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Membrane-bound glucocorticoid receptors on distinct nociceptive neurons as potential targets for pain control through rapid non-genomic effects

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ABSTRACT

Glucocorticoids were long believed to primarily function through cytosolic glucocorticoid receptor (GR) activation and subsequent classical genomic pathways. Recently, however, evidence has emerged that suggests the presence of rapid non-genomic GR-dependent signaling pathways within the brain, though their existence in spinal and peripheral nociceptive neurons remains elusive. In this paper, we aim to systemically identify GR within the spinal cord and periphery, to verify their putative membrane location and to characterize possible G protein coupling and pain modulating properties. Double immunofluorescence confocal microscopy revealed that GR predominantly localized in peripheral peptidergic and non-peptidergic nociceptive C- and Aδ-neurons and existed only marginally in myelinated mechanoreceptive and proprioreceptive neurons. Within the spinal cord, GR predominantly localized in incoming presynaptic nociceptive neurons, in pre- and postsynaptic structures of the dorsal horn, as well as in microglia. GR saturation binding revealed that these receptors are linked to the cell membrane of sensory neurons and, upon activation, they trigger membrane targeted [^{35}S]GTPγS binding, indicating G protein coupling to a putative receptor. Importantly, subcutaneous dexamethasone immediately and dose-dependently attenuated acute nociceptive behavior elicited in an animal model of formalin-induced pain hypersensitivity compared to naive rats. Overall, this study provides firm evidence for a novel neuronal mechanism of GR agonists that is rapid, non-genomic, dependent on membrane binding and G protein coupling, and acutely modulates nociceptive behavior, thus unraveling a yet unconsidered mechanism of pain relief.

1. Introduction

It is well established that glucocorticoid receptors (GR) function through classical genomic pathways in which the glucocorticoid is bound to the receptor, this complex then acts as a nuclear transcription factor and finally regulates the transcription of specific genes, a process that usually takes several hours (De Kloet et al., 1998). One of the best examples of this genomic pathway is the immunosuppressive effect of glucocorticoids, which inhibits inflammatory processes and is commonly used in the treatment of painful arthritis (Garg et al., 2014). However, apart from these rather long-term properties, glucocorticoids are also able to elicit very rapid effects, e.g, on ion channels within the hippocampus of the brain (Chatterjee and Sikdar, 2014; Groeneweg et al., 2012). For example, it has been reported that corticosterone inhibits NMDA

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(*N*-methyl-D-aspartic acid) receptor currents in cultured hippocampal neurons by using patch-clamp techniques (Liu et al., 2007). Moreover, it has been shown that topical corticosteroid application to peripheral neurons immediately blocked the transmission of nerve impulses (Johansson et al., 1990). These actions occur rapidly and independently of gene expression and, therefore, support the notion that GR may also act via non-genomic pathways on specific membrane receptors to influence cell signaling (Chatterjee and Sikdar, 2014; Groeneweg et al., 2012; Lösel and Wehling, 2003).

Since the first report on the successful treatment of sciatic pain by the administration of hydrocortisone (Lievre et al., 1953), glucocorticoids have been well perceived and widely used for their pain relieving effects (Rijsdijk et al., 2014). In addition to their systemic application, they have been successfully used both perineurally and intrathecally in combination with local anesthetics, but there is a lack of knowledge concerning the true mechanism and site of action (Knezevic et al., 2015; Albrecht et al., 2015). Until recently, it was believed that the therapeutic effect is mainly due to glucocorticoids' antiphlogistic mechanism of action either on immune cells or on activated glia cells (Albrecht et al., 2015). Therefore, previous animal studies focused primarily on the late assessment of glucocorticoids' antinociceptive effects, e.g., after several weeks, (Kingery et al., 2001; Pinto-Ribeiro et al., 2009). However, emerging evidence suggests that glucocorticoids may also have a direct effect on neurons (Chatterjee and Sikdar, 2014; Groeneweg et al., 2012; Johansson et al., 1990).

To date there has been no demonstration of membrane-bound GR on sensory neurons being responsible for alterations in nociceptive behavior through rapid, non-genomic effects. Therefore, we have set out to identify the distinct nociceptive neurons as well as glia cells that express GR in the periphery and spinal cord of naive rats. Our goal is to determine the neurons' functional role under resting conditions and situations of acute nociceptive stimulation, to characterize GR-specific membrane binding sites, and finally to test whether activation of these membrane located GR results in [^{35}S]GTPγS binding, indicating G protein coupling to a putative membrane receptor, thus unraveling a yet unconsidered mechanism of pain relief.

2. Methods

2.1. Reagents

The following drugs were used: isoflurane (1.0–2.5 vol%, Abbott, Wiesbaden, Germany); GR agonist dexamethasone (Sigma-Aldrich, St. Louis, MO, USA); dexamethasone was dissolved in a vehicle composed of 10% ethanol and saline by volume as described previously (Coderre and Yashpal, 1994). Routes and volumes of drug administration were i.t. 20 μL, i.pl. 100 μL and s.c. 100 μL. Intrathecal injections were administered through a Hamilton syringe under inhalation anesthesia. The drugs or its solvent were injected into the L3–L4 intrathecal interspace (spontaneous tail movement being a positive indication for correct i.t. positioning) with a 30-gauge needle connected to a 50 μL syringe (Shaqura et al., 2016). Intraplantar injections were given under inhalational anesthesia into the subcutaneous tissue of the glabrous skin directly proximal to the callosities of the toes. Subcutaneous injections were given under the loose skin of the back between the shoulder blades. In accordance with previous studies (Coderre and Yashpal, 1994), separate groups of animals for each dose and injection technique received i.pl., i.t. or s.c. administrations of different doses of dexamethasone (i.t. 2–20 μg, i.pl. 5–100 μg or s.c. 0.5–5 mg/kg) or vehicle in a blinded fashion.

2.2. Animals

Experiments were conducted in male Wistar rats (200–250 g) (breeding facility, Charité-Universitätsmedizin Berlin, Germany and Semmelweis University Budapest) after approval by the local animal care committee and in accordance with the European Directive introducing new animal welfare and care guidelines (2010/63/EU).

2.3. GR mRNA detection by RT-PCR

Total RNA was extracted from the kidney, spinal cord and L3-5 DRG (L3-L5 DRG pooled) of Wistar rats (n = 6) using RNeasy Kit (Qiagen, Hilden, Germany) as previously described (Mousa et al., 2016; Shaqura et al., 2016). The following specific primers for GR were used: Forward primer: 5′-CATCTTCAGAACAGCAAAATCGA-3′, Reverse primer: 5′-AGGTGCTTTGGTCT GTGGGATA-3′ (Ensembl, Accession Nr: NM_012576.2). Taqman® Real-Time PCR (Taqman® 7500, Applied Biosystems) was performed with a SYBR® Green master mix following the manufacturer's instructions (Applied Biosystems, Carlsbad, CA). Amplification was carried out for 40 cycles, each consisting of 15 s at 95 °C for GR and 18S of 30 s at 60 °C. A temperature just below the specific melting temperature (Tm) was employed for detection of fluorescence specific products (GR: Tm 83 °C, 18S: Tm 88 °C). GR mRNA was quantified using triplicates of each sample using the delta-delta CT method (Shaqura et al., 2016). The housekeeping gene 18S (Accession No. NR_046237, Forward primer: CGGCTA CCACATCCAAGGAA Reverse Primer: GCTGGAATTACCGCGGCT) was used as an internal reference gene. Experiments were done in triplicate.

2.4. Western blot

Kidney, DRG (L3-L5 DRG pooled), spinal cord and sciatic nerve from adult rats (n = 4) were solubilized according to Mousa et al. (2016) to obtain total cell protein. Western blot analyses were performed in duplicate as previously described (Mousa et al., 2016; Shaqura et al., 2016). After blotting the membranes were blocked in 3% BSA for 2 h and incubated with rabbit anti-GR (a gift from M. Kawata, Kyoto Prefectural University of Medicine, Japan; 1:4000 in 3% BSA) overnight at 4 °C. This antibody has been proven in different cell lines to be highly specific following GR-transfection and knock-down (Ito et al., 2000; Han et al., 2005). After incubation with the secondary antibody (peroxidase-conjugated goat anti-rabbit, 1:40.000, Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature, reactive protein bands were digitally visualized using ECL solutions (SuperSignal West Pico, Thermo Scientific) in ChemiDoc MP Imager.

