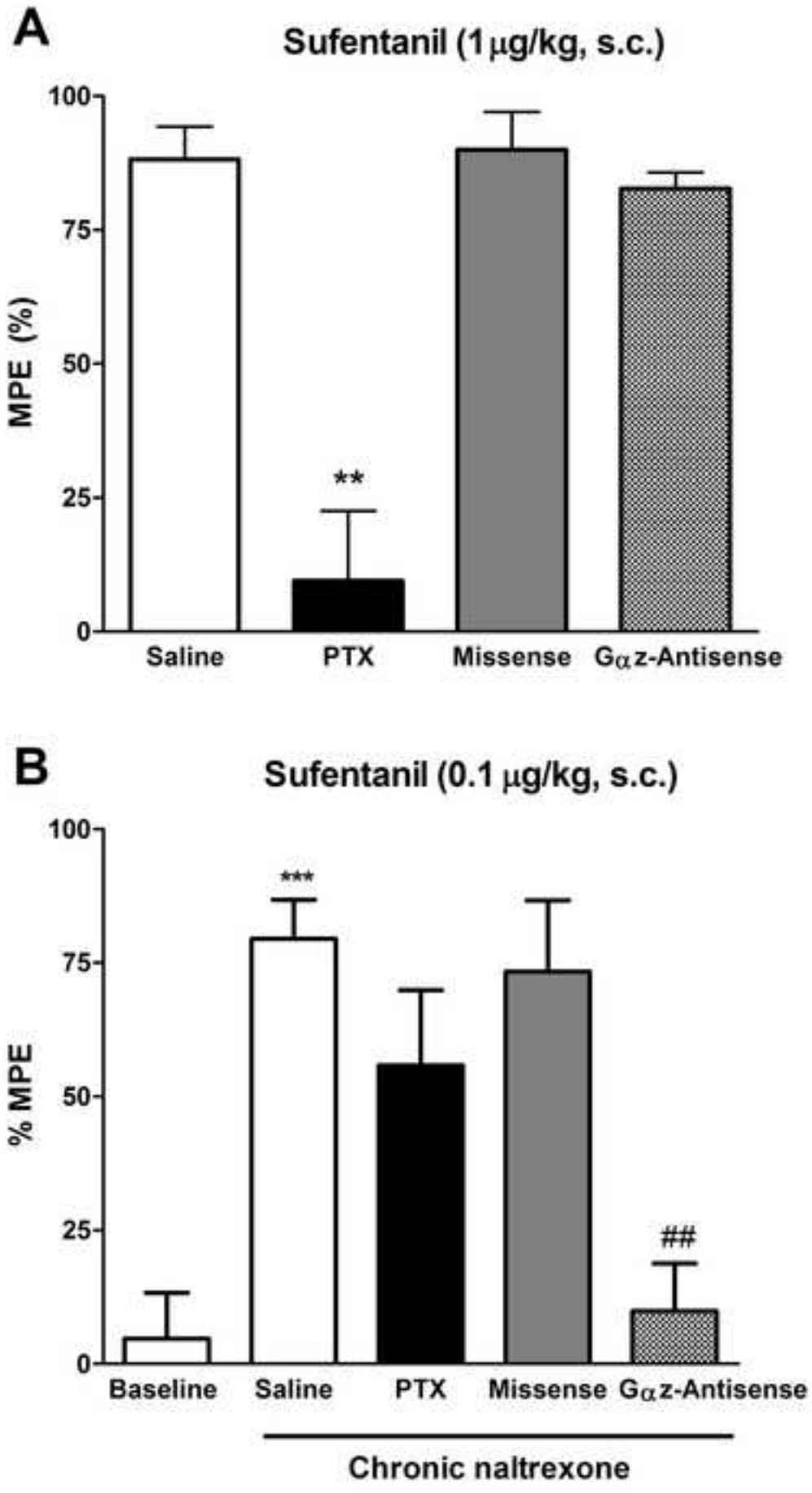

