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Original experimental

Effect of intrathecal glucocorticoids on the central glucocorticoid receptor in a rat nerve ligation model

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HIGHLIGHTS

- The efficacy of neuraxial glucocorticoids for neuropathic pain is subject to debate.
- Glucocorticoids act mainly through their receptor (GR).
- Spinal nerve ligation increases spinal GR protein levels but not GR mRNA levels.
- After intrathecal glucocorticoid treatment only spinal GR mRNA decreases.
- This does not result in decreased ligation-induced mechanical hypersensitivity.

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ABSTRACT

Background and aims: Despite widespread use, the efficacy of neuraxial glucocorticoids for neuropathic pain is subject to debate. Since most glucocorticoid actions are mediated through its receptor, we explored the effects of intrathecal methylprednisolone acetate (MPA) on total glucocorticoid receptor (tGR) levels and activation of the glucocorticoid receptor (phosphorylated state = pGR) within the spinal dorsal horn (SDH) and dorsal root ganglion (DRG) in a spinal nerve ligation (SNL) model in rats.
Methods: Rats received unilateral ligation of the L5/L6 spinal nerves and were treated with two intrathecal doses of either 400 μg MPA or 0.9% saline with a 72-h interval. Plantar tactile thresholds were measured over time. Seven days after drug treatment, DRG and SDH were harvested to assess tGR and pGR levels using immunohistochemistry and qPCR.
Results: Allodynia, defined by lowered tactile withdrawal thresholds after SNL, was unaltered by intrathecal MPA. In saline controls, mRNA levels of *tGR* did not change after SNL in the DRGs or SDH. tGR and pGR protein levels in the SDH however, significantly increased on the ipsilateral side of SNL compared to the contralateral side and to naïve tissue. When treating rats with MPA, *tGR* mRNA levels were significantly reduced in the SDH compared to saline controls. tGR and pGR protein levels, however were not significantly lower compared to saline controls.
Conclusions: In intrathecal MPA treated rats, *tGR* mRNA levels decreased after SNL. However this did not result in lower tGR and pGR protein levels compared to saline controls, and did not decrease ligation-induced mechanical hypersensitivity.
Implications: Intrathecal MPA treatment after SNL did not result in lower tGR and pGR levels within the SDH and DRG compared to saline controls. In present study we did not differentiate between the various isoforms of the GR which might clarify this finding.

1. Introduction

As glucocorticoids act upon a variety of crucial targets in pain pathways [1], they should be potent long acting analgesic agents. However, despite widespread use of neuraxial glucocorticoids in pain medicine, their efficacy is subject to debate. There is consensus

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only on a short lasting analgesic effect in low back pain patients [2], but not in sustained pain states or for use in neuropathic pain syndromes. Preclinical and clinical results, in fact show varying effects of glucocorticoids from analgesic to hyperalgesic effects [3–10].

Our research group conducted a randomized controlled clinical trial in which we encountered disappointing analgesic effects with intrathecal methylprednisolone acetate (MPA) in patients suffering from postherpetic neuralgia [6]. Four intrathecal injections with MPA with 7-day intervals were administered in patients with intractable neuropathic pain. Patients treated with intrathecal MPA reported increased pain and with statistical evidence of futility, the trial was ended early. Our results were in sharp contrast with results of an earlier trial published in the *New England Journal of Medicine* with a similar drug and dosing regime, showing pain reduction in 92% of patients in the intrathecal MPA treated group [11]. Since we did not understand the differences in results between the two trials, we decided to conduct a preclinical study using a similar MPA formulation. In that study we researched the effect of intrathecal MPA in three established rodent pain models; carrageenan, formalin or spinal nerve ligation (SNL) model [12]. No acute analgesic effects were observed with intrathecal MPA in any of the three models. One of the questions that rose was if the glucocorticoid receptor is involved in the absence of the analgesic effects of intrathecal glucocorticoids.

On a cellular level, glucocorticoids mediate their actions primarily by binding to the glucocorticoid receptor (GR). The GR also known as NR3C1, is a ligand-driven transcription factor. Upon binding with a glucocorticoid, GR phosphorylates into an active form (pGR) and translocates to the nucleus where it affects expression of specific sets of genes by transcriptionally activating or repressing them [13]. In addition, glucocorticoids may evoke fast non-genomic neuronal responses by binding to membrane-bound or cytosolic GR or by effects not mediated by a receptor [13].

It is unclear exactly how glucocorticoids would act to regulate or modify pain signalling. After nerve injury, plasma cortisol levels and GR expression in the spinal cord are increased, indicating an elevated glucocorticoid activity [3,10,14]. Exogenous glucocorticoids may influence the endogenous increased plasma cortisol and GR expression in the spinal cord in several ways. They may increase GR binding and activity and stimulate its downstream actions, and down regulate endogenous cortisol levels and GR expression via a negative feedback mechanism. It is not known what the net effect of exogenous glucocorticoids on spinal GR levels in an acute pain state is. Therefore we examined if an intrathecal administered glucocorticoid, methylprednisolone acetate (MPA), alters (i) pain-like behaviour and (ii) total (tGR) and activated (pGR) glucocorticoid receptor levels within the spinal dorsal horn (SDH) and dorsal root ganglion (DRG) in a SNL model in rats.

2. Materials and methods

The protocol of the present study has been approved by the AAALAC accredited (International Association for Assessment and Accreditation of Laboratory Animal Care) Institutional Animal Care and Use Committee (IACUC) of the University of California, San Diego, USA.