2.5. Immunohistochemistry

After perfusion of the rats spinal cord, DRG, sciatic nerve and subcutaneous paw tissue were removed and further processed as described previously (Mousa et al., 2016; Shaqura et al., 2016). The sections were then incubated over night with the following primary antibodies (see antibodies information's in Table 1): polyclonal rabbit antibodies against GR in combination with a polyclonal guinea pig anti-CGRP, goat polyclonal anti-trkA, anti-trkB, anti-trkC, anti-RET, monoclonal mouse anti-TH, anti-NF200, anti-S100b, anti-CD11b, anti-GFAP, anti-PSD-95, anti-EAAC1, anti-GluN1/NR1, anti-GluR1 or FITC-conjugated IB4. The GR antibody (M. Kawata) has previously been shown in COS-1 cells with or without GR transfection to be highly specific (Han et al., 2005). Finally, the tissues were washed in PBS, mounted in vectashield (Vector Laboratories) and imaged on a confocal laser scanning microscope, LSM510 as described previously (Shaqura et al., 2016).

Table 1
Characterization of primary antibodies used.

Antigen	Immunogen	Manufacturer, species, type, Catalogue number	Dilution used
GR	A part of the rat GR transcription modulation domain	A gift from M. Kawata (Kyoto Prefectural University of Medicine, Japan), rabbit polyclonal, # Ito et al., 2000	1:3.000
GR	A peptide mapping at the N-terminus of GR of mouseorigin	(Santa Cruz Biotechnology, California) GR (M-20), rabbit polyclonal # sc-1004, # McKlveen et al., 2013	1:200
CGRP	Synthetic entire calcitonin gene-related peptide	Peninsula Laboratories (CA, USA), guinea pig polyclonal, # T-5027 # Mousa et al., 2013	1:1.000
NF200	Carboxy terminal tail segment of dephosphorylated NF200	Sigma-Aldrich (USA), mouse monoclonal #N0142/N52 # Kestell et al., 2015	1:1.000
SV2	Synaptic vesicle glycoprotein 2A, clone SV2A	Developmental Studies Hybridoma Bank, (IA, USA), mouse monoclonal # AB-2315387 # Wahlin et al., 2010	1:1.000
S-100 (b-subunit)	Bovine brain S-100b clone SH-B1	Sigma-Aldrich (USA), mouse monoclonal, #S2532 # Tanga et al., 2006	1:1.000
TH	An epitope in the midportion of the TH molecule	Immunostar Inc. (WI, USA), mouse monoclonal, #22941 # Berglöf et al., 2007	1:2.000
trkA	Extracellular domain Ala33-Pro418 of rat trkA	R&D Systems (USA), goat polyclonal, # AF1056 # Matsumoto et al., 2012	1:500
trkB	Extracellular domain Cys32-Thr429 of recombinant mouse trkB	R&D Systems (USA), goat polyclonal, # AF1494 # Matsumoto et al., 2012	1:500
trkC	Extracellular domain Cys32-Thr429 of recombinant mouse trkC	R&D Systems (USA), goat polyclonal, # AF1404 # Matsumoto et al., 2012	1:500
Ret	Mouse myeloma cell line NS0 derived recombinant mouse Ret Leu29Arg637	R&D Systems (USA), goat polyclonal, # AF482 # Young et al., 2003	1:300
EAAC1	Fusion protein amino acids 115-208 (extracellular domain) of rat EAAC1	NeuroMab (University of California, Davis/NIH), mouse monoclonal, #73-428 #RRID: AB_2532046	1:1.000
GluN1/NR1 (NMDAR1)	Fusion protein amino acids 42-361 (extracellular N-terminus) of rat GluN1/NR1	NeuroMab (University of California, Davis/NIH), mouse monoclonal, #75-272, #RRID:AB_11000180	1:1.000
PSD-95	Fusion protein amino acids 77-299 of human PSD-95	NeuroMab (University of California, Davis/NIH), mouse monoclonal, #75-028, # RRID: AB_2292909	1:1.000
GFAP	clone G-A-5	Sigma-Aldrich (USA), mouse monoclonal #G3893 # Liu et al., 2012	1:1.000
CD11b	clone OX-42	AbD Serotec (Germany), mouse monoclonal # MCA275R # Robinson et al., 1986	1:1.000

To demonstrate staining specificity, the following controls were included as described in our previous studies (Mousa et al., 2016): 1) omission of either the primary antisera or the secondary antibodies.

The method of quantification for DRG staining has been described previously (Mousa et al., 2016; Shaqura et al., 2016). For counting of the total number of neurons, only those immunostained neurons containing a distinct nucleus were counted using the microscope (40× objective). In a similar way, the number of GR/total DRG, mineralocorticoid receptor (MR)/total DRG, GR/MR-, CGRP/GR-, trkA/GR-, trkB/GR-, trkC/GR-, NF200/GR-, GFAP/GR- or OX42/GR-IR neurons were counted and divided by the total number of GR-IR neurons in each DRG section and represented as percentages. Data show the means of 2–3 fields of n = 5 rats by a blinded observer.

2.6. Von Frey filament testing

Rats were placed in a plastic cage for half an hour to acclimate until cage exploration and major grooming activities ceased. At the bottom of the cage, a wire mesh allowed full access to the paws. Mechanical hind paw withdrawal thresholds were assessed by the application of a calibrated series of von Frey filaments of logarithmic incremental stiffness (Stoelting, Wood Dale, IL, USA) as described previously (Shaqura et al., 2016). Briefly, the resulting pattern of positive and negative responses were tabulated using the convention, X = withdrawal; 0 = no withdrawal, and the 50% response threshold was interpolated using the formula: 50% g threshold = $(10^{(xf+k\delta)})/10{,}000$, where x_f = value (in log units) of the final von Frey filament used; k = tabular value for the pattern of positive/negative responses; and δ = mean difference (in log units) between stimuli. After baseline measurements, von Frey thresholds were reevaluated at different time intervals before and 0–60 min after i.pl. or i.t. injection of vehicle or the GR agonist dexamethasone. The mechanical paw withdrawal thresholds were defined as the mean of 5–6 animals performed before and after i.pl. or i.t. drug injections.

2.7. Formalin test

As an animal model of acutely evoked nociceptive behavior, the formalin test was performed as described by Coderre and Yashpal (1994). Briefly, to habituate the animals (n = 28) to the procedure, rats were wrapped in cloth with only their hindlimb free for three consecutive days prior to the experiments ("handling"). On the day of formalin injection, the rats were similarly wrapped in cloth and restrained by one researcher, while the other researcher injected a 2.5% solution of formalin into the plantar surface of the right hind paw in a volume of 50 μl/rat. Then, animals were moved into Plexiglas observation chambers (25 × 25 × 25 cm^3) placed on glass fixed over a mirror of 45 angle to allow free viewing of the paws. Immediately after formalin injection, the number of shaking, biting and licking behavior of the injected paw was counted. Two observation phases, phase I (0–10 min) and phase II (11–60 min) were chosen to assess the accumulated pain behavior. Different doses (0.1–5.0 mg/kg body weight) of dexamethasone or vehicle were s.c. administered 15 min prior the formalin challenge.

2.8. Radioligand binding assay

Membranes were obtained from the lumbar (L3-5) DRG, and [^{3}H]Corticosterone and [^{35}S]GTPγS binding assays were performed as described previously (Mousa et al., 2016; Shaqura et al., 2016). Saturation binding experiments were performed using GR agonist

[^{3}H]Corticosterone (specific activity 40 Ci/mmol, Hartmann Analytic, Germany). 50–100 μg of membrane protein was incubated with various concentrations of 1.25–40 nM [^{3}H]Corticosterone and 10 μM of the unlabeled GR ligand corticosterone for 1 h at 22 °C in a total volume of 1 ml of binding buffer (50 mM Tris–HCl, 5 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 0.2% bovine serum albumin). All experiments were performed in duplicate and carried out at least five times. B_{max} and K_d values in saturation binding assays were determined by nonlinear regression analysis of concentration-effect curves using GraphPad Prism (GraphPad Software Inc., CA, USA).

