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RESEARCH PAPER

Inhibition of the regulator of G protein signalling RGS4 in the spinal cord decreases neuropathic hyperalgesia and restores cannabinoid CB_1 receptor signalling

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BACKGROUND AND PURPOSE

Regulators of G protein signalling (RGS) are major determinants of metabotropic receptor activity, reducing the lifespan of the GTP-bound state of G proteins. Because the reduced potency of analgesic agents in neuropathic pain may reflect alterations in RGS, we assessed the effects of CCG 63802, a specific RGS4 inhibitor, on pain hypersensitivity and signalling through cannabinoid receptors, in a model of neuropathic pain.

EXPERIMENTAL APPROACH

The partial sciatic nerve ligation (PSNL) model in male Sprague Dawley rats was used to measure paw withdrawal thresholds to mechanical (von Frey hairs) or thermal (Hargreaves method) stimuli, during and after intrathecal injection of CCG 63802. HEK293 cells expressing CB_1 receptors and conditional expression of RGS4 were used to correlate cAMP production and ERK phosphorylation with receptor activation and RGS4 action.

KEY RESULTS

Treatment of PSNL rats with CCG 63802, twice daily for 7 days after nerve injury, attenuated thermal hyperalgesia during treatment. Spinal levels of anandamide were higher in PSNL animals, irrespective of the treatment. Although expression of CB_1 receptors was unaffected, HU210-induced CB_1 receptor signalling was inhibited in PSNL rats and restored after intrathecal CCG 63802. In transfected HEK cells expressing CB_1 receptors and RGS4, inhibition of cAMP production, a downstream effect of CB_1 receptor signalling, was blunted after RGS4 overexpression. RGS4 expression also attenuated the CB_1 receptor-controlled activation of ERK1/2.

CONCLUSIONS AND IMPLICATIONS

Inhibition of spinal RGS4 restored endogenous analgesic signalling pathways and mitigated neuropathic pain. Signalling through CB_1 receptors may be involved in this beneficial effect

Abbreviations

GFAP, glial fibrillary acidic protein; Iba 1, ionized calcium-binding adapter molecule 1; PSNL, partial sciatic nerve ligation; PWL, paw withdrawal latency; PWT, paw withdrawal threshold; RGS, regulators of G protein signalling

Tables of Links

Targets
GPCRs[a]
CB1 receptors
CB2 receptors
Other proteins[b]
RGS4, regulator of G protein signalling 4

Ligands
2-AG, 2-arachidonoylglycerol
AEA, anandamide
HU210
JWH133
PEA, N-palmitoylethanolamine

These Tables list key protein targets and ligands in this article, corresponding to entries in http://www.guidetopharmacology.org, the common portal for data from IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a] [b] Alexander *et al.*, 2013a,b).

Introduction

Injury to the somatosensory nervous system leads to highly intractable neuropathic pain that negatively affects the life of nearly 7% of the general population (Bouhassira *et al.*, 2008; van Hecke *et al.*, 2014). The use of currently available drugs is still overshadowed by the relative lack of efficacy as well as adverse effects that limit any therapeutic benefit. Even the most powerful analgesic drugs, such as opioids, suffer from reduced analgesic efficacy in neuropathic pain, possibly due to nerve lesions altering the activity of endogenous analgesic systems (Przewlocki and Przewlocka, 2005). A better understanding of the pathophysiological mechanisms that drive neuropathic pain and underlie the reduced efficacy of otherwise potent analgesic systems is crucial to the development of more effective and specific mechanism-based treatments for this debilitating condition (Baron *et al.*, 2010; Mayer *et al.*, 1999).

Nerve injury elicits maladaptive plasticity throughout the nociceptive system (Tao *et al.*, 2003; Wang *et al.*, 2011), particularly in the dorsal horn of the spinal cord, leading to a pathological amplification in the synaptic processing of peripherally originating signals before these signals are gated to the brain (Costigan *et al.*, 2009). The modulation of synaptic transmission at this site is largely driven by signalling through a variety of GPCRs (Pan *et al.*, 2008). Nerve injury can dramatically affect GPCR systems such as the opioid system, effectively reducing analgesic potency after nerve injury (Przewlocki and Przewlocka, 2005). Signalling through many GPCRs is controlled by proteins known as regulators of G protein signalling (RGS) (Ding *et al.*, 2006; Rahman *et al.*, 2003), a family of proteins whose main action is to terminate receptor signalling by accelerating GTP hydrolysis at the G_α-subunit of the G protein. RGS are known to negatively modulate opioid receptors and opioid-mediated analgesia (Psifogeorgou *et al.*, 2007; Psifogeorgou *et al.*, 2011; Rodriguez-Munoz *et al.*, 2007; Zachariou *et al.*, 2003), but the analgesic modulatory properties appear as being site-specific (Han *et al.*, 2010; Stratinaki *et al.*, 2013).

Within the spinal cord, RGS4 is the only RGS isoform showing an up-regulation after peripheral nerve injury and this up-regulation was proposed to explain reduced spinal opioid receptor activity (Garnier *et al.*, 2003). RGS4 decreased signalling through cannabinoid receptors (Sutor *et al.*, 2011), which are engaged in tonic modulation of the basal thermal nociceptive threshold (Richardson *et al.*, 1998). This modulation is mediated through the cannabinoid CB_1 receptors that are abundantly expressed in the dorsal horn (Hohmann, 2002). There is strong evidence that nerve injury specifically compromises the efficacy by which CB_1 receptor signalling inhibits thermo-sensitive C-fibre-induced activity of spinal wide dynamic range neurons (Chapman, 2001; Hohmann *et al.*, 1999). These findings, coupled with evidence for the specific up-regulation of RGS4 in the lumbar spinal cord after nerve injury (Garnier *et al.*, 2003), led us to our main study objectives.

Here, we have investigated whether the pharmacological inhibition of spinal RGS4 could effectively attenuate neuropathic pain and counteract the silencing of endogenous analgesic systems such as spinal CB_1 receptor signalling. In order to address these objectives we selected the model of partial sciatic nerve ligation (PSNL) of adult male Sprague Dawley rats in which spinal RGS4 up-regulation had been previously described (Garnier *et al.*, 2003).

Methods

Rats

All animal care and experimental procedures used in these studies complied strictly with the European Community Council directive of 24 November 1986 (86-609/ECC) and the decree of 20 October 1987 (87-848/EEC).and were approved by the Ethical Committee on Animal Research of the Université catholique de Louvain (LA2230419). Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 145 animals were used in the experiments described here.

Animals were housed socially (two to three animals per standard Makrolon type III cages) at a timer-controlled normal 12 h–12 h light–dark cycle with *ad libitum* access to food and water. Only during the period of catherization, animals were housed individually. For the *in vivo* experiments, 10-week-old male Sprague Dawley rats (Charles River) were subjected to PSNL or sham surgery using a modification of the procedure described earlier (Berger *et al.*, 2011). A subset of animals was implanted with an indwelling intrathecal catheter for spinal treatment with the RGS4 inhibitor CCG 63802

(0.05 mg·mL^{-1}, 10 μL injection) or vehicle solution. The specificity of CCG 63802 for RGS4 has been validated previously by a multiplex flow cytometry protein interaction assay (Blazer *et al.*, 2010). The concentration of CCG 63802 used here was based on unpublished experiments in which this concentration of the inhibitor effectively modulated calcium concentrations induced by activation of phospholipase C-coupled glutamatergic GPCRs in primary astrocyte cultures.