2.1. Animals

Male Harlan Sprague-Dawley rats, 80–100 g (Indianapolis, IN, USA), were maintained 2 per cage in standard cages at room temperature on a 12:12 h light/dark cycle with free access to food and water. After arrival at the housing facility, they were allowed at least 2–3 days of acclimation before use. Experiments have been carried out during light cycle.

2.2. Spinal nerve ligation (SNL) model

Spinal nerve injury was induced by the procedure described by Kim and Chung [15]. Briefly, the left L5 and L6 lumbar spinal nerves were exposed in isoflurane 2.4%/oxygen-anesthetized rats and tightly ligated with 6.0 silk suture at a point distal to their DRGs and proximal to their conjunction to form the sciatic nerve. Rats were given post-operative subcutaneous fluids including analgesics (lactated Ringers + 5 mg/kg Carprofen) and then housed 2 per cage for post-operative recovery. Withdrawal thresholds were obtained at 0, 1, 3, 7, 18, 21 and 25 days after SNL for all rats.

2.3. Behavioural measurements

All behavioural measurements were made by observer (MR) blinded to the treatment groups and were conducted at fixed times (9:00 a.m. to 5:00 p.m.). The thresholds for mechanical allodynia were measured with a series of calibrated von Frey filaments (Stoelting, Wood Dale, IL, USA), ranging from 3.16 to 5.18 (0.41–15.0 g). The animals were acclimated for 45 min in the test chamber with mesh floors and von Frey filaments were applied perpendicularly against the plantar surface of the paw. The "up–down" method of Dixon as described by Chaplan [16] was used to determine the value at which paw withdrawal occurred 50% of the time, interpreted as the mechanical threshold.

2.4. Intrathecal catheter implantation and drug administration

On postoperative day 13 after SNL, when all rats were weighing more than 200 g, intrathecal catheters were implanted for drug injections. Rats were surgically implanted with intrathecal catheters under isoflurane 2.4%/oxygen inhalation anaesthesia as described previously by Yaksh and Rudy [17]. The catheter tip was located at the lumbar level of the rat spinal cord. Intrathecal catheters were externalized for injection. Rats were given post-operative subcutaneous fluids including analgesics (lactated Ringers + 5 mg/kg Carprofen) and then housed individually for post-operative recovery. Following implantation, catheters were flushed with saline and rats were monitored daily for viability, allowing 5 days of recovery before testing. Animals showing any evidence of motor dysfunction or distress after catheter placement were immediately euthanized using a carbon dioxide chamber.

On postoperative day 18 after SNL, rats were randomized to either the methylprednisolone acetate (MPA) group or the saline control group. Before administration, the presence of preservatives in the MPA preparation (depo-medrol® from Pfizer) was minimized as described in more detail before using saline as a vehicle [18]. The MPA group received 400 μg (10 μl) of the suspension followed by 10 μl 0.9% saline flush through the intrathecal catheter. In the saline group a total of 20 μl of 0.9% saline was injected.

Intrathecal injections were given twice with a 3-day interval, on postoperative days 18 and 21. This dosing interval was chosen based on pharmacokinetic data from our previous study showing that after intrathecal MPA administration, MP plasma levels went below the level of detection after 72 h [12]. We decided to expose rats to two periods of high levels of MPA before sacrifice, since the transcriptional activation or repression of genes by glucocorticoids can take 24–48 h [13].

2.5. Tissue collection

On postoperative day 25 after SNL, 7 days after the start of intrathecal drug treatment, spinal cord and dorsal root ganglia were collected from all rats and processed for either immunohistochemistry or quantitative real time PCR (qPCR). For immunohistochemistry, tissues were collected from rats subjected

to (i) SNL only, (ii) SNL + intrathecal saline, and (iii) SNL + intrathecal MPA ($n = 3$ rats/group). Three additional control groups were added: (i) naive rats ($n = 4$), (ii) naive rats + intrathecal saline ($n = 3$), and (iii) naive rats + intrathecal MPA ($n = 3$). Naive rats that received intrathecal drug treatment followed the same drug dosing protocol as the SNL rats. For qPCR analysis, SNL + intrathecal saline ($n = 5$), SNL + intrathecal MPA ($n = 6$) and naive rats ($n = 6$) were included.