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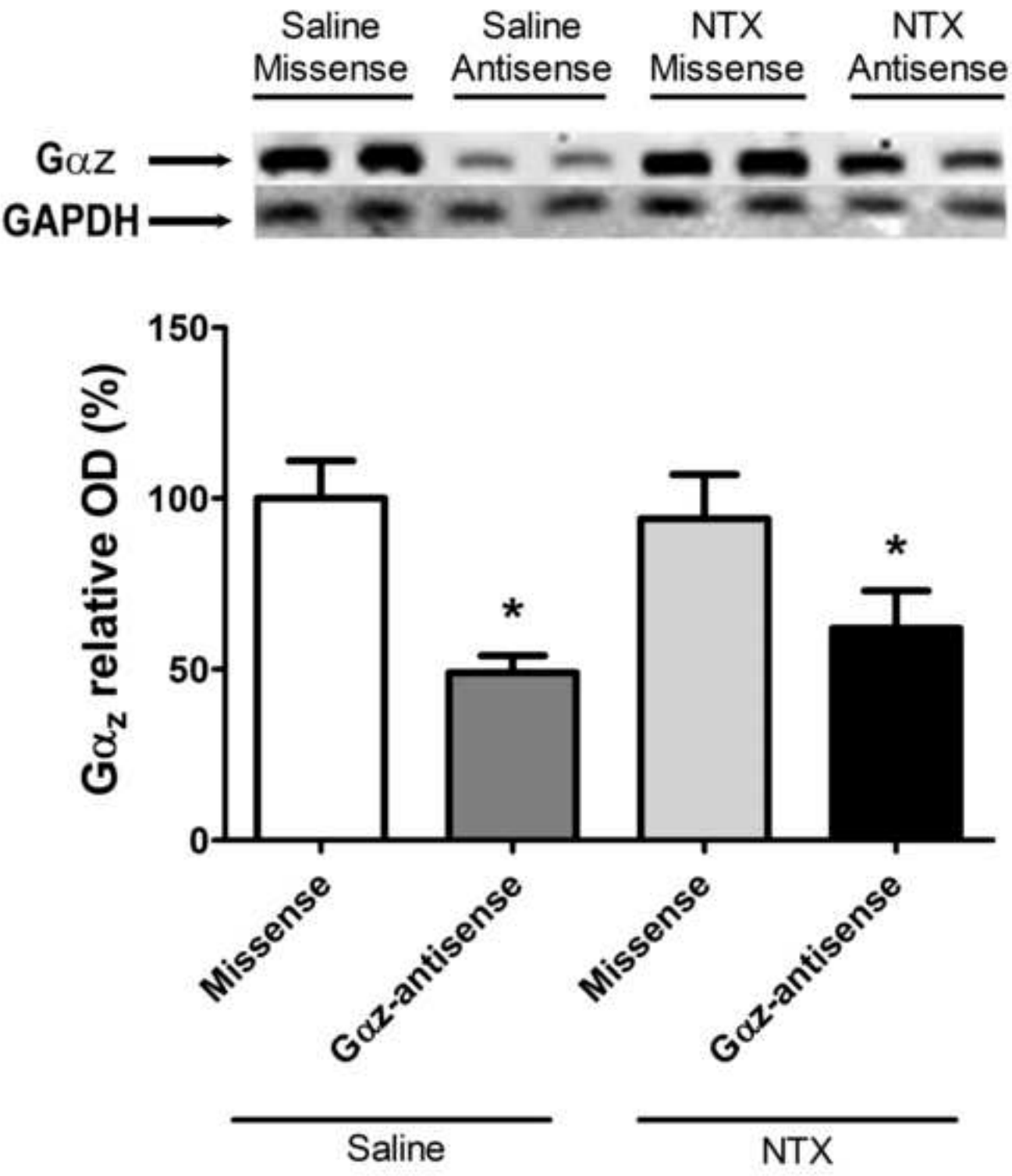