For [^{35}S]GTPγS binding assay DRG membranes were incubated in [^{35}S]GTPγS (specific Activity 1000 Ci/mmol, Hartmann Analytic, Germany) assay buffer containing 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, and 1 mM dithiothreitol. Concentration-effect curves were generated by incubating the appropriate concentration of membranes (30–50 μg) and varying concentrations of dexamethasone (10^{-12}–10^{-4} M), with 30 μM GDP and 0.05 nM [^{35}S]GTPγS in a total volume of 800 μl. Basal values were obtained in the absence of agonist, and nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS. The reaction mixture was incubated for 2 h at 30 °C. Bound and free [^{35}S]GTPγS were separated by vacuum filtration through GF/B filters and quantified by liquid scintillation counting. All experiments were performed in duplicate and carried out five times.

2.9. Statistical analysis

All tests were performed using Sigma Stat 2.03 software (SPSS Inc., Germany). GR mRNA data are expressed as means ± s.e.m. Paw withdrawal thresholds determined by von Frey filament testing as well as nociceptive behavioral events determined by formalin testing were expressed as means ± s.e.m and statistically analyzed by repeated measurement ANOVA and one-way ANOVA plus post-hoc Dunnett's test, respectively. For all statistical tests, significance was assumed at $P < 0.05$.

3. Results

3.1. GR specific mRNA and protein identification in DRG and spinal cord

GR mRNA transcripts were detected in kidney, spinal cord and DRG tissue of naive rats using quantitative Taqman® RT-PCR. PCR products showed primer specific hybridization curves and characteristic crossing points for GR mRNA in rat spinal cord and DRG similar to rat kidney (Fig. 1A). Translation of this mRNA into GR protein was revealed by our Western blot analysis of respective tissue extracts showing GR-immunoreactive bands at the expected molecular weight of 87 kDa (Fig. 1B), consistent with previous GR protein demonstration at a similar molecular weight in rat brain neurons (Han et al., 2005).

3.2. GR localization profile of distinct DRG neurons

Identification of DRG neuron subtypes that reveal GR immunoreactivity in naive rats by use of several neuron specific markers such as CGRP, trkA (peptidergic nociceptive C-type neurons), IB4 and/or RET (non-peptidergic nociceptive neurons), NF-200 (neurofilament 200 as marker for myelinated A-type neurons in DRG), trkB (Aδ-type neurons) and trkC (Aβ-type neurons) (Le Pichon and Chesler, 2014) demonstrated that GR-IR neurons co-localized predominantly with non-peptidergic IB4- (54.2 ± 3.9%) and RET-IR neurons (47.1 ± 7.1%) and to a lesser degree with peptidergic CGRP-IR neurons (36.8 ± 12.7%) (Fig. 2).

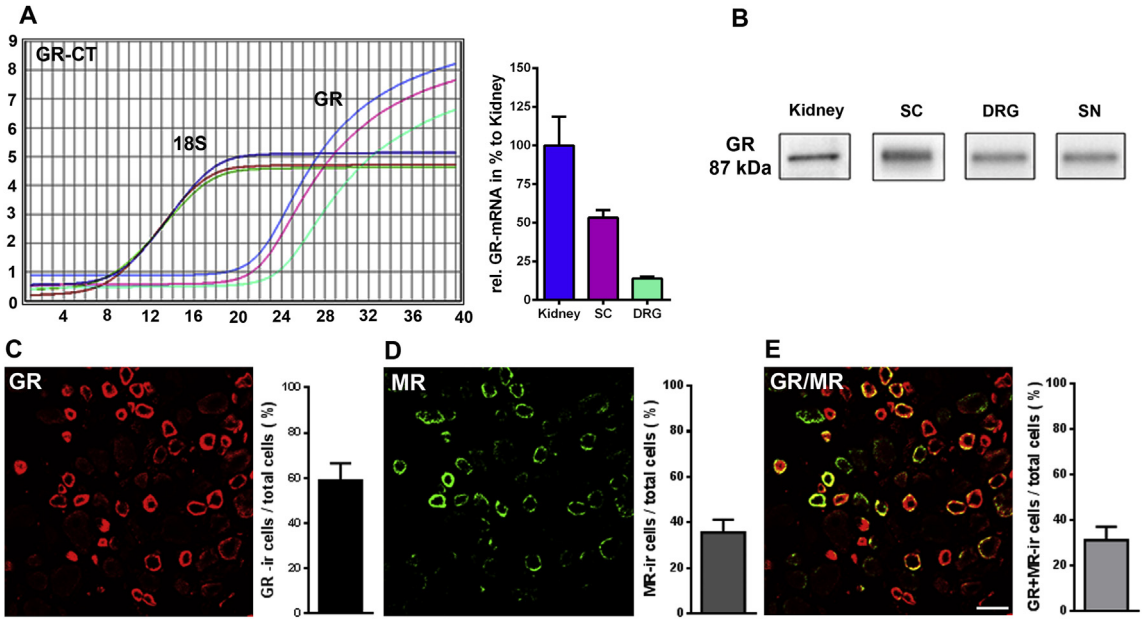

Fig. 1. Detection of GR mRNA and protein in rat kidney, spinal cord (SC), dorsal root ganglia (DRG) and sciatic nerve (SN). Quantification of GR mRNA using Taqman® Real-Time PCR in kidney, spinal cord, dorsal root ganglia of naive rats. **A**: the amplification profiles of the 18S- and GR-specific cDNA of naive rats and the column graph representing % GR mRNA expression relative to the expression in kidneys of naive rats (experiments were done in triplicate from n = 6 rats). **B**: Western blot analysis with a rabbit polyclonal anti-GR antibody shows GR specific protein bands from kidney, SC, DRG and SN of naive rats with an expected molecular weight of 87 kDa. **C-E**: Confocal microscopy of double immunofluorescence of GR (*red fluorescence*) with MR (*green fluorescence*) (Bar = 40 μm). **C and D** show the number (means ± SD) of GR-IR and MR-IR neurons in relation to the total number of DRG neurons per visual field being 58.9 ± 7.5% and 35.6 ± 5.6%, respectively (data show means ± SD of 2–3 fields of n = 5 rats). Also, note that DRG contained 1.7-fold more GR-IR than MR-IR neurons and 31.3 ± 5.9% of total DRG neurons showed colocalization of GR and MR (**E**). Bar = 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

70% of GR-IR neurons did not show colocalization with NF200 (Fig. 2) indicating that they are unmyelinated. We confirmed the colocalization of GR with CGRP using another GR polyclonal antibody (M-20, Santa Cruz) (Supplemental Fig. 1). Similar to the extent of GR colocalization with CGRP- and NF200, GR colocalized with trkA (33.6 ± 7.0%) and trkB (26.3 ± 3.9%) (Fig. 3). There was only little colocalization between GR and trkC (12.6 ± 4.1%) (Fig. 3). Moreover, the number of GR-IR and MR-IR DRG neurons in relation to the total number of DRG neurons per visual field were 58.9 ± 7.5% and 35.6 ± 5.6%, respectively (Fig. 1C, D), indicating that DRG contained 1.7-fold more GR-IR than MR-IR neurons. Approximately 31.3 ± 5.9% of total DRG neurons showed a colocalization of GR with MR (Fig. 1E). GR were not identified in satellite cells of DRG. These findings indicate that – in addition to GR expressing peptidergic CGRP-/trkA-IR sensory neurons – they are localized to a major part in nociceptive non-peptidergic IB4- and RET-IR DRG neurons (Honda et al., 1999), however, much less in mechanoreceptive and proprioceptive neurons (Le Pichon and Chesler, 2014).