2.5.1. Immunohistochemistry

Animals were anesthetized with isoflurane 4.0% in a room air/oxygen mixture and transcardially perfused with saline 1 ml/g bodyweight followed by freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffered saline 1 ml/g bodyweight. Spinal cord and DRGs of L5 and L6 roots were isolated, post-fixed overnight in the same fixative and moved to 30% sucrose for at least 72 h. Free floating transverse sections (30 μm) were taken from the spinal cord using a microtome. DRGs were embedded in Tissue-Tek® (O.C.T. Compound, Sakura® Finetek, PA, USA) frozen and cut (10 μm) on a Leica CM1800 Cryostat (IMEB, CA, USA) and directly mounted on glass slides. Both free floating spinal sections and mounted DRG sections were permeabilized with 0.3% Triton X-100 (Sigma, St. Louis, MO, USA), blocked with 5% goat serum in phosphate buffered saline and incubated with antibodies raised against the glucocorticoid receptor (3D5) in every form (tGR = total GR) (primary antibody made in mouse, 1:1500, cat. no. sc-56851, Santa Cruz Biotechnology Inc, CA, USA) and its phosphorylated state pGR (Ser211) (made in rabbit, 1:1500, cat. no. #4161, Cell Signaling Technology Inc, MA, USA) for 48 h at 4 °C. Both antibodies have been previously used for immunohistochemistry and western blotting [19,20]. Binding sites were visualized with secondary antibodies conjugated with fluoro-Alexa-594 (goat anti-mouse, 1:1000, cat. no. A11032, Invitrogen, NY, USA) and streptavidin conjugated fluoro-Alexa-488 (goat anti-rabbit, 1:1000, cat no. S-32354, Life Technologies, CA, USA). A streptavidin/biotin blocking kit (cat. No. SP-2002, Vector Labs, CA, USA) was utilized as appropriate before biotinylated pGR. tGR and pGR antibodies were incubated simultaneously and with markers for astrocytes (GFAP) (made in mouse, 1:4000, cat. no. #MAB360, Chemicon, USA), microglia (Iba1) (made in rabbit, 1:2000, cat. no. #019-19741, WAKO, VA, USA), satellite glial cells (Vimentin) [21] (made in mouse, 1:100, cat. no. 180052, Invitrogen, NY, USA), and neurons (NeuN) (made in mouse, 1:1000, cat. no. MAB377, EMD Millipore Corporation, MA, USA) to examine cellular localization. The tGR and pGR location within the different cell types was also noted since the activated pGR is mainly found within the nucleus of cells. Images were captured using a Nikon TE300 fluorescence microscope (Nikon Corp, Tokyo, Japan) and overlay performed with Adobe Photoshop Creative suite (CS6; Adobe Systems Incorporated) or confocal microscopy. The investigator (MR) was blinded for the experimental conditions during quantification of tGR and pGR staining. Quantification of tGR in the spinal cord was performed by measuring the total integrated signal intensity of pixels in lamina I–II of the SDH after subtraction of the background signal intensity in this area using ImageJ 1.47 software. The total signal intensity of tGR, expressed as relative expression units (REU), was measured in at least 6 sections of the L5 to L6 area of the ipsi- and contralateral SDH (total of 12 sections per animal). Per section, 3 background signal intensity measurements were performed in the lamina I–II area and pooled to a mean for subtraction. An increase in the signal intensity for tGR was interpreted as receptor upregulation. In addition, the number of pGR positive cells in lamina I and II were manually counted in at least 6 sections of the L5 to L6 area in the ipsi- and contralateral SDH (total of 12 sections per animal) and the results expressed as the mean count per side per group.

2.5.2. Western blot

Animals were anesthetized deeply, and spinal cords and DRGs were harvested by laminectomy. Tissues were immediately flash frozen and stored at −70 °C until analysis. Total protein was extracted from spinal cord and DRGs, and separated by gel electrophoresis (NuPAGE 3–8% Tris acetate gel and Tris acetate running buffer, Invitrogen) and transferred to nitrocellulose membrane (Invitrogen). Membranes were blocked with 5% non-fat milk in Tris based buffer (50 mM/L NaCl with 0.1% Tween 20) and incubated with primary antibody pGR (Ser211) (made in rabbit, 1:1000, cat. no. #4161, Cell Signaling Technology Inc, MA, USA) and (tGR = total GR) (primary antibody made in mouse, 1:200, cat. no. sc-56851, Santa Cruz Biotechnology Inc, CA, USA) followed by secondary antibody conjugated to horse radish peroxidase (1:10,000, Cell Signaling Technology). Chemiluminescent reagent (Supersignal Pierce, Rockford) was used to visualize the protein antibody complex.

2.5.3. Quantitative real-time PCR (qPCR)

For qPCR measurements, rats were anesthetized with isoflurane 4.0% in a room air/oxygen mixture and spinal cords harvested from the vertebral column by hydro-extrusion using a saline-filled syringe after decapitation. The lumbar spinal cord (L3–L6) was divided into four parts; the ipsilateral ventral and dorsal parts and the contralateral ventral and dorsal parts. The ipsi and contralateral L5 and L6 DRGs were also immediately harvested at necropsy. Both spinal and DRG tissues were rapidly frozen on dry ice after dissection and kept at −70 °C until analysis. For analysis, samples were homogenized using pestle and mortar techniques in TRIzol and RNA extracted according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). All samples exhibited 260/280 absorbance ratios of approximately 2.0. Complementary DNA was prepared using MultiScribe reverse transcriptase (Applied Biosystems, CA, USA) according to the manufacturer's protocol. To determine RNA levels of tGR a SYBR® Green-Based Gene Expression Analysis (Applied Biosystems, CA, USA) was used. The primer for tGR costume made by Eurofins MWG Operon (Ebersberg, Germany) with a design based on a paper by DuBois et al. [22] had the following sequence:

Common forward primer: GCCCTGGGTTGGAGATCATAC
Common Reverse primer: CATGCAGGGTAGAGACATTCTC

Serially diluted cDNA samples synthesized from C6 cell line collected after 24 h of TNFalpha stimulation, which expresses tGR, were used as standard curve material. The threshold cycle values were determined and the number of cell equivalents in each sample calculated with the standard curve method [23]. Data was normalized to *Hprt1* values and expressed as Relative Expression Unit (REU).

2.6. Data analysis

Data are presented as means with 95% confidence intervals. Significance was ascribed for $p < 0.05$. Behavioural time-course data in the SNL model (tactile thresholds) was analyzed using two way ANOVA with repeated measures across time. Differences in signal intensity expressed as REU for immunohistochemistry tGR and qPCR mRNA levels, and pGR cell counts on the ipsilateral side between treatment groups were calculated with a *t*-test. Differences within groups between the ipsilateral and contralateral sides were analyzed with a paired *t*-test. Graphics and statistical analyses were carried out using Prism 6 for Windows.