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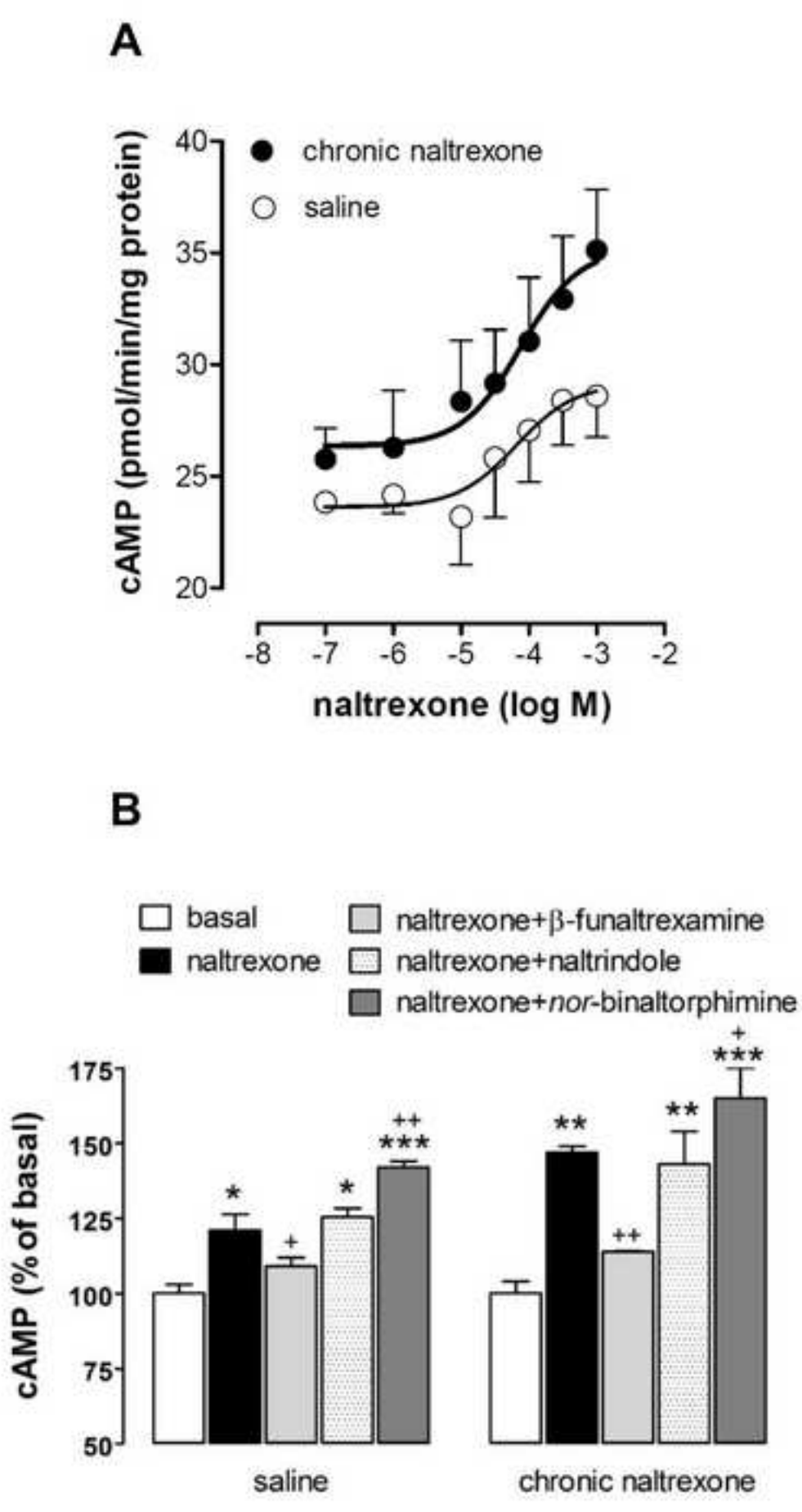

Supplementary Material

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Highlights

1. Chronic treatment with opioid antagonists increases agonist-mediated antinociception

2. We examine spinal cord changes in μ-opioid receptor signalling underlying this effect

3. Opioid receptor-coupling to G-proteins and inhibition of cAMP pathway are enhanced

4. μ-opioid receptors experience a transduction shift from $G\alpha_{i/o}$ to $G\alpha_z$ proteins

5. The inverse agonist effect of naltrexone on cAMP accumulation is enhanced