3.3. GR localization profile of nociceptive neurons in sciatic nerve and skin

In sections of the sciatic nerve and longitudinal sections of skin nerves GR-immunoreactive nerve fibres colocalized with CGRP almost to the same extent as was shown for DRG sections (Fig. 4A). Consistently, the majority of nerve fibres of the sciatic nerve and skin showed immunoreactivity for CGRP or GR alone. GR-IR nerve

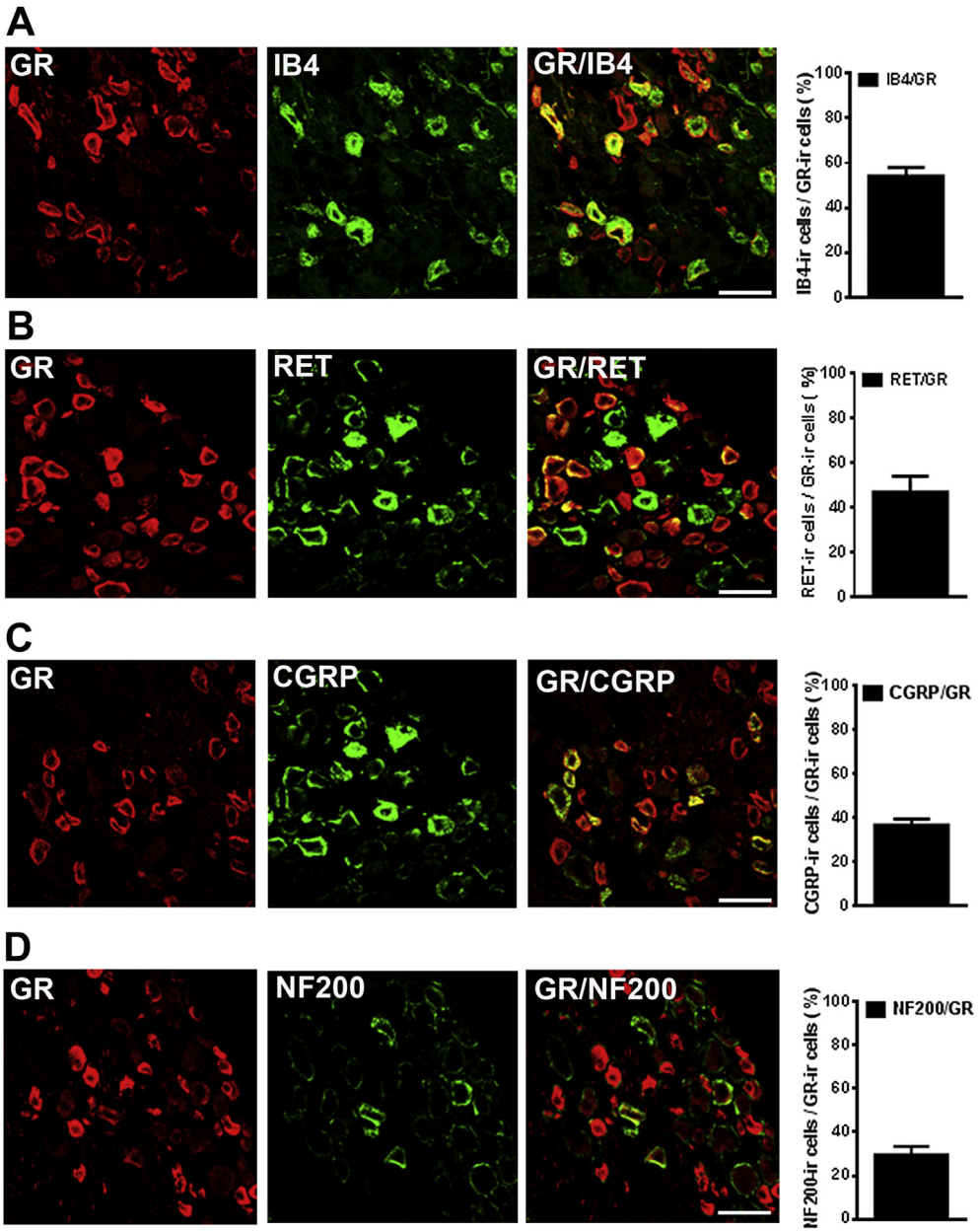

Fig. 2. Confocal microscopy of double immunofluorescence of GR (*red fluorescence*) with IB4, RET, CGRP, or NF200 (*green fluorescence*) in DRG of naive rats. A–D images: coexistence of GR (*red*) with IB4, RET, CGRP, or NF200 (*green*) with some cells showing IB4, RET, CGRP, or NF200 staining alone. **A–D columns**: Quantitative analysis shows the number of DRG neurons coexpressing GR with IB4 (54.2 ± 3.9%), RET (47.1 ± 7.1%), CGRP (36.8 ± 12.7%) or NF200 (29.5 ± 3.9%). Data show means ± SD of 2–3 fields of n = 5 rats. Bar = 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fibres mostly ended in branching nerve terminals within the subepidermal layer, where they usually extended into the epidermis (Fig. 4B). Most notably, GR-immunoreactivity strongly colocalized with S100-IR Schwann cells in both sciatic nerve and skin (Fig. 4C, D). In the skin GR-IR cells were also identified as keratinocytes within the basal layer of the epidermis (Fig. 4D) as well as TH-ir nerve fibres in the immediate vicinity of arterial vessels (data not shown).

3.4. GR expression profile of nociceptive neurons and glia in the spinal cord

In the spinal cord, GR colocalized to some degree (approximately 30%) with the neuropeptide CGRP in presynaptic nociceptive nerve terminals incoming from DRG to Rexed laminae I and II of the dorsal horn (Fig. 5A), whereas the majority of spinal CGRP staining did not colocalize with GR. In addition, GR-IR nerve fibres colocalized with non-peptidergic RET-IR neurons from the periphery in lamina I and II of the dorsal horn (see Jongen et al., 2007), however, there was no overlap with NF200 (Fig. 5B).

Next, we examined the co-localization of GR with several neuronal markers for synaptic proteins: glutamate receptor subtype *N*-methyl-D-aspartate (NMDA) (GluN1/NR1) and Glutamate receptor 1 (GluR1), glutamate transporters EAAC-1 (excitatory amino acid carrier). Interestingly, a large number of cell profiles expressing GR also expressed GluN1/NR1, GluR1 and EAAC-1 within the superficial laminae of the spinal dorsal horn (Figs. 5 and 6). Consistently, GR immunorectivity co-localized with postsynaptic density protein 95 (PSD-95) in the dorsal horn of the spinal cord indicating a close cellular association between GR and GluN1/NR1 and GluR1 on postsynaptic neurons (Fig. 6). In addition, GR colocalized with the microglia marker OX-42 but only scarcely with the astrocyte marker GFAP in lamina I and II of the grey matter as well as within the white matter of the spinal cord (Fig. 7).

3.5. GR impact on nociception in naive rats and in rats with acute nociceptive stimulation

To assess the functional relevance of GR on nociceptive neurons, we evaluated possible alterations in mechanical withdrawal thresholds during von Frey filament testing following the i.pl. and i.th. administration of increasing doses of the GR agonist dexamethasone in naive rats. The results showed that over a large dose range of dexamethasone there was no significant change in mechanical thresholds of naive rats (Fig. 8A, B). However, when we tested s.c. dexamethasone administration in a rat model of acute nociceptive stimulation by i.pl. injection of formalin we observed a dose-dependent inhibition of characteristic pain behavior such as shaking, biting and licking particularly in the second phase (11–60 min), but not in the first phase (0–10 min) of the formalin test ($P < 0.05$; Fig. 8C).

3.6. GR specific binding sites and [^{35}S]GTPγS coupling in membranes of rat DRG neurons

In membrane fractions of DRG extracts of naive rats, increasing concentrations of [^{3}H]corticosterone in the presence of 10 μM unlabeled corticosterone led to the identification of GR specific binding sites with a saturation curve that showed a B_{max} value of 112 ± 18.5 fmol/mg protein and a K_d value of 16.9 ± 6.1 nM (Fig. 8D). More interestingly, increasing concentrations of the GR agonist dexamethasone resulted in saturated [^{35}S]GTPγS coupling most likely