PSNL surgery

Anaesthesia was induced and maintained through a face mask with sevoflurane (3% in oxygen) connected to a sevoflurane vaporizer (19.3 Sevoflurane Vaporizer, Abbott, Germany). Adequate depth of anaesthesia was confirmed by the absence of a response to a toe pinch. After shaving of the surgical area at mid-thigh level and a skin incision (2 cm long), blunt dissection was performed to expose the right sciatic nerve and isolate it from surrounding tissues. A 6/0 prolene suture (Ethicon, Livingstone, Scotland) was used to tightly ligate 1/3–1/2 of the nerve trunk rostral to its bifurcation in peroneal and tibial branches (Seltzer *et al.*, 1990). Muscle and skin layers were then closed using 2/0 prolene suture (Ethicon), and animals were returned to their cage. Postoperative well-being of the animals was routinely monitored.

Implantation of indwelling intrathecal catheters and intrathecal treatment

The anaesthetized animal (sevoflurane anaesthesia as before) was positioned in a stereotaxic frame with its head secured between ear bars. After incision of the skin overlying the midline of the skull, the atlanto-occipital membrane was exposed. A custom-made catheter was inserted into the subarachinoid space through a slit in the atlanto-occipital membrane and advanced subdurally for 8.5 cm thereby reaching the lumbar spinal cord level. The proximal, exterior part of the catheter was guided to a separate exit point through the skin overlying the skull. Subsequently, the catheter was flushed using 10 μL of saline solution, and the skin was sutured using 4/0 prolene sutures (Ethicon). As long as animals were catheterized, they were individually housed. The RGS4 inhibitor CCG 63802 was diluted in saline containing 5% DMSO to a final concentration of 0.05 mg·mL^{-1}, and 10 μL of this solution was bolus-injected through the catheter followed by a 10 μL flush with saline solution for two groups of animals (sham/CCG 63802 and PSNL/CCG 63802). Vehicle treatment consisted of two subsequent injections with 10 μL of saline solution containing 5% DMSO and was given to two groups of animals (sham/vehicle and PSNL/vehicle). Drug treatment was commenced immediately after surgery and thereafter at 12 h intervals (at 7a.m. and 7p.m.) for seven consecutive days.

Spinal cord slice preparation and assay to determine cannabinoid receptor signalling

Rats were killed by CO_2-induced asphyxiation and lumbar spinal cord samples were excised. Samples were quickly rinsed in ice-cold Ringer's solution (120 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 6 mM glucose, pH 7.4), dried and cut into 350 μm transverse sections using a McIlwain Tissue Chopper. After separating ipsilateral and contralateral sides, alternative sections were incubated in Ringer's solution with or without HU210 (1 μM) for 15 min at 37°C under light shaking. This concentration of HU210 has been frequently used (see Lauckner *et al.*, 2005; Millns *et al.*, 2001), and concentration–response experiments have shown that this concentration reaches the maximum effect in the [^{35}S]-GTPγS assay, described below (data not shown). In control experiments, alternative sections were incubated in Ringer's solution with or without JWH133 (100 nM) for 15 min at 37°C under light shaking. The concentration of JWH133 was selected to ensure specificity for the CB_2 receptor. The reaction was stopped by quickly transferring slices into ice-cold PBS buffer and immediate homogenization in lysis buffer containing 50 mM HEPES, 50 mM KF, 1 mM Na_3PO_4, 1 mM EDTA, 1 mM EGTA, 0.5% 2-mercaptoethanol, 5 mM β-glycerolphosphate, 5 mM $Na_4P_2O_7$, 1 mM phenylmethylsulphonyl fluoride, 100 μM Na_3VO_4, 1% Triton X100, Halt protease and phosphatase cocktail inhibitor (Thermo Scientific, Aalst, Belgium) and phosphatase inhibitor cocktail III (Sigma-Aldrich, Bornem, Belgium).

HEK Flp-In™ T-Rex™ cell line in culture and generation of the inducible RGS4 system

Cells were purchased from Invitrogen (Merelbeke, Belgium) and used either as wild type or as specialized cells expressing RGS4 in a tetracycline-inducible way (HEK Flp-In™ T-Rex™ RGS4 cells). CB_1 receptors were introduced by nucleofection. The tetracycline-inducible HEK Flp-In™ T-Rex™ RGS4 cell line was generated by introducing an RGS4-myc tagged sequence in the HEK Flp-In™ T-Rex™ 293 cell line. The cDNA sequence encoding rat myc-tagged RGS4 was obtained by KpnI and PmeI restrictions of the pcDNA3.1myc-His-RGS4 vector, a generous gift from G. Willars (Tovey and Willars, 2004), and cloned into KpnI and EcoRV restriction sites in the pcDNA5/FRT/TO vector (Invitrogen), allowing genomic recombination in the HEK Flp-In™ T-Rex™ cell line. HEK Flp-In™ T-Rex™ cells were double transfected with both pcDNA5/FRT/TO-RGS4 and pOG44 vectors (Invitrogen), the latter coding for the Flp recombinase. HEK Flp-In™ T-Rex™ cells stably expressing myc-tagged RGS4 proteins were obtained by maintaining appropriate antibiotic selection pressure with hygromycin and blasticidin. HEK Flp-In™ T-Rex™ RGS4 cells were routinely grown in DMEM (41965, Invitrogen) supplemented with 10% foetal bovine serum, 100 U·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin, 2mM L-glutamine, 100 μg·mL^{-1} hygromycin and 15 μg·mL^{-1} blasticidin. When required, RGS4 expression was induced over 24 h by supplementing culture medium with 1 μg·mL^{-1} doxycycline (total of 24 h) and with 10 μM of the proteasome inhibitor MG132 (last 8 h of induction period).

Expression of CB_1 receptors in HEK Flp-In™ T-Rex™ cell lines

Wild type HEK Flp-In™ T-Rex™ cells or HEK Flp-In™ T-Rex™ RGS4 cells (4.10^6 cell count) were resuspended in antibiotic-free culture medium supplemented with sodium pyruvate 1 mM, pelleted in polypropylene tubes and nucleofected using Amaxa cell line nucleofector kit V (Lonza, Verviers, Belgium) following the manufacturer's instructions. Five micrograms

of hCB_1 pCDNA3.1 vector (UMR cDNA Resource Centre, www.cdna.org) was used for each nucleofection. After nucleofection, cells were grown for 24 h in antibiotic-free, sodium pyruvate supplemented culture medium.

Treatment of HEK Flp-In™ T-Rex™ cell lines with CB_1 receptor agonist

The two cell lines, either induced for expression of RGS4 or non-induced, were plated at a density of 300 000 per well in 6-well culture plates and maintained for a total duration of 48 h (24 h before and 24 h after induction with doxycycline and MG132). Then, cells were treated for 10 min either with HU210 diluted to 1 μM in culture medium or with culture medium (control). For Western blot analysis, cells were rapidly rinsed with cold PBS, scraped and homogenized in lysis buffer containing 50 mM HEPES, 50 mM KF, 1 mM Na_3PO_4, 1 mM EDTA, 1 mM EGTA, 0.5% 2-mercaptoethanol, 5 mM β-glycerolphosphate, 5 mM $Na_4P_2O_7$, 1 mM phenylmethylsulphonyl fluoride, 100 μM Na_3VO_4, 1% Triton X100, Halt protease and phosphatase cocktail inhibitor (Thermo Scientific) and phosphatase inhibitor cocktail III (Sigma).