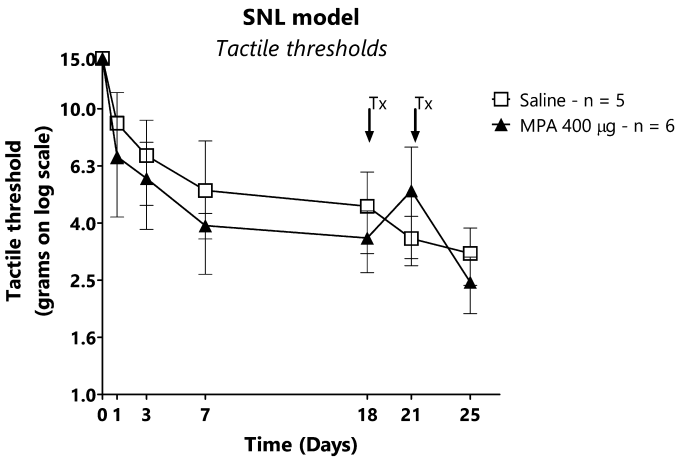

Fig. 1. Effect of intrathecal methylprednisolone acetate (MPA) treatment on pain-like behaviour in the spinal nerve ligation (SNL) model. Tactile thresholds in grams on a log scale (*y*-axis) over time in days (*x*-axis). Tx with arrow indicates drug treatment on day 18 and on day 21 after SNL. White square, saline controls; black triangle, MPA treatment, n, number of animals; data are plotted as mean; error bars are the standard error of the mean.

3. Results

3.1. Intrathecal MPA does not reduce pain-like behaviour after SNL

After SNL, reduced tactile thresholds were observed from post-operative day 1, remaining decreased throughout the study. Pain-like behaviour was well established on postoperative day 18, when the drug treatment was initiated. Intrathecal administration of MPA did not increase tactile thresholds in the allodynic hind-paw of SNL rats as compared to saline controls in the following 6 days (two-way rm-ANOVA; drug $p = 0.67$, interaction $p = 0.87$; Fig. 1).

3.2. Intrathecal MPA decreased mRNA levels of the GR in the SDH rats after SNL

In DRGs, *tGR* mRNA levels did not significantly change after SNL, comparing levels in naïve rats (mean *tGR* mRNA 0.014; 95% CI 0.003–0.024) to levels in the ipsilateral DRGs in saline controls (mean 0.019; 95% CI 0.011–0.027; $p = 0.35$) (Fig. 2A). When treating SNL rats with intrathecal MPA, *tGR* mRNA levels in the ipsilateral DRGs did not change (mean 0.023; 95% CI 0.009–0.036; $p = 0.58$) compared to saline controls (Fig. 2A).

In the SDH, there was no significant increase in the ipsilateral *tGR* mRNA levels after SNL in intrathecal saline rats (mean 0.24; 95% CI 0.21–0.27) compared to naïve tissue (mean 0.25; 95% CI 0.17–0.33; $p = 0.80$) (Fig. 2B). However, the ipsilateral *tGR* mRNA levels following intrathecal MPA (mean 0.15; 95% CI 0.11–0.20) significantly decreased compared to saline controls ($p = 0.003$) and naïve tissue ($p = 0.017$) (Fig. 2B).

3.3. tGR and pGR immunoreactive signal intensity increased after SNL in both intrathecal saline and MPA treated animals

In the SDH, tGR immunoreactive signal intensity did not significantly increase after SNL on the ipsilateral side in intrathecal saline treated rats (mean 12.8; 95% CI 8.6–16.9) compared to naïve tissue (mean 9.9; 95% CI 7.1–12.6; $p = 0.076$). However, using a paired comparison for assessing tGR immunoreactive signal intensity on the ipsilateral versus the contralateral side (mean 10.0; 95% CI 3.7–16.2) in intrathecal saline treated rats, a significant increase was observed ($p = 0.030$), indicating a nerve ligation effect (Fig. 3A).

Intrathecal MPA treatment by itself, in naïve rats, did not change tGR signal intensities (mean 11.8; 95% CI 8.5–15.1; $p = 0.17$). After SNL, a significant increase in tGR signal intensities was observed comparing the ipsilateral (mean 11.7; 95% CI 10.6–12.8) versus the contralateral (mean 8.2; 95% CI 6.0–10.4) sides in intrathecal MPA treated rats ($p = 0.040$). There were no significant differences in ipsi-