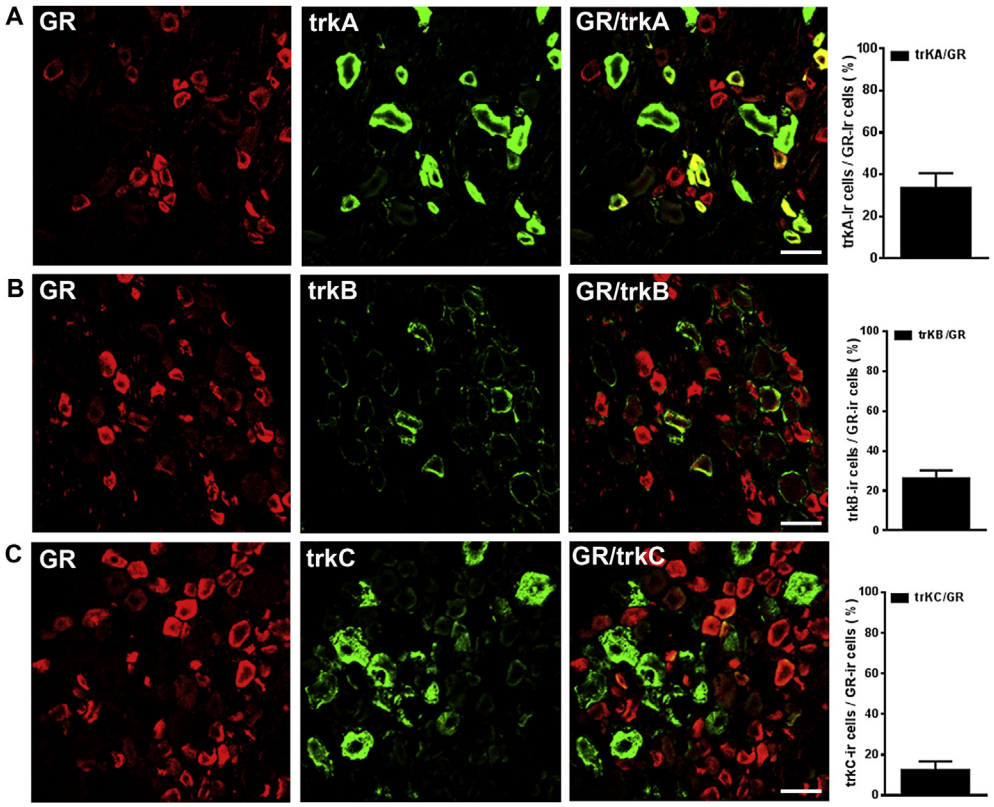

Fig. 3. Confocal microscopy of GR (*red fluorescence*) and trkA, trkB or trkC (*green fluorescence*) double immunofluorescence in DRG of naive rats. Note that the population of DRG neurons coexpressing GR with trkA (33.6 ± 7.0%) is much higher than those coexpressing trkB (26.3 ± 3.9%) or trkC (12.6 ± 4.1%). Data show means ± SD of 2–3 fields of n = 5 rats). Bar = 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to a putative G protein coupled receptor (EC_{50} = 0.46 ± 0,03 μM) ($P < 0.05$; one-way ANOVA and Dunnett's test) (Fig. 8E).

4. Discussion

This study provides first evidence for acute pain inhibition by the GR-selective agonist dexamethasone through rapid non-genomic effects. These effects are most likely mediated by a putative, membrane-bound G protein-coupled receptor on peripheral nociceptive neurons. Here we show that GR predominantly localize on peripheral nociceptive C- and Aδ-neurons and only scarcely on populations of myelinated mechanoreceptive and proprioceptive neurons. In the spinal cord, GR exist in presynaptic nociceptive nerve terminals incoming from DRG to Rexed Laminae I and II, as well as postsynaptic structures within the superficial dorsal horn. In contrast to a lack of MR localization in glia cells

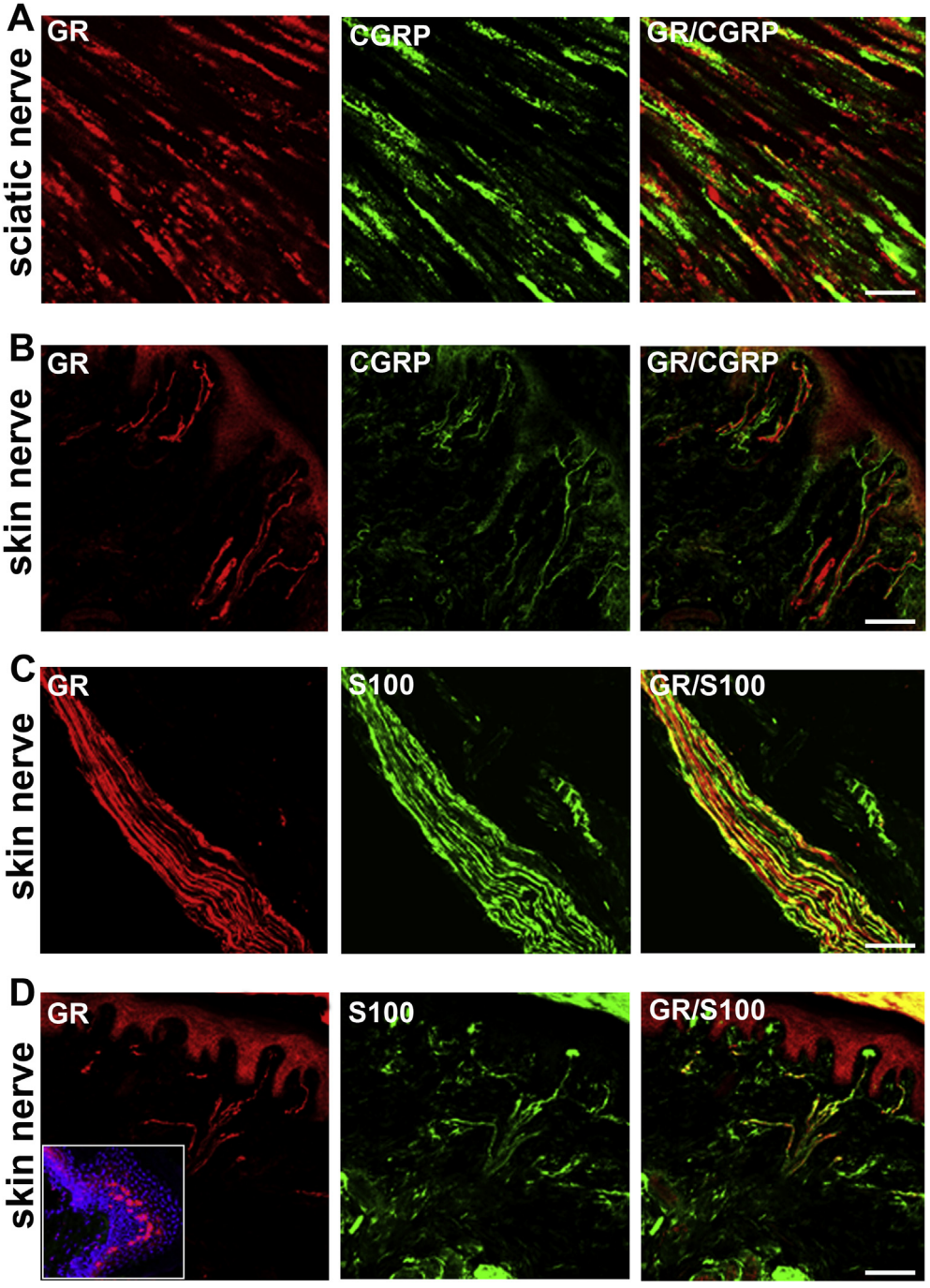

Fig. 4. Confocal microscopy of double immunofluorescence of GR (*red fluorescence*) with CGRP or S100 (*green fluorescence*) in sciatic nerve and subcutaneous tissue. A–B: colocalization of GR immunoreactive nerve fibres (red) with the neuropeptide CGRP (green) in nerve fibres of the sciatic nerve (**A**) and in branching nerve terminals within the sub-epidermal layer of the skin which usually extend into the epidermis (**B**); **C-D**: colocalization of GR immunoreactivity (*red*) with the Schwann cell marker S100 (*green*) accompanying nerve fibres of subcutaneous tissue (**C**) and branching nerve terminals into the sub-epidermal layer of the skin (**D**); note that the magnified insert shows GR-IR keratinocytes in the basal layer of the epidermis (**D**). Representative sections of n = 5 rats. Bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Shaqura et al., 2016), GR are prominent in Schwann cells of peripheral nerves and in spinal microglia but only rarely present in astrocytes. Importantly, i.pl. or i.t. application of dexamethasone does not alter mechanical sensitivity in naive animals, but its s.c. application immediately inhibits acute nociceptive behavior elicited in an animal model of formalin-induced pain hypersensitivity. Consistently, the activation of GR specific membrane binding sites in peripheral sensory neurons leads to saturated [^{35}S]GTPγS coupling.