Western blotting

Protein extracts (40 μg) were separated on a 12% SDS polyacrylamide gel, transferred to nitrocellulose membrane and probed as previously described (Bosier *et al.*, 2008). ERK1/2 phospho-specific antibody (p-ERK1/2) (1:2000 dilution, overnight, 4°C; Cell Signalling, Leiden, Netherlands) and peroxidase-linked anti-rabbit IgG (Sigma, 1:3000, 1 h, room temperature) were used for primary labelling. The blots were then stripped and re-probed with antibodies that recognize both the phosphorylated and non-phosphorylated forms of ERK1/2 (Tot-ERK1/2) (1:2000, overnight, 4°C; Cell Signalling) and the same anti-rabbit antibody. Bands were visualized by chemiluminescence using the Western Lightning Plus ECL Reagents (Perkin Elmer NEN, Zaventem, Belgium), and films were scanned and quantified using image J software.

cAMP assay

After nucleofection, HEK Flp-In™ T-Rex™ RGS4 cells were seeded in 96-well plates ($2x10^4$ cells per well). Twenty four hours later, cells were either induced with doxycycline ($1\ \mu g \cdot mL^{-1}$ for 24 h; Westburg, Leusden, Netherlands), and the proteasome inhibitor MG132 (10 μM for last 8 h; Sigma) or not induced. Cells were then pre-treated with 0.1 mM IBMX for 30 min at 37°C. Subsequently, HU210, diluted to 1 μM in culture medium, was added to the culture medium for another 30 min at 37°C in the presence of 1 μM forskolin. Controls were treated with culture medium alone. cAMP levels were measured using cAMP Biotrak Enzyme Immunoassay (EIA; Amersham Pharmacia Biotech, Munich, Germany) following the manufacturer's instructions.

[^{35}S]-GTPγS assay

Membranes fractions from ipsilateral lumbar spinal cord samples were prepared in 50 mM Tris–HCl pH 7.4, as previously described (Govaerts *et al.*, 2004). [^{35}S]-GTPγS binding experiments were performed at 30°C in plastic tubes containing the membrane homogenate (5 μg of protein) in a 0.3 mL final volume of binding buffer (50 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 0.1% BSA, pH 7.4) supplemented with 20 μM GDP and varying concentrations of test compounds. The assay was initiated by the addition of [^{35}S]-GTPγS (0.05 nM, final concentration). The tubes were incubated for 1 h in a shaking water bath. The incubations were terminated by rapid filtration through GF/B filters using a 48-well Brandell cell harvester. Filters were washed three times with ice-cold washing buffer (50 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl), and trapped radioactivity was counted by liquid scintillation using Aqualuma. Non-specific binding was measured in the presence of 100 μM Gpp(NH)p.

Immunohistochemistry

Glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba 1) were stained using 20-μm-thick transversal cryosections of L4/L5 lumbar spinal cord as described previously (Goursaud *et al.*, 2015). Antibodies were rabbit anti-Iba 1 (1:1000; Wako, Osaka, Japan), goat anti-rabbit IgG (H + L) Alexa Fluor®488 (Invitrogen, 1:500) and the monoclonal mouse anti-GFAP-Cy3™ (Sigma-Aldrich, 1:500) diluted in a working solution containing 1% NGS and 1% Triton X-100 in TBS pH 7.4. Sections were examined with an AMG Evosfl Digital Inverted Microscope (Westburg); the dorsal horn was delineated as the region of interest and, after subtraction of background signals, the area fraction occupied with specific immunoreactivity was quantified using Image J (NIH) software and normalized to that of control animals.

RT-PCR

Total RNAwas isolated from the dorsal quadrant of the ipsilateral lumbar spinal cord using TriPure isolation reagent (Roche Diagnostics, Vilvoorde, Belgium), treated with the RQ1 RNase-free DNase kit (Promega) and reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Nazareth, Belgium). Real-time PCR amplifications were carried out using the iCycler IQ™ multicolour real time PCR detection system (Bio-Rad), in a total volume of 25 μL containing 10 ng cDNA template, 0.3 μM of the primers (forward primer: 5′taacttgccagagggtgagc3′; reverse primer: 5′aaagctgccagtccacattc3′) and the IQ™ SYBR Green Supermix using an annealing temperature of 60°C. For quantitative analysis, a relative standard curve was generated using same amplification conditions, with dilutions of a mix of cDNA templates (from 20 to 0.078 ng). Each sample was normalized to the relative amplification of GAPDH. Quantification of mRNA in the samples was performed using the post-run data analysis software provided with the iCycler system.

HPLC-MS quantification of endocannabinoids and related compounds

Anandamide (AEA), 2-arachidonoylglycerol (2-AG) and *N*-palmitoyethanolamine (PEA) were quantified from ipsilateral and contralateral dorsal lumbar spinal cord samples, as previously described (Alhouayek *et al.*, 2013). Lipid tissue fractions were analysed by HPLC-MS using a LTQ Orbitrap mass spectrometer (ThermoFisher Scientific) coupled to an Accela HPLC system (ThermoFisher Scientific). The endocannabinoids and related compounds were quantified by isotope dilution using

their respective deuterated standards (showing identical retention times). Data were normalized relative to values of sham vehicle-treated rats.

Behavioural tests

Assays were performed without knowledge of the treatments. In order to assess allodynia, the mechanical paw withdrawal threshold (PWT) was determined using the von Frey hair filament test, according to the up-down method previously described (Chaplan *et al.*, 1994). The filament set used (Stoelting, Wood Dale, IL) consisted of the following filaments: 0.4, 0.7, 1.2, 2.0, 3.6, 5.5, 8.5, 15.1 and 28.8 (indicated in their calibrated gram-values). Rats were placed in separate transparent, bottom-free plastic boxes positioned on an elevated metal-wire-mesh floor. After about 30 min of habituation, the assay started by placing the 2.0 g filament perpendicular to the midplantar surface of the hind paw. The filament was held in a slightly buckled position for no longer than 8 s. In case of a positive withdrawal response (brisk withdrawal and keeping the paw in an elevated position, shaking and/or licking of the stimulated paw), the next closest lower filament was used in the same way. Upon a negative response, the next closest higher filament was used, and this procedure was continued until either a cut-off value was reached or until four-five filament applications after the first positive response. Through this method, each paw is stimulated minimally four times (in case a minimal cut off is reached) and maximally nine times. An inter-stimulation interval of about 5 s was used, to avoid sensitization. The 50% PWT was then calculated as described previously (Chaplan *et al.*, 1994).

In order to assess hyperalgesia, the thermal paw withdrawal latency (PWL) was determined using the Hargreaves test as previously described (Berger *et al.*, 2011). Rats were placed in separate transparent, bottom-free plastic box positioned on the Hargreaves Paw Thermal Stimulator (University of California San Diego) and habituated for about 30 min. To determine the PWL, each animal was stimulated three times with an inter-stimulus interval of about 4 min, and the latencies were averaged per animal. A cut-off was fixed at 20 s of paw stimulation to prevent tissue damage.

For assessment of locomotor activity, the open field test was used according to previously described procedures (Bosier *et al.*, 2010). Rats were placed in the centre of a 60 × 60 cm arena and allowed to explore the arena for a duration of 10 min. EthoVision video tracking system (Noldus Information Technology, Wageningen, Netherlands) was used to measure the total distance moved and the velocity of movement.

Data analysis

Data were checked for normality, processed and analysed using GraphPad Prism-4.0. All data are expressed as means ± SEM and analysed using a two-way ANOVA followed by Bonferroni's *post hoc* analysis (data on endocannabinoids and related compounds, hyperalgesia and allodynia, and spinal glial reactivity) or a two-tailed Student's *t* test (other assays). The criterion for statistical significance was $P < 0.05$.

Materials

The suppliers of compounds used were as follows: CCG 63802, HU210 and JWH133 (Tocris, Bristol, UK); [^{35}S]-GTPγS (Perkin-Elmer NEN, Zaventem, Belgium; specific activity 1000 Ci mmol^{-1}).

Results

Effect of spinal RGS4 inhibition on PSNL-induced pain hypersensitivity and glial activation

Based on the known modulatory properties of RGS4 on spinal opioid-mediated analgesia (Garnier *et al.*, 2003), we hypothesized that augmented spinal RGS4 expression could decrease endogenous analgesic tone and thereby support neuropathic pain. We first examined whether PSNL altered RGS4 mRNA level in the ipsilateral dorsal spinal cord. Here, a nearly two-fold increase in RGS4 gene expression was observed at 7 days after the surgery (Figure 1A), without any effect on the contralateral side (Figure 1B). We then designed a treatment regimen consisting of twice-a-day intrathecal bolus injections of the RGS4 inhibitor CCG 63802 for the first week after PSNL. Hyperalgesia was potently reduced by spinal treatment with the RGS4 inhibitor (Figure 1C), an effect that was restricted to the period of treatment (Figure 1C,E) as the hyperalgesic state progressively returned to the level of vehicle-treated PSNL animals upon cessation of treatment (Figure 1C). The observed effects were not due to a sedative effect of the drug as open field locomotor scores were unaffected by the treatment with CCG 63802 (Figure 2A,B). At variance with the effect on hyperalgesia, allodynia was not affected by spinal RGS4 inhibition (Figure 1D,F).