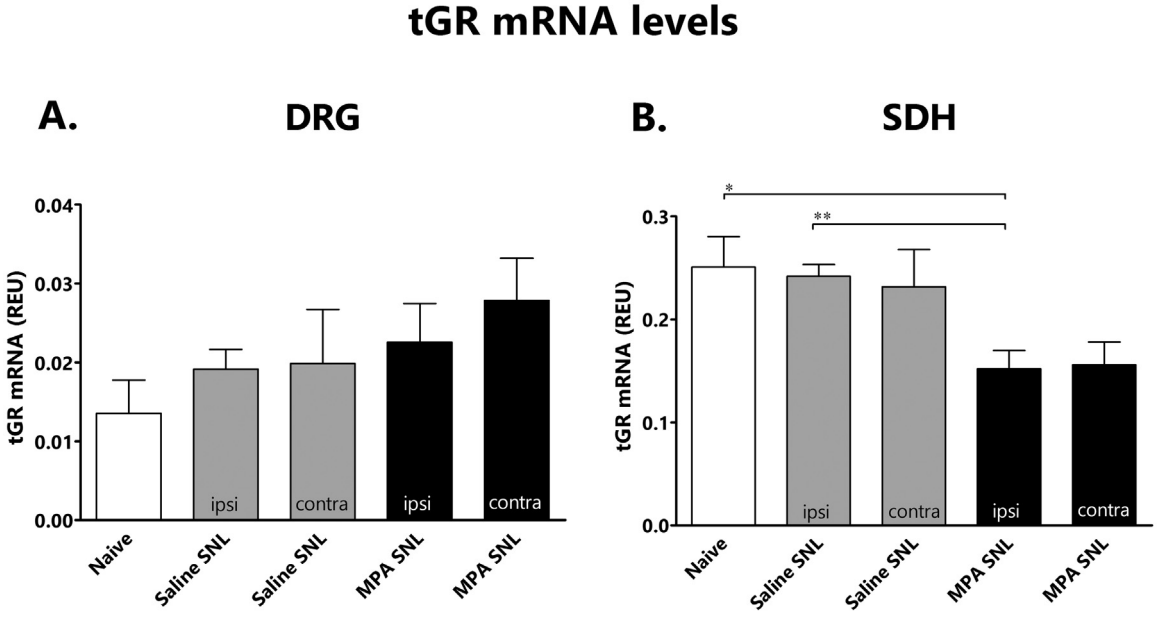

Fig. 2. Effect of intrathecal methylprednisolone acetate (MPA) on glucocorticoid receptor mRNA levels (*tGR* mRNA) in the spinal nerve ligation (SNL) model in the (A) dorsal root ganglion (DRG) and (B) spinal cord dorsal horn (SDH). *tGR* mRNA levels expressed as relative expression units (REU) (*y*-axis) are plotted for the different groups; white, naïve animals (number of animals (n) = 6); grey, intrathecal saline treated animals after SNL ($n = 5$); black, intrathecal MPA treated animals after SNL ($n = 6$). ipsi, ipsilateral side of the nerve ligation; contra, contralateral side of the nerve ligation. Data are plotted as mean; error bars are the standard error of the mean. *p-value < 0.05, **p < 0.01.

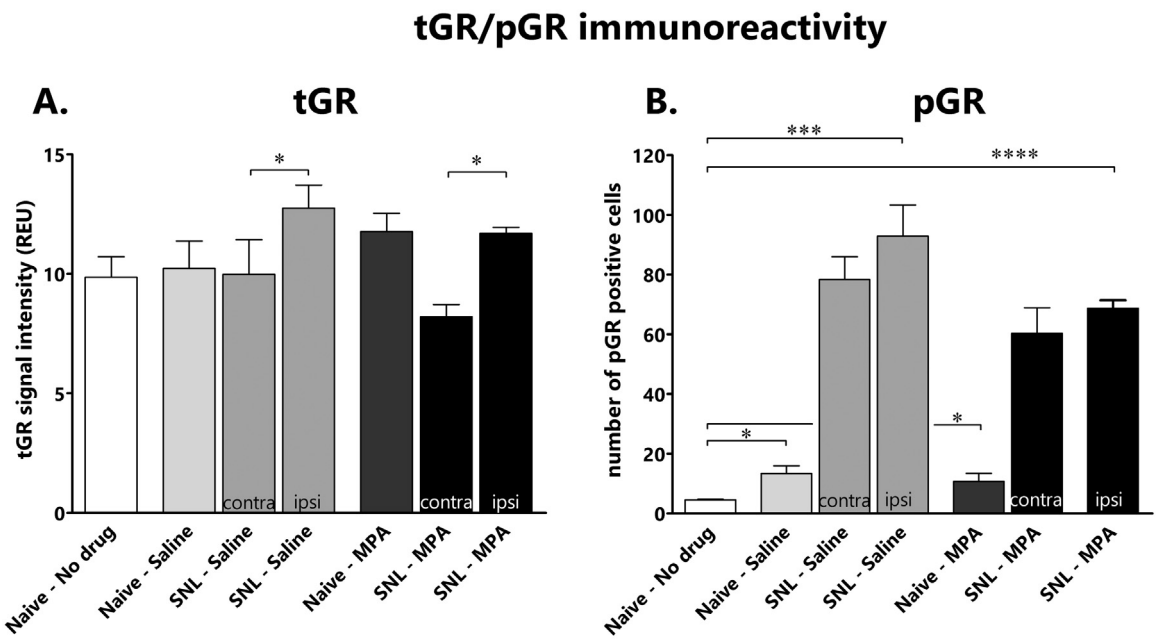

Fig. 3. Effect of intrathecal methylprednisolone acetate (MPA) in the spinal nerve ligation (SNL) model on glucocorticoid receptor (GR) immunoreactive signal intensity of (A) the total GR (tGR) and (B) phosphorylated GR (pGR) in the spinal cord dorsal horn. (A) Signal intensity of tGR immunohistochemistry staining expressed as relative expression units (REU) (*y*-axis) and (B) number (*n*) of pGR positive cells are plotted for the different groups; white, naïve animals (naïve – no drug, $n = 4$); light grey, intrathecal saline treated animals without SNL (naïve – saline, $n = 3$); darker grey, intrathecal saline treated animals after SNL (SNL – saline, $n = 3$); off black, intrathecal MPA treated animals without SNL (naïve – MPA, $n = 3$); black, intrathecal MPA treated animals after SNL (SNL – MPA, $n = 3$). ipsi, ipsilateral side of the nerve ligation; contra, contralateral side of the nerve ligation. Data are plotted as mean; error bars are the standard error of the mean. $^{*}p$-value < 0.05, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

lateral tGR signal intensities after SNL between intrathecal MPA treated rats and saline controls ($p = 0.35$) (Fig. 3A).