4.1. GR localization profile in sensory DRG neurons and periphery

Using RT-PCR we were able to isolate GR specific transcripts from rat spinal cord and DRG similar to control tissue such as

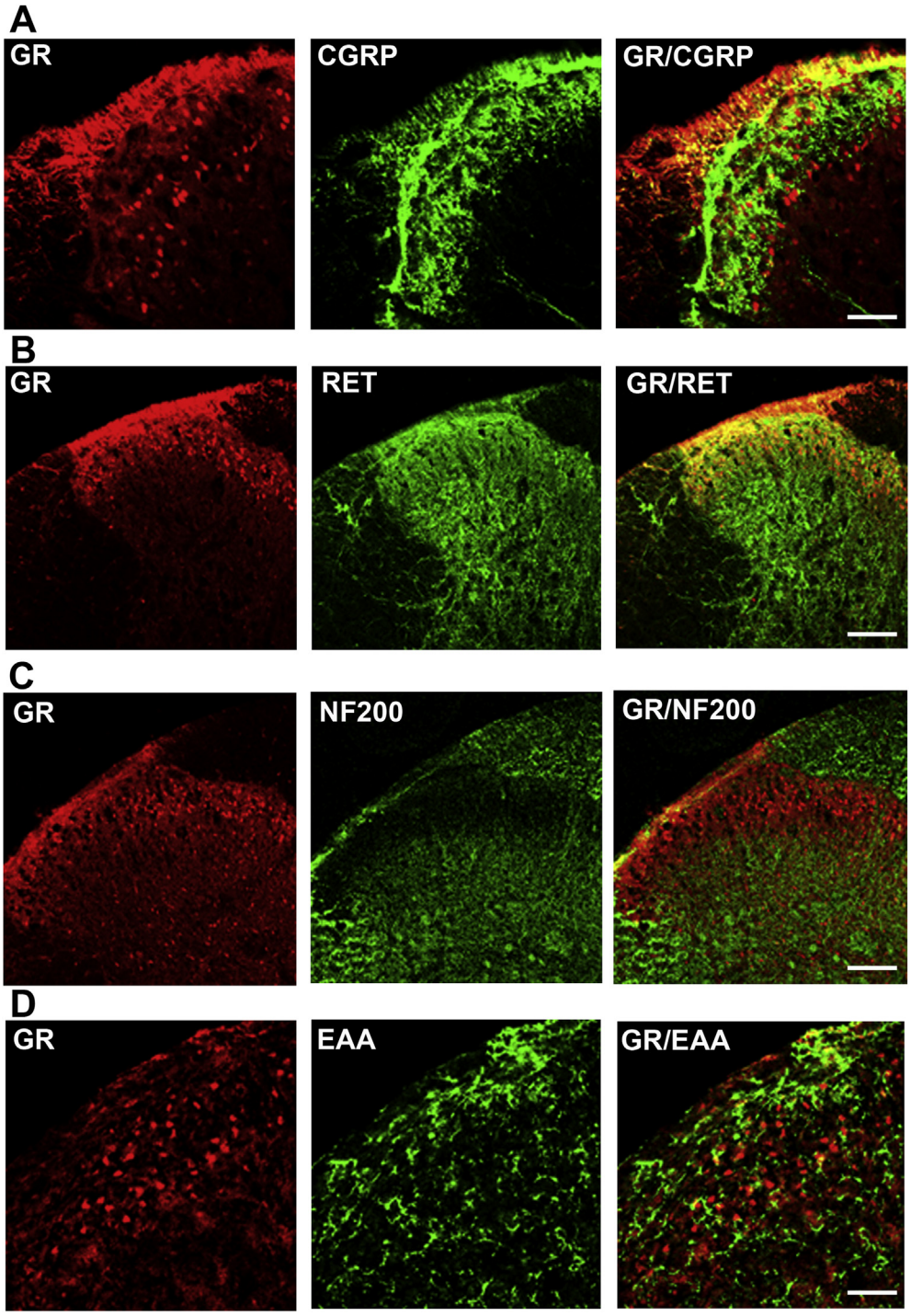

Fig. 5. Confocal microscopy of double immunofluorescence of GR (*red fluorescence*) with CGRP, RET, NF200 or EAA (*green fluorescence*) in the dorsal horn of the spinal cord. A, B: show that GR colocalized to some degree with CGRP or RET in presynaptic nociceptive nerve terminals incoming from DRG to Rexed laminae I and II of the dorsal horn, whereas the majority of spinal CGRP or RET immunoreactivity did not colocalize with GR. **C**: There was almost no overlap with NF200. **D**: shows that some cell profiles expressing GR also expressed EAAC-1 within the superficial laminae of the spinal dorsal horn. Representative sections of n = 5 rats. A–C Bar = 20 μm, D Bar = 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

kidney tissue. These findings are consistent with former in situ hybridization studies demonstrating GR-hybridization products in the brain and spinal cord (Marlier et al., 1995; Aronsson et al., 1988) and GR mRNA in the spinal cord (Patacchioli et al., 1998; Marlier et al., 1996). To prove that GR transcripts were indeed translated into receptor proteins, we performed Western Blot analysis that showed a single GR specific protein band at the expected molecular weight in kidney, spinal cord and DRG neurons, consistent with GR protein demonstration at a similar molecular weight in rat brain neurons (Han et al., 2005).

Previous immunohistochemical studies stated that GR coexist with the neuropeptides substance P and CGRP in a specific subpopulation of DRG neurons (DeLeón et al., 1994). Extending these earlier findings, we demonstrate now that GR do not only colocalize with peptidergic CGRP-IR nociceptive neurons but more so with non-peptidergic IB4-IR/RET-IR DRG neurons. The former have been