In addition to regulating nociceptive systems, GPCR-associated signalling is known to control the reactivity of spinal cord glia which play a major role in neuropathic pain (Kavelaars *et al.*, 2011). Therefore we assessed astrocyte and microglia activation. PSNL strongly induced the expression of the astrocytic marker GFAP (Figure 3A) and the macrophage/microglial-specific marker Iba 1 (Figure 3B). Intrathecal treatment with CCG 63802 for the first 7 days after PSNL strongly reduced immunoreactivity of both of these glial markers, highlighting a potent modulation of glial activation by spinal RGS4 inhibition.

Signalling through spinal CB_1 receptors is decreased after PSNL, by an RGS4-dependent mechanism

Because the endocannabinoid system plays an important role in the tonic modulation of basal thermal nociceptive thresholds, we examined the effect of peripheral nerve injury on the expression and functionality of CB_1 receptors in the lumbar spinal cord. PSNL did not alter the expression of CB_1 receptors in the ipsilateral or contralateral lumbar spinal cord 1(Figure 4A–B), but substantially affected the functionality of these receptors, as reflected by a decreased biochemical response to the agonist HU210 (Figure 4C). Indeed, the E_{max} value obtained in the [^{35}S]-GTPγS binding assay revealed that HU210-stimulated G protein activation in

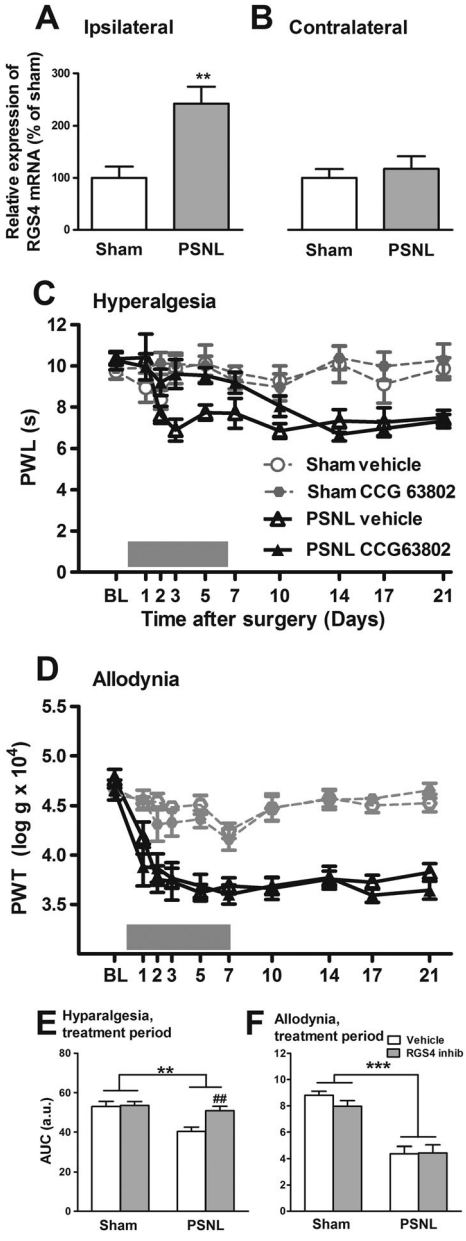

Figure 1

Spinal RGS4 is required for PSNL-induced hyperalgesia. (A,B) RGS4 mRNA expression in the ipsilateral and contralateral dorsal lumbar spinal cord at 7 days after sham or PSNL (at least $n = 6$ per group). Expression was normalized against the level of GAPDH and shown as a percentage of sham-operated animals. ** $P < 0.01$, significantly different from sham-operated animals; two-tailed *t*-test. (C,D) Pain hypersensitivity was determined for the ipsilateral hind paw at baseline (BL; before surgery) and during three weeks after sham surgery ($n = 7$) or PSNL ($n = 7$). Treatment with CCG 63802 (intrathecal; 2 times daily) or vehicle was restricted to the first week after surgery. Paw withdrawal latency (PWL) to thermal stimulation was used as a read-out of hyperalgesia (C), while the 50% paw withdrawal threshold (PWT) was used as a read-out of allodynia (D). AUC analysis was performed for the treatment period only (E,F). Two-way ANOVA revealed a general group effect for PSNL (*** $P < 0.001$; **, $P < 0.01$, significantly different from sham-operated animals); ## $P < 0.01$, significantly different from vehicle-treated PSNL; Bonferroni *post hoc* correction.

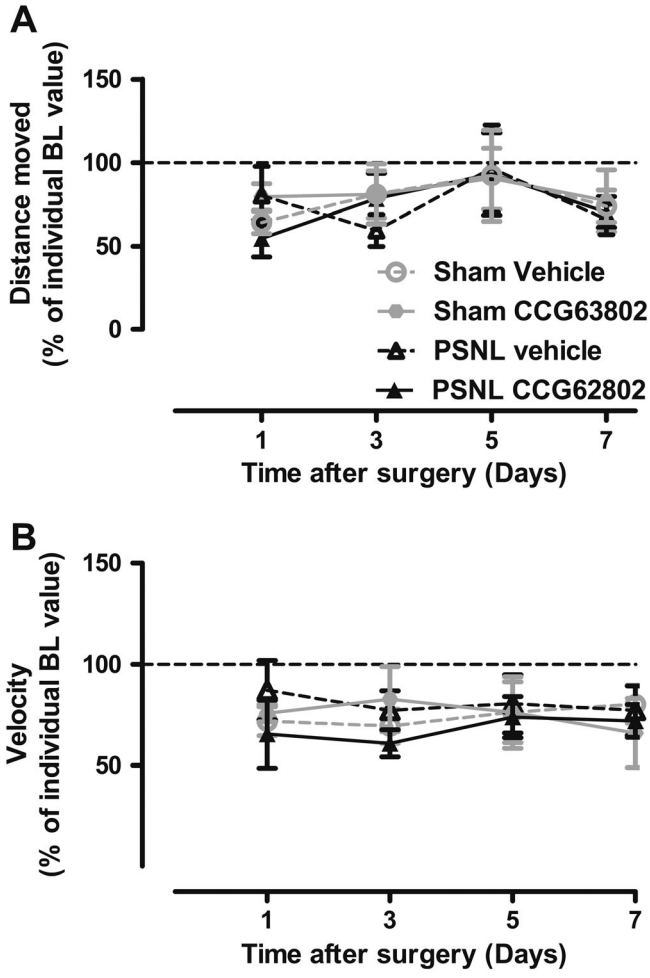

Figure 2

Intrathecal CCG 63802 treatment does not affect locomotor scores after surgery. (A,B) open field locomotor scores for distance moved and velocity were determined at baseline (BL: before surgery) and in the first week after sham surgery or PSNL ($n = 5$ per group), corresponding to the pharmacological treatment period. Values were calculated as a percentage of the baseline for each individual animal. No effect of PSNL or CCG 63802 treatment was found for the two locomotor measures.