In the SDH, the number of pGR immunopositive cells significantly increased after intrathecal catheter implantation and saline (13.3; 95% CI 2.1–24.5; $p = 0.010$) or MPA (mean 10.7; 95% CI −1.1 to 22.4; $p = 0.044$) treatment compared to naïve tissue (mean 4.5; 95% CI 3.6–5.4). After SNL, the number of pGR immunopositive cells increased significantly on the ipsilateral side in both intrathecal saline (mean 92.8; 95% CI 47.7–138; $p = 0.0002$) and MPA treated animals (mean 68.8; 95% CI 57.4–80.1; $p < 0.0001$) compared to naïve tissue. Intrathecal MPA treatment did not change the number of SNL evoked pGR immunopositive cells compared to saline controls ($p = 0.090$) (Fig. 3B).

3.4. pGR and GR co-localization

pGR and tGR localization and signal intensity was assessed by immunohistochemistry. The antibodies used for these studies were examined by western blot analysis of naïve tissues and the pGR antibody detected one strong band in DRGs and spinal cord samples around 80 kDa. The antibody used for tGR detection also showed the strongest band around 80 kDa, but also a heavier band around 100 kDa in the DRG samples (Fig. 4A). Some very faint lighter bands were also observed for pGR and tGR in DRGs. Using immunohistochemistry pGR co-localized with neurons (Fig. 4B) and satellite glial cells (Fig. 4C) in DRGs. In neurons on the contralateral side of the nerve ligation, pGR was observed in the cytosol and barely in the nucleus. On the ipsilateral side, pGR staining in the neuronal nucleus was observed. In the SDH, tGR and pGR co-localized as expected (Fig. 5). tGR predominantly co-localized with the neuronal marker NeuN, and had only minor co-localization with the microglial marker Iba1. Co-localization of tGR with GFAP, the astrocyte marker, was not observed (Fig. 6). pGR expression was also localized in a similar fashion.

4. Discussion

4.1. Overview of results

In the present study, no analgesic effect with two doses of intrathecal MPA was observed 18–25 days after SNL when ligation-induced mechanical hypersensitivity was well established in rats. Seven days after drug treatment, rats were euthanized and decreased *tGR* mRNA levels in intrathecal MPA treated animals compared to saline controls were observed. However this did not result in lower tGR and pGR immunoreactive signal intensities in intrathecal MPA treated animals compared to saline controls.

4.2. Efficacy of intrathecal MPA in neuropathic pain states

The lack of an analgesic effect with intrathecal glucocorticoid treatment in rats with pain-like behaviour after SNL and spared nerve injury model has been described before [7,10]. In agreement with our findings in rats, there are also clinical studies showing no effect of intrathecal MPA treatment in postherpetic neuralgia patients and patients suffering from complex regional pain syndrome [5,6].

4.3. Glucocorticoid receptor expression after nerve injury

After nerve injury in rats, plasma cortisol levels are significantly increased compared to baseline, lasting at least 21 days [3,10]. In our study, intrathecal MPA was administered on day 18 and day 21 after SNL, when plasma cortisol levels are still known to be high. Also an increased GR expression in the SDH has been reported after nerve injury with a time course parallel to the development of pain behaviour [10,14,24,25]. In our study we show that tGR and pGR immunoreactivity is still upregulated 25 days after SNL in both saline controls and intrathecal MPA treated animals. Surprisingly, there was no significant difference between both groups in tGR and pGR immunoreactivity signal intensities 7 days after initiation