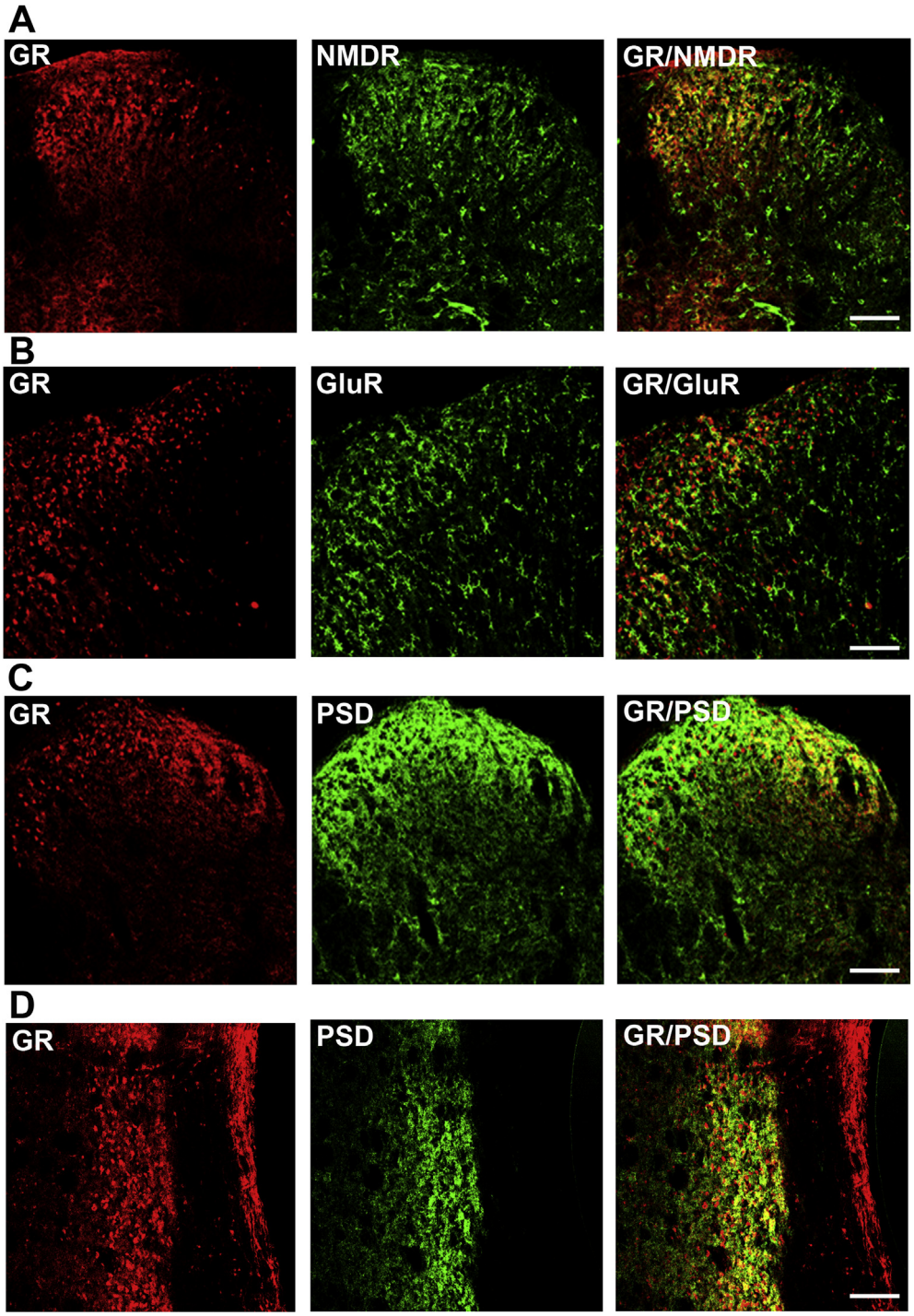

Fig. 6. Confocal microscopy of double immunofluorescence of GR (*red fluorescence*) with NMDR, GluR or PSD (*green fluorescence*) in the dorsal horn of the spinal cord. A–C: Transverse sections of the spinal cord showing the majority of cell profiles coexpress GR (*red*) with NMDR, GluR or PSD (*green*) within the superficial laminae of the spinal dorsal horn. **D**: longitudinal sections of the spinal cord showing colocalization of GR- (*red*) with PSD- (*green*) immunoreactivity. Representative sections of n = 5 rats. A, B Bar = 40 μm, C, D Bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

suggested to address rather the sensory discriminative component of pain, whereas the latter focuses on the affective emotional component of the pain experience (Braz et al., 2005). Therefore, GR distribution in DRG neurons is quite distinct from MR-IR sensory neurons in the way that MR colocalize to a much higher degree (approximately 75%) with CGRP (Shaqura et al., 2016). In the present study, 34% of GR-IR DRG neurons show trkA immunoreactivity to a similar degree as their CGRP expression, defining them as nociceptive C-fibres (Matsumoto et al., 2012). On the other hand, much less GR-IR neurons seem to colocalize with trkB as well as trkC, thus suggesting that only few GR-IR neurons function as cutaneous Aδ nociceptors or Aβ mechanoreceptors (Matsumoto et al., 2012). This is consistent with the notion that the majority of GR-IR nociceptive neurons (~70%) prove to be unmyelinated neurons.

In addition to sensory neurons, we identified GR in numerous S100-IR Schwann cells that accompanied peripheral nerves in the skin in contrast to MR, which showed no colocalization with Schwann or glia cells (Shaqura et al., 2016). Earlier reports have suggested a functional role of GR in glucocorticoid-induced Schwann cell proliferation (Neuberger et al., 1994), myelination (Morisaki et al., 2010), regeneration (Morisaki et al., 2010) and gene expression (Girard et al., 2010). In the skin, we also identified GR-IR cells in keratinocytes within the basal layer of the epidermis, consistent with the long history of glucocorticoids as therapeutic agents for numerous skin diseases (Stojadinovic et al., 2007). Taken together, our findings indicate that GR are predominantly expressed in nociceptive peptidergic and nonpeptidergic nociceptive C- and Aδ-neurons, yet much less in mechanoreceptive and proprioceptive neurons (Matsumoto et al., 2012). This implies the receptors' crucial role in modulating various aspects of nociception.

4.2. GR expression profile of nociceptive neurons and glia of the spinal cord

At the spinal level, double fluorescence confocal microscopy predominantly colocalized GR with incoming peptidergic CGRP-IR and non-peptidergic RET-IR nerve fibres in Rexed Lamina I and II of the dorsal horn. Earlier studies reported GR localization especially in substance P- and CGRP-IR DRG neurons (DeLeón et al., 1994). Further supportive evidence, showed that corticosterone deprivation by adrenalectomy alters the content of both neuropeptides in primary afferent neurons (Smith et al., 1991). However, GR colocalization with non-peptidergic RET-IR neurons was still unknown.

In contrast to a former study that reported an overlap between GR and EAAC1 expression in the spinal cord (Wang et al., 2006), our results show that EAAC1 immunoreactivity in GR-expressing spinal

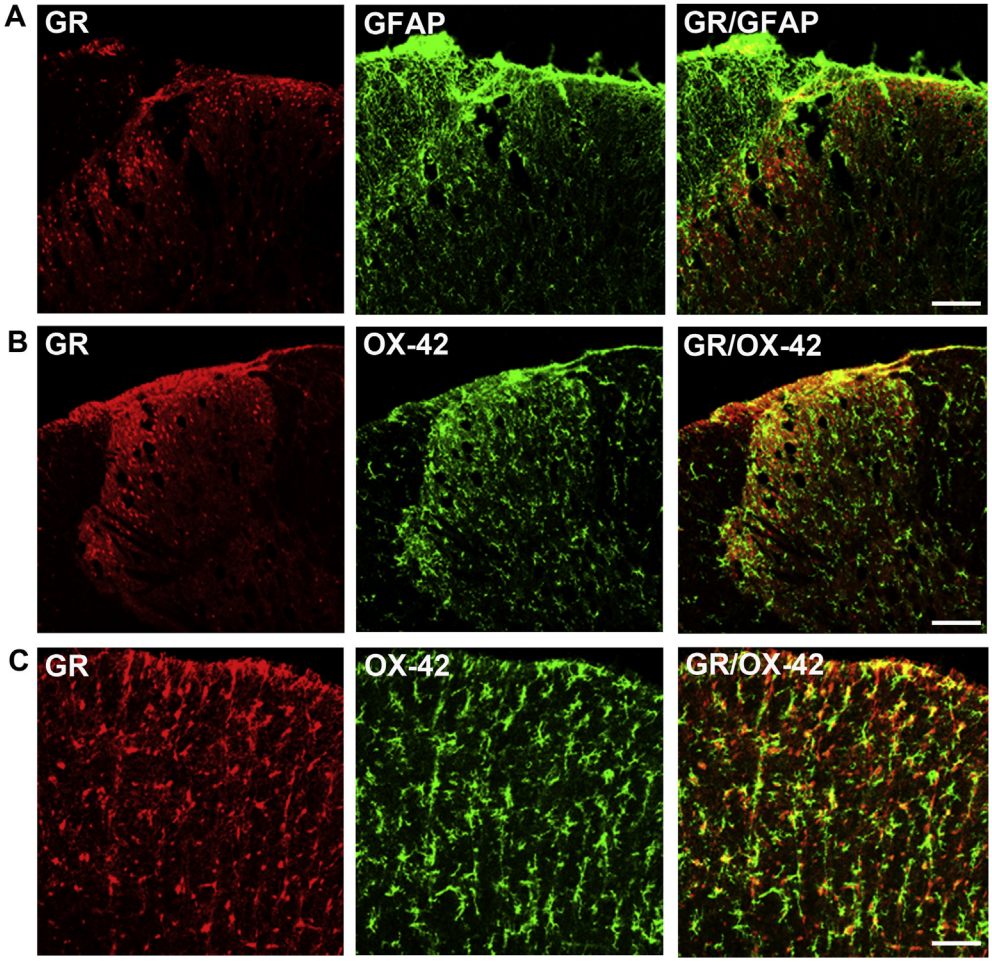

Fig. 7. Confocal microscopy of double immunofluorescence of GR (*red fluorescence*) with GFAP or OX-42 (*green fluorescence*) in the dorsal grey and white matter of the spinal cord. A: scarce colocalization of GR-immunoreactivity (red) with the astrocyte marker GFAP (green) throughout the dorsal horn; **B, C**: abundant colocalization of GR-immunoreactivity (red) with the microglia marker OX-42 (green) in the dorsal grey and white matter of the spinal cord. Representative sections of n = 5 rats. A, B Bar = 20 μm, C Bar = 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