membranes from the dorsal lumbar spinal cord was significantly decreased by PSNL to about 85% of the level of sham-operated rats. However, no significant differences were noted for the estimated EC_{50} values, suggesting a specific alteration in the ability of the receptor to induce cellular responses to the nerve lesion without change in agonist potency. Accordingly, the HU210-induced phosphorylation of ERK, a signalling kinase downstream of CB_1 receptor activation, was abolished in lumbar spinal cord slices from PSNL rats, contrasting with preserved downstream signalling in slices from sham-operated animals (Figure 4E). Indeed, HU210-treatment increases ERK phosphorylation by approximately 30% in slices obtained from sham-operated animals. Because HU210 is not selective for the CB_1 receptor, we performed additional experiments to investigate whether the CB_2 receptors could be involved in any of the effects of

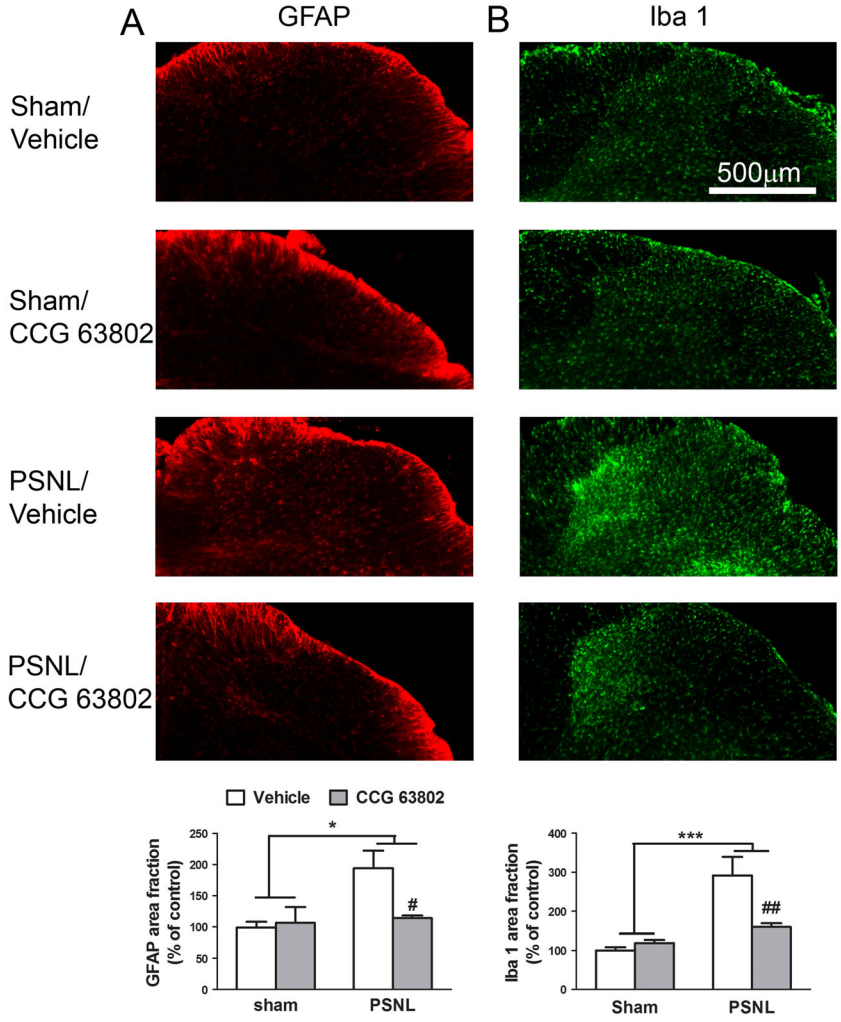

Figure 3

Inhibition of RGS4 modulates spinal glial reactivity. (A,B) GFAP and Iba 1 expression in the ipsilateral dorsal horn of the lumbar spinal cord at 7 days after sham or PSNL; treatment consisted of twice daily intrathecal injection with CCG 63802 or vehicle solution. The fraction of dorsal horn area covered by immunoreactivity for the two markers was quantified and expressed as percentage of sham-operated vehicle treated animals ($n = 4$ per group). Two-way ANOVA revealed a general group effect for PSNL (***$P < 0.001$; *$P < 0.05$, significantly different from sham-operated animals); ## $P < 0.01$, significantly different from vehicle-treated PSNL; Bonferroni *post hoc* correction.

HU210 in the PSNL model. Compound JWH133 is a potent CB_2 receptor agonist, showing selectivity for the CB_2 receptor up to a concentration of at least 100 nM. When tested at relevant concentrations, JWH133 failed to promote significant [^{35}S]-GTPγS binding, suggesting a lack of functional CB_2 receptors in ipsilateral spinal cord samples of PSNL or sham-operated rats (Figure 4D) in the tested conditions. Moreover, JWH133 was ineffective in inducing phosphorylation of ERK in lumbar spinal cord slices of either PSNL or sham-operated rats (Figure 4G).

GPCRs form signalling platforms with many associated proteins that regulate their responses. Based on previous data showing the tissue-specific increase of RGS4 in the spinal cord in response to nerve lesion (Garnier *et al.*, 2003), and considering the influence of RGS4 on the GTPase activity of some CB_1 receptor-activated G proteins (Sutor *et al.*, 2011), we hypothesized that RGS4 could directly interfere with GPCR downstream signalling, thereby affecting CB_1 receptor-dependent cascades and altering the functional response to the agonist. We therefore examined CB_1 receptor signalling after intrathecally treating animals twice a day with CCG 63802 for seven consecutive days after PSNL. This treatment completely restored HU210-mediated ERK phosphorylation, to levels comparable to those measured in samples from sham-operated animals (Figure 4F).

Spinal RGS4 does not influence endocannabinoid levels

Peripheral nerve injury has been shown to not only alter expression levels of cannabinoid receptors, but also of endocannabinoids (Lim *et al.*, 2003; Petrosino *et al.*, 2007). Here, we found that only anandamide was increased in the dorsal horn of the ipsilateral lumbar spinal cord at