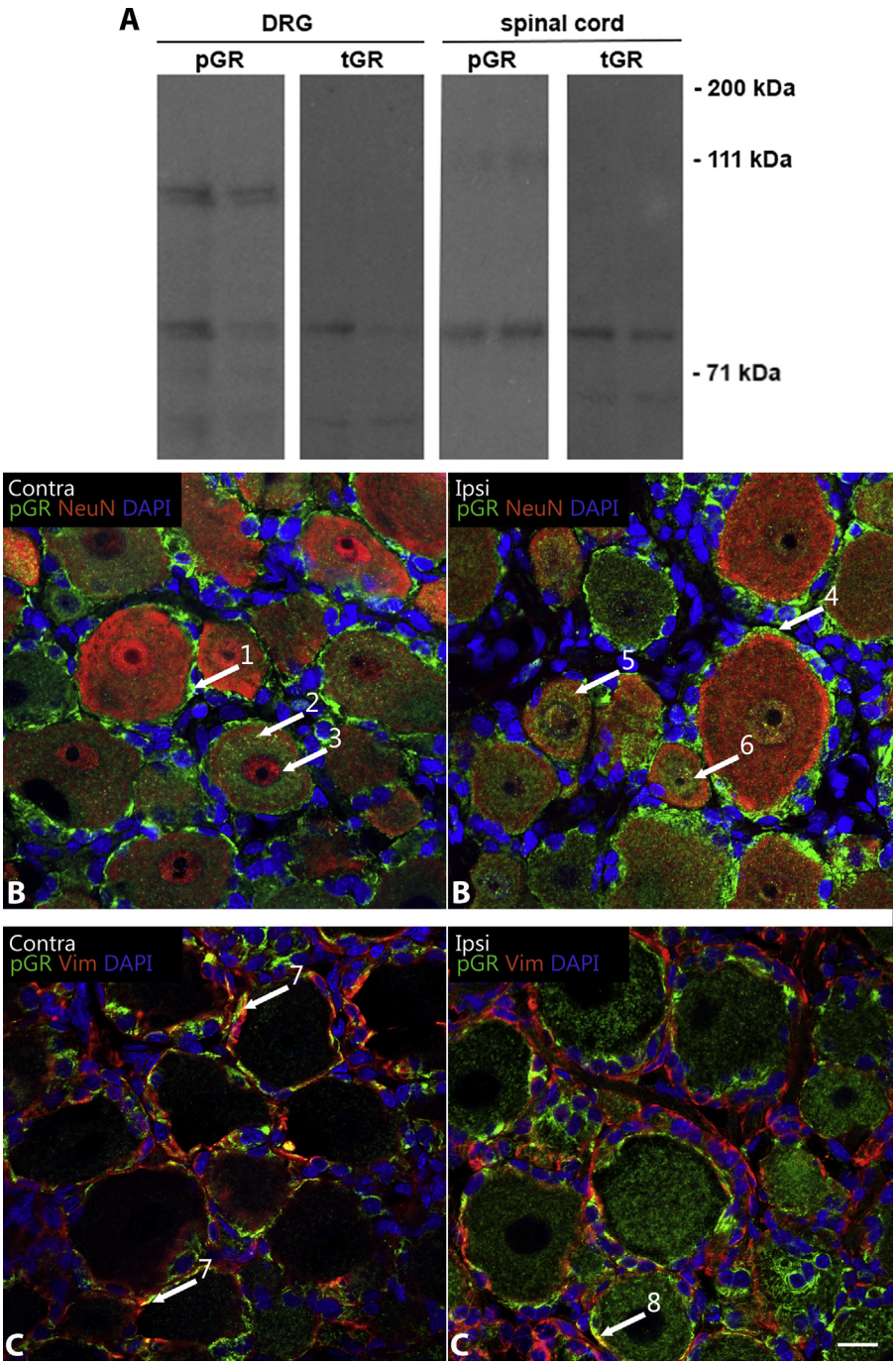

Fig. 4. Co-localization of the activated (phosphorylated) glucocorticoid receptor (pGR) in the dorsal root ganglion (DRG) of a spinal nerve ligated saline control rat. (A) Western blot film for pGR and tGR to represent the specificity of antibody in DRGs and in spinal cord. pGR (green) co-localizes with (B) neurons (NeuN = red) and (C) satellite glial cells (Vimentin (Vim) = red). Contra B, contralateral DRG to the nerve ligation site showing (1) pGR outside the neuron, (2) pGR in the neuronal cytosol and (3) nearly no pGR in neuronal nucleus. Ipsi B, ipsilateral DRG to the nerve ligation site showing (4) pGR outside the neuron similar to the contralateral side, (5) increased pGR staining in the neuronal cytosol compared to contralateral side and (6) pGR located in the neuronal nucleus. Contra C, contralateral DRG to the nerve ligation site showing (7) co-localization of pGR with satellite glial cells. Ipsi C, ipsilateral DRG in the nerve ligation site showing (9) co-localization of pGR with satellite glial cells not visibly increased compared to the contralateral side. Nuclei staining with DAPI = blue.

of intrathecal glucocorticoid treatment. We did however measure a decreased *tGR* mRNA level in intrathecal MPA treated animals. The reduced *tGR* mRNA expression after intrathecal MPA administration might be the result of a feedback mechanism after relative high doses of glucocorticoids at the target tissue. It however did not lead to significantly less pGR protein expression compared to saline controls.

We did not measure the effect of intrathecal MPA on downstream markers of GR activity in the present study. Several studies report effects of intrathecal glucocorticoids on surrogate markers such as microglial and astrocyte activation and changes in pro-inflammatory cytokine (tumour necrosis factor α, interleukin-1β) levels after nerve injury with varying and sometimes contradicting results summarized in our review [13].

The pGR we measured is one of several post-translational modifications of the GR. GR consists of a constant component, from exon 2 through 8, and a variable component exon 9. Alternative splicing of exon 9 gives rise to 5 different (human) protein subtypes that