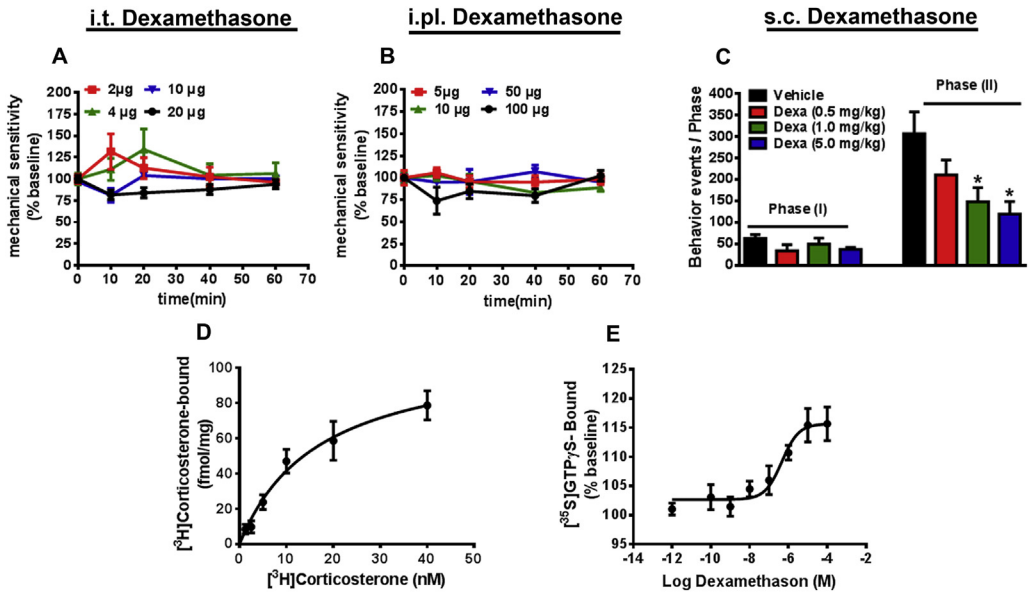

Fig. 8. Role of GR in pain behavior, membrane binding and [^{35}S]GTPγS coupling. A–C: von Frey filament or formalin testing of mechanical sensitivity following the local i.pl., i.t. or s.c. application of dexamethasone. A, B: show that there was no significant change in mechanical thresholds of naive rats (n = 5–6) in response to i.pl. (**A**) or i.t. (**B**) injection of increasing doses of the GR agonist dexamethasone ($P > 0.05$, repeated measurement ANOVA). **C**: shows that s.c. dexamethasone significantly and dose dependently reduced formalin-induced pain behavior (shaking, biting and licking responses) in the second phase (11–60 min) but not in the first phase (0–10 min) of the formalin test ($P < 0.05$, ANOVA and Dunnett's test, n = 7). **D, E**) Saturation binding analysis of GR specific binding sites (D) and [^{35}S]GTPγS coupling (E) to cell membranes of DRG neurons. (**D**) In naive rats (n = 5), increasing concentrations of [^{3}H]Corticosterone in the presence of 10 µM unlabeled specific GR agonist corticosterone led to a specific saturation curve with a B_{max} value of 112 ± 18.5 fmol/mg protein and a K_d value of 16.9 ± 6.1 nM (**E**) shows that increasing concentrations of the GR agonist dexamethasone resulted in saturated [^{35}S]GTPγS-coupling most likely due to GR-induced G protein coupling (EC50 = 0.46 ± 0.03 µM). Data represent experiments from 5 rats in duplicate and are expressed as means ± SEM.

neurons within the dorsal horn of the spinal cord is scarce in naive rats. In addition, we were able to demonstrate that GR colocalize to a great extent with pre- and postsynaptic NMDA receptors, GluR1, and PSD, consistent with the known modulatory role of glucocorticoids in synaptic processes such as neurotransmission, biogenic amine content, lipid metabolism, and specific enzyme activity (De Nicola et al., 1998). In addition to spinal neurons, GR are also expressed in OX-42-IR microglial cells both in the grey and white matter of the spinal cord, though much less in GFAP-IR astrocytes. Evidence for the presence of GR in primary cultures of spinal glia cells has been demonstrated in earlier studies (Chantong et al., 2012; Sierra et al., 2008), and immunohistochemical evidence has previously only been shown in various brain areas, such as the hippocampus (Cintra et al., 1994).

4.3. Functional role of GR in nociception

In oder to examine the functional role of these spinal and peripheral GR, we applied increasing doses of the GR-selective agonist dexamethasone into the hindpaw or intrathecal space of naive rats and tested possible alterations in mechanical sensitivity with von Frey filaments. The doses were chosen based on previous publications that showed significant effects on nociceptive behavior in different animal models of pain (Min et al., 2014; Zhang et al., 2014; Gu et al., 2011). However, in naive rats we did not find any significant change in mechanical thresholds following either i.pl. or i.th. administration of dexamethasone. This contrasts with the i.pl. and i.t. application of MR agonists which resulted in acute mechanical sensitization (Shaqura et al., 2016) yet is in line with previous *in vivo* evidence that dexamethasone applied to silent nociceptive neurons does not modulate A- or C-fiber conduction (Johansson et al., 1990; Devor et al., 1985). On the other hand, dexamethasone substantially reduced the incidence of spontaneous ectopic discharge generated in experimental nerve end neuromas (Devor et al., 1985) or inhibited the transmission of electrical impulses in C-, but not Aβ-nerve fibres (Johansson et al., 1990). We therefore tested the modulatory effects of the GR-selective agonist dexamethasone in an animal model of acute nociceptive stimulation such as the formalin test. Indeed, s.c. injection of dexamethasone resulted in the dose-dependent inhibition of formalin-evoked nociceptive behavior corroborating previous findings of antinociceptive effects following the spinal application of dexamethasone (Min et al., 2014; Coderre and Yashpal, 1994). Since the inhibition of nociceptive behavior occurred within 15–20 min and persisted for approximately 60 min after dexamethasone application, this questions the traditionally held presumption that the inhibition occurs through genomic mechanisms. Instead, recent evidence shows that neuronal GR agonists may also elicit their effects through non-genomic ways (Chatterjee and Sikdar, 2014; Groeneweg et al., 2012). These non-genomic pathways can be elicited either by directly interfering with intracellular signaling pathways or by interfering with membrane-bound structures such as ion channels and/or G-protein coupled receptors (Chatterjee and Sikdar, 2014; Groeneweg et al., 2012).

4.4. Membrane localization and G protein coupling of sensory neuron GR

Thus, we further corroborated the evidence for a putative non-genomic pathway by demonstrating GR binding sites in membrane fractions of DRG neurons of naive rats similar to its previous demonstration in the brain (Orchinik et al., 1992). Our immunofluorescence confocal microscopy consistently showed that GR immunoreactivity was not identifiable in the nuclear but rather in the cytosol/plasma membrane compartment of DRG neurons in naive rats. The presence of GR has also been shown in synaptosome extracts of the brain (Komatsuzaki et al., 2005; Wang and Wang, 2009) and, as enriched densities, at neuronal plasma membranes

using electron microscopy (Johnson et al., 2005; Prager et al., 2010). In addition to GR membrane binding, we observed for the first time that increasing concentrations of the GR agonist dexamethasone resulted in saturated [^{35}S]GTPγS coupling, most likely due to a putative G protein coupled receptor. This coupling has been previously postulated for neurons in the brain as there is accumulating evidence for the formation of a ternary complex of steroid, receptor and guanine nucleotide-binding protein (Orchinik et al., 1992) and for inhibitors of G proteins that abolish many glucocorticoid effects (Groeneweg et al., 2012). In addition, dexamethasone has been shown to activate postsynaptic, membrane-associated receptors and G protein signaling. This elicits a rapid suppression of excitatory postsynaptic inputs or a rapid nitric oxide-dependent increase in inhibitory postsynaptic inputs (Naher et al., 2015). Taken together, these findings strongly suggest a potential rapid, non-genomic mechanism for GR mediated pain control most likely occurring through the involvement of a G protein coupled receptor.

5. Conclusions

Taken together, this study provides firm evidence for the expression of GR being predominantly in nociceptive (unmyelinated C-fibres and Aδ−fibres) but much less in mechanoreceptive and proprioceptive neurons (myelinated large diameter Aβ-fibres) of DRG and the periphery of naive rats. In addition, GR co-localize with incoming presynaptic nociceptive nerve terminals, glutamatergic pre- and postsynaptic structures, however much less with microglia and astrocytes within the dorsal horn of spinal cord. The results furnish functional evidence for a novel neuronal mechanism of GR agonists in nociceptive neurons that has been previously explored in other neuronal networks. This mechanism in nociceptive neurons is rapid, non-genomic, dependent on membrane binding and G protein coupling, and acutely modulates nociceptive behavior, thus unraveling a yet unconsidered mechanism of pain relief.

Conflicts of interests

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2016.08.019.

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