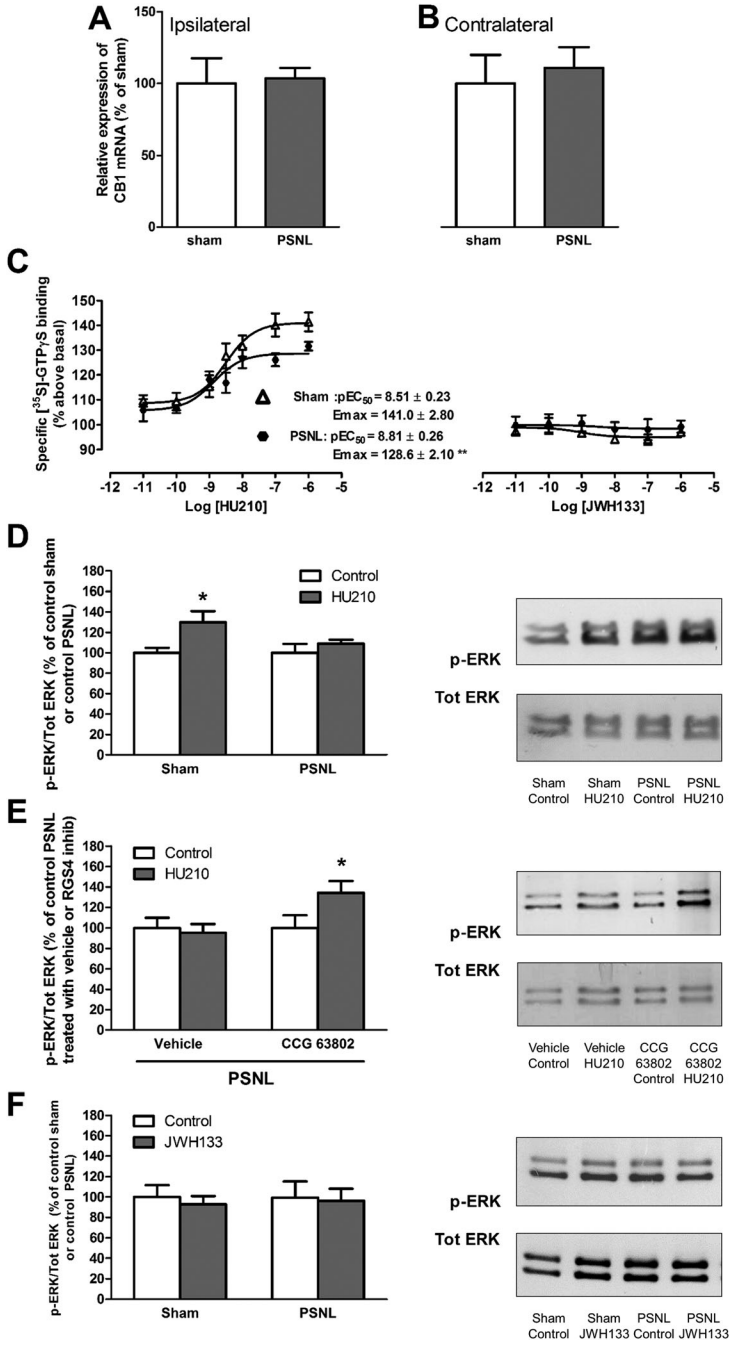

Figure 4

PSNL negatively modulates CB_1 receptor signalling through an RGS4-dependent mechanism. (A,B) CB_1 receptor mRNA expression in the ipsilateral and contralateral dorsal lumbar spinal cord at 7 days after sham surgery or PSNL (at least $n = 6$ per group). Expression was corrected for the level of GAPDH and shown as a percentage of the respective shams. (*C*) Influence of HU210 and JWH133 on [^{35}S]-GTPγS specific binding in membrane-containing homogenates from the ipsilateral lumbar spinal cord at 7 days after sham surgery or PSNL ($n = 5$ per group for HU210, $n = 6$ per group for JWH133). Values were calculated in relation to basal levels (set at 100%). E_{max} values were estimated through single-slope non-linear regression (Hill slope set at 1). (D,E) HU210-induced ERK activation in ipsilateral lumbar spinal cord slices prepared from sham-operated and PSNL animals (D) or PSNL animals intrathecally treated with vehicle or CCG 63802 (E). Slices were treated for 15 min with or without HU210 (1μM). Western blots for p-ERK expression were corrected to total ERK expression and expressed as a percentage of respective controls (at least $n = 5$ per group). Although not shown in these pharmacological control-corrected graphs, PSNL also induced a non –significant change ($P = 0.061$) in the p-ERK/Tot ERK ratio (132 ± 16%), relative to sham-operated animals. Moreover, in PSNL animals, CCG 63802 did not induce a further increase in p-ERK/Tot ERK (94 ± 9.4% relative to PSNL-vehicle treated; not significant). (F) p-ERK/Tot ERK Western blot data obtained from slices of ipsilateral lumbar spinal cords at 7 days after sham surgery ($n = 7$) or PSNL ($n = 6$), which were treated for 15 min with or without JWH133 (100 nM). * $P < 0.05$, ** $P < 0.01$; significantly different from sham-operated animals (*C*) or controls (*D,E*); two-tailed Student's *t*-test.

7 days after PNSL, while 2-arachidonoylglycerol and *N*-palmitoylethanolamine remained unaltered (Figure 5A–F). The contrast between an increase in anandamide levels and a reduction in CB_1 receptor signalling after PSNL suggests that maladaptive plasticity in the endocannabinoid system after nerve injury is a receptor-dependent process.

We then tested whether inhibition of spinal RGS4 would induce changes in these three most studied members of endocannabinoids and related compounds. RGS4 inhibition fully preserved spinal endocannabinoid levels at 7 days after PSNL (Figure 5A–F). The fact that CCG 63802 treatment does not change the levels of endocannabinoids and *N*-palmitoylethanolamine further suggests that the restoration of CB_1 receptor signalling following spinal RGS4 inhibition is receptor-mediated rather than related to changes in levels of endogenous ligands.

RGS4 directly influences the signalling efficacy of CB_1 receptors

In order to study the functional relevance of RGS4 expression on CB_1 receptor signalling, we generated a stable HEK Flp-In™ T-Rex™ RGS4 cell system in which RGS4 expression was doxycycline-inducible and CB_1 receptors were introduced by transfection with a CB_1 expression vector. The CB_1 receptor is negatively coupled to adenylyl cyclase via the $G_{i/o}$ protein and is known to reduce the accumulation of cAMP. A decrease in the HU210-induced accumulation of cAMP was observed in non-induced HEK Flp-In™ T-Rex™ RGS4 cells, i.e., HEK cells lacking RGS4 expression (Figure 6A), an effect that was abolished by the overexpression of RGS4 (Figure 6B). Similarly, HU210-induced ERK phosphorylation was completely suppressed upon induction of RGS4 expression in HEK Flp-In™ T-Rex™ RGS4 cells (Figure 6C,D). The

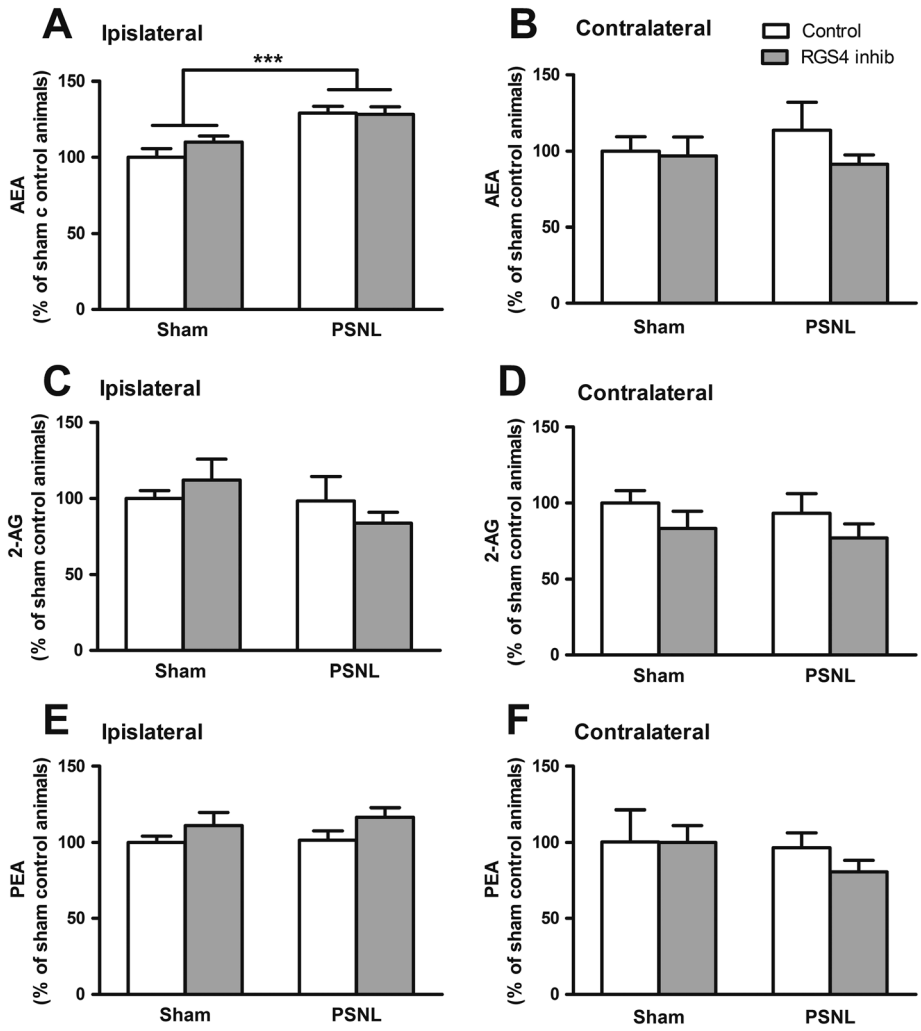

Figure 5

Effects of PSNL and RGS4 inhibition on levels of spinal endocannabinoids and related compound. Animals were subjected to PSNL or sham surgery and intrathecally treated for one week (twice per day) with CG 63802 or vehicle solution. Directly after treatment, the endocannabinoids anandamide (AEA) (A,B), 2-arachidonoylglycerol (2-AG) (C,D) and the analgesia-related *N*-palmitoylethanolamine (PEA) (E,F) were determined in the ipsilateral and contralateral dorsal lumbar spinal cords and expressed as a percentage of sham-operated vehicle-treated animals (at least $n = 3$ per group). Two-way ANOVA revealed a general group effect for PSNL. *** $P < 0.001$, significantly different from sham-operated animals, for AEA only.

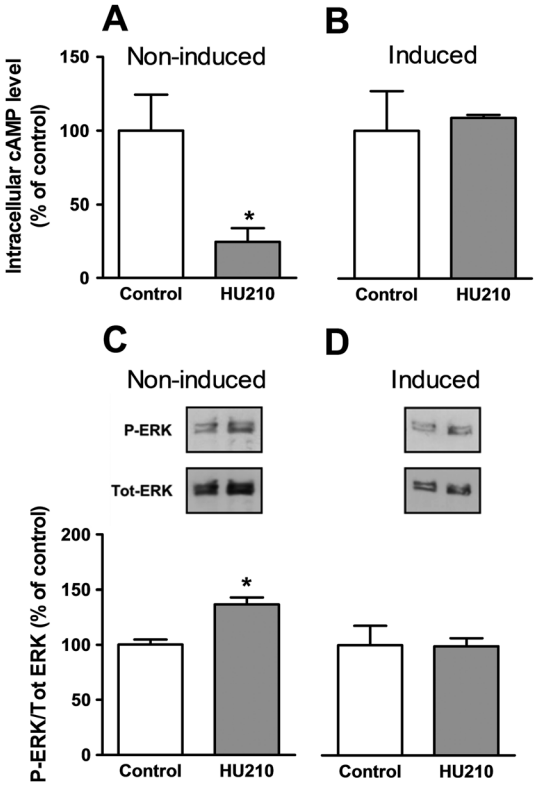

Figure 6

Functional interaction between RGS4 and CB_1 receptor signalling. cAMP levels ($n = 3$, performed in duplicate; A,B) and phosphorylation of ERK ($n = 3$; C,D) were evaluated as downstream signals upon CB_1 activation. HEK Flp-In^{TM} T-Rex^{TM} RGS4 cells were used in which RGS4 is inducible upon incubation with doxycycline (1 μg·mL^{-1}; 24 h incubation) and the proteasome inhibitor MG132 (10 μM; 8 h incubation). HU210 was diluted to 1 μM, and incubation was done for 30 min and 15 min for cAMP and phosphorylated ERK assessments, respectively. Western blots for p-ERK expression were corrected to total ERK expression. Values for both cAMP and p-ERK/Tot ERK were expressed as a percentage of control-treated cells. * $P < 0.05$, significantly different from control-treated cells; two-tailed Student's *t*-test.

specific requirement of both RGS4 and CB_1 receptors for the observed effects was confirmed in control experiments. Indeed, reduced cAMP levels and increased phosphorylation of ERK upon HU210 treatment were fully preserved when the induction protocol was applied to wild type HEK Flp-In^{TM} T-Rex^{TM} cells transfected for CB_1 receptor expression (Figure 7A,B,D,E). Moreover, the lack of CB_1 receptor expression abolished HU210-mediated effects on cAMP levels or ERK phosphorylation (Figure 7C,F).

Discussion

A growing body of evidence indicates that, along the nociceptive neuraxis, RGS4 influences analgesic systems (Han *et al.*, 2010; Ibi *et al.*, 2011; Psifogeorgou *et al.*, 2011; Zachariou *et al.*, 2003), and it is such systems that typically show reduced efficacy in neuropathic conditions. In this report, a potent up-regulation of RGS4 in the lumbar spinal dorsal horn ipsilateral to the injured nerve was confirmed and we now show that inhibition of RGS4 attenuated thermal hyperalgesia. The signalling through spinal CB_1 receptors, extensively compromised by nerve lesion, was restored by RGS4 inhibition, placing this signalling pathway in a series of possible mechanisms by which RGS4 inhibition effectively reduced hyperalgesia.

Nerve lesions trigger maladaptive plasticity throughout the nociceptive system leading to a pathological amplification of signalling (Costigan *et al.*, 2009). Particularly at the level of the spinal cord dorsal horn, an important site for the integration of nociceptive information (Wu *et al.*, 2010), hypoactivity of endogenous analgesic systems may essentially contribute to the aetiology of hyperalgesic states (Genzen and McGehee, 2003). In a first attempt to understand how a specific spinal up-regulation of RGS4 may contribute to reduce the efficacy of analgesic systems, we focused on signalling through the CB_1 receptors. Indeed these receptors are principally used by endocannabinoids and exogenously administered cannabinoids to modulate the noxious heat-evoked activity of dorsal horn nociceptive neurons (Hohmann *et al.*, 1999). Furthermore, administration of the CB_1 receptor agonist HU210 directly to wide dynamic range dorsal horn neurons potently reduced the activity of these cells that had been triggered by Aδ and C fibres (Chapman, 2001). After nerve injury, this CB_1 receptor-mediated effect was specifically compromised for the thermosensitive C fibre-evoked response (Chapman, 2001). In our investigations, spinal RGS4 inhibition was =also found to modulate thermal hyperalgesia, making it tempting to speculate about the direct role of rescued CB_1 receptor signalling in the analgesic effect of spinal RGS4 inhibition. Recent work has already suggested an important role for RGS4 in endocannabinoid-dependent neuroplasticity (Lerner and Kreitzer, 2012). Moreover, a direct interaction between RGS4 and CB_1 receptor-dependent G protein activity has been demonstrated *in vitro* using a fusion protein between the cannabinoid receptor and $G_{\alpha i2}$-protein (Sutor *et al.*, 2011). We, now, show for the first time a functional interaction between RGS4 and CB_1 receptors that directly affects downstream signalling cascades. Thus, RGS4 appears here as a negative modulator of signalling through CB_1 receptors in addition to its effects on signalling through other receptors as proposed earlier (Ruiz de Azua *et al.*, 2010; Schwendt *et al.*, 2012; Song *et al.*, 2009; Tokudome *et al.*, 2008). Even though it remains unknown which cells show RGS4 expression within the spinal cord, spinal RGS4 has been linked to reduced activity of the μ-subtype of opioid receptors after peripheral nerve injury (Garnier *et al.*, 2003). In our study, also a marked reduction in nerve injury-induced reactivity of spinal glial cells was noted after spinal RGS4 inhibition. These glial cells are strongly implicated in neuropathic pain (Scholz and Woolf, 2007; Zhuang *et al.*, 2006) even though there is not always an evident link between glial reactivity states and neuropathic pain symptoms such as allodynia (Gallo *et al.*, 2015; Leinders *et al.*, 2013). It remains to be determined whether the effect of spinal RGS4 inhibition on glial reactivity is directly or indirectly linked to the anti-hyperalgesic effects in our investigation.

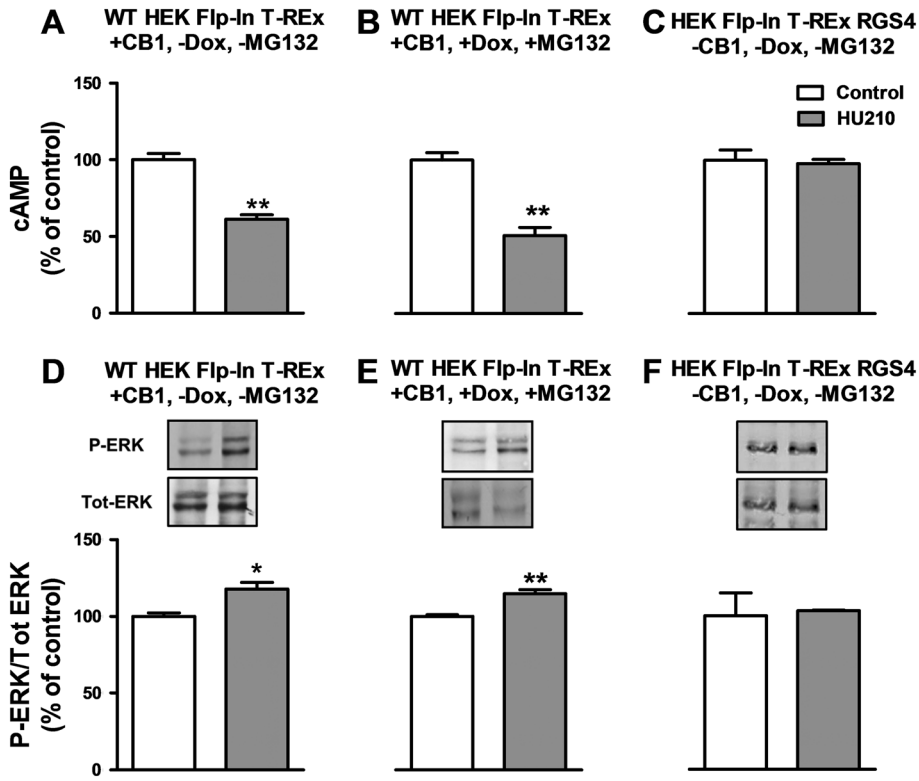

Figure 7

HU210-induced signalling is dependent on CB_1 receptors and not influenced by the presence of doxycycline nor by the proteasome inhibitor MG132 *per se*. cAMP levels ($n = 3$, performed in duplicate; A–C) and phosphorylation of ERK ($n = 3$; D–F) were evaluated as downstream signals upon CB_1 receptor activation. In order to confirm that the observed effects reported in Figure 3 were specific to the presence of RGS4 and were not related to interference with the pharmacological agents doxycycline (Dox) and MG132, we kept wild type HEK Flp-In™ T-Rex™, transfected with the vector for CB_1 receptor expression, for 24 h in the absence (A, D) or presence (B, E) of doxycycline (1 $\mu g \cdot mL^{-1}$; full incubation period) and MG132 (10 μM; last 8 h of incubation period). HU210 was diluted to 1 μM, and incubation was conducted for 30 min and 15 min for cAMP and phosphorylated ERK assessment, respectively. Western blots for p-ERK expression were corrected for total ERK expression. Values for both cAMP and p-ERK/Tot ERK were expressed as a percentage of control-treated cells. As expected, the magnitude of HU210-mediated effects on both cAMP levels and p-ERK/Tot ERK expression were the same in the absence or presence of Dox and MG132. To confirm the specific involvement of CB_1 receptors in the HU210-induced effects on cAMP and p-ERK/Tot ERK, we used the generated HEK Flp-In™ T-Rex™ RGS4 cells, non-transfected for CB_1 receptor expression. As expected, HU210 failed to induce any of the two investigated downstream signalling responses (C, F). * $P < 0.05$, **, $P < 0.01$; significantly different from control-treated cells; two-tailed Student's *t*-test.

The specific induction of spinal RGS4 expression after peripheral nerve injury suggests that the targeting of this regulatory protein can be considered a disease-modifying strategy. Evidence suggests that RGS4 expression does not directly influence acute pain, making it an even more attractive therapeutic target for pathological conditions. Specifically, RGS4 knock-out mice show normal sensitivity thresholds to acute noxious heat (Grillet *et al.*, 2005). We found that RGS4 modulated pain after nerve injury, but with a specific role in chronic hyperalgesia rather than allodynia. The lack of anti-allodynic effect in this investigation may be due to the time delay between drug delivery and behavioural assessments. Indeed, independent investigations in our laboratory show that the RGS4 inhibitor has anti-allodynic effects that are relatively short-lived (around 40–60 min after intrathecal bolus injection; data not shown). As such, RGS4 could be considered of more direct relevance to hyperalgesia and its treatment than to allodynia. In line with this, allodynia was largely unaffected in nerve-injured RGS4 knock-out mice (Stratinaki *et al.*, 2013). In our study, hyperalgesia was abolished by spinal RGS4 inhibition while CB_1 receptor function was restored, suggesting that the potentiation or rescue of tonic analgesic systems such as the endocannabinoid system is sufficient to abolish hyperalgesia. Apart from merely modulating endogenous analgesic systems, targeting RGS may have wider therapeutic relevance. Indeed, RGS4 was previously associated with an altered responsiveness to exogenously administered therapeutic agents including tricyclic anti-depressants, selective 5-HT re-uptake inhibitors, noradrenaline re-uptake inhibitors, NMDA receptor antagonist and even synthetic opioids (Han *et al.*, 2010; Stratinaki *et al.*, 2013). The anti-allodynia and/or anti-depressant properties of these agents, used in treatment of neuropathic pain (Baron *et al.*, 2010; Dworkin *et al.*, 2010), were either positively or negatively influenced by RGS4, depending on the site of RGS4 action (Han *et al.*, 2010; Stratinaki *et al.*, 2013).

As spinal RGS4 inhibition *per se* left allodynia unaffected in our study, it remains a scientific goal to study whether this therapeutic approach could be used in combination with anti-allodynia agents. Spinal RGS4 inhibition might even influence the effect of such exogenously delivered agents.

The endocannabinoid system acting both at peripheral and central sites has been extensively involved in modulation of nociceptive transmission (Agarwal *et al.*, 2007; Hsieh *et al.*, 2011; Ibrahim *et al.*, 2003; Ibrahim *et al.*, 2005; Piomelli *et al.*, 2014; Quartilho *et al.*, 2003; Walker *et al.*, 1999). The existence of an endocannabinoid system influencing the nociceptive system has been considered particularly useful for the treatment of neuropathic pain where opiates are relatively ineffective. Analgesic effects have been observed upon administration of receptor agonists (Landry *et al.*, 2012; Romero-Sandoval *et al.*, 2008; Wallace *et al.*, 2003; Wilkerson *et al.*, 2012), inhibitors of enzymes catalysing endocannabinoid hydrolysis (Jhaveri *et al.*, 2006; Starowicz *et al.*, 2013) and drugs that interfere with endocannabinoid transport (La Rana *et al.*, 2006). In 2014, several states in the US legalized the medical use of cannabis. Thus, it will be crucial for any such treatment strategy to retain functional signalling through cannabinoid receptors. Obviously, the up-regulation of spinal endocannabinoids and their receptors after peripheral nerve injury (Lim *et al.*, 2003; Petrosino *et al.*, 2007) loses biological relevance when receptor signalling is severely compromised. In light of the still ongoing debate on the analgesic potential of cannabinoids, our data on the negative link between RGS4 expression and CB_1 signalling provides important insights. Earlier reports of spinal RGS4 expression decreasing opioid receptors highlight the relevance of our observations, reported here, on the anti-hyperalgesic effect of spinal RGS4 inhibition (Garnier *et al.*, 2003). The cannabinoid and opioid systems show functional interactions within the spinal cord (Desroches *et al.*, 2014), and reduced signalling through their respective receptors after nerve injury may be related to the injury-induced up-regulation of RGS4 (Garnier *et al.*, 2003). As a newly identified key mediator of reduced signalling through receptors for analgesic agents, RGS4 represents a promising target for the potentiation of several analgesic systems and the effective treatment of neuropathic pain.

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Author contributions

B.B., E.H. and R.D. designed research; B.B., P.J.D., A.B., G.G. M., E.A., N.D. and R.D. performed research; B.B. and A.B. analysed data and B.B. and R.D. wrote the paper.

Conflict of interest

The authors declare that there are no conflicts of interest.

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