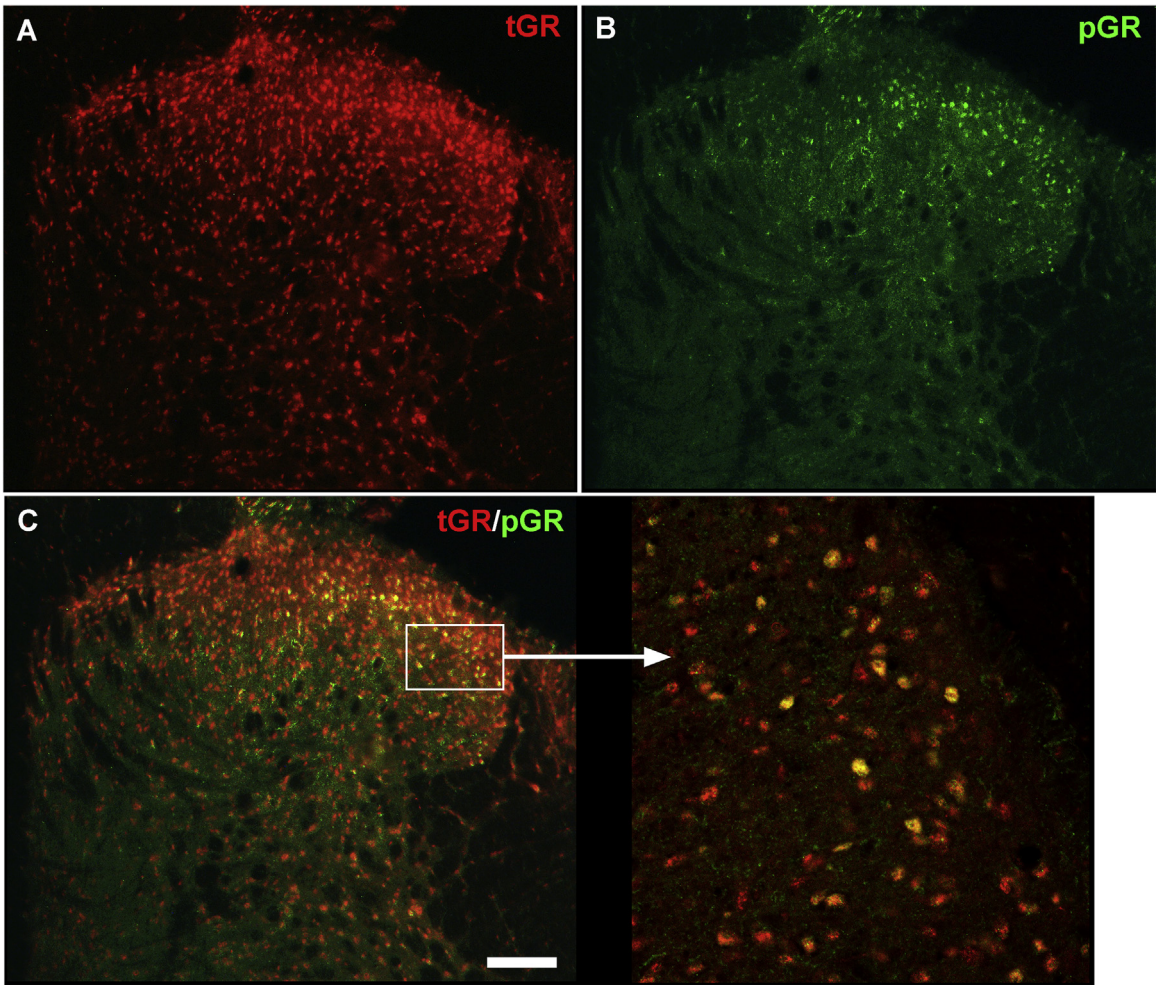

Fig. 5. Co-localization of the total glucocorticoid receptor (tGR) with the activated (phosphorylated) glucocorticoid receptor (pGR) on the ipsilateral side of the spinal dorsal horn in a spinal nerve ligated (SNL) intrathecal saline treated rat. tGR, red; pGR, green; co-localization of tGR/pGR, yellow. The picture on the far right is an enlargement of the area surrounded by the white box on the picture on the mid right. All pGR staining is co-localized with tGR staining.

have been termed hGRα, hGRβ, hGRγ, hGR-A and hGR-P [26]. These protein subtypes have several different subisoforms (e.g. GRα-A, GRα-B, GRα-C1) [27] which are subject to post-translational modifications for example phosphorylation, which further modulates the transcriptional activity of the receptor. In the present study, we have chosen to focus on the total GR receptor (tGR), using an antibody directed to the constant component of the GR staining all the present glucocorticoid receptor subtypes. The activated GR (pGR), we studied is a GR phosphorylated at serine 211 in humans (corresponding with phosphorylation at serine 232 in the rat) [20,28]. Phosphorylation at this site occurs to a greater extent in the presence of glucocorticoids and leads to translocation of the receptor complex to the nucleus [20]. Ser211 phosphorylation is therefore seen as a biomarker for activated GR in vivo [28]. An explanation for the observed reduction in *tGR* mRNA expression after intrathecal MPA administration, not leading to reduced pGR immunoreactive signal intensities, is that other pGR posttranslational modificated isoforms were downregulated after intrathecal MPA administration.

4.4. Colocalization of the glucocorticoid receptor in the central nervous system

The GR is located in every cell in the body but higher GR expression in the neuraxis is observed in the hippocampus, hypothalamus, and in the spinal cord in the substantia gelatinosa [29]. Pain is modulated in these areas and could indicate that pain pathways are under regulation of these receptors. Focusing on the spinal cord, GR was primarily increased in lamina I/II of the SDH in the development of pain and a majority of GR-expressing cell profiles expressed NeuN, corresponding with previous findings [10,29]. We find it surprising that GR is mainly expressed in neurons since most inflammatory processes have been studied in microglia and astrocytes. GR co-localization with astrocytes and microglial cells and oligodendrocytes has also been observed [29].

4.5. Study strength and weaknesses

Although larger sample size per group for measurement of GR activity would have been useful, we did find significant differences in tGR and pGR immunoreactive signal intensities between the contra and ipsilateral side after SNL. Regarding the lack of difference in pGR immunoreactive signal intensities between MPA treated and saline control rats, it would be interesting to study different posttranslational modificated glucocorticoid receptor isoforms in nerve injury models to shed light on the varying and contradicting results of glucocorticoid therapy in neuropathic pain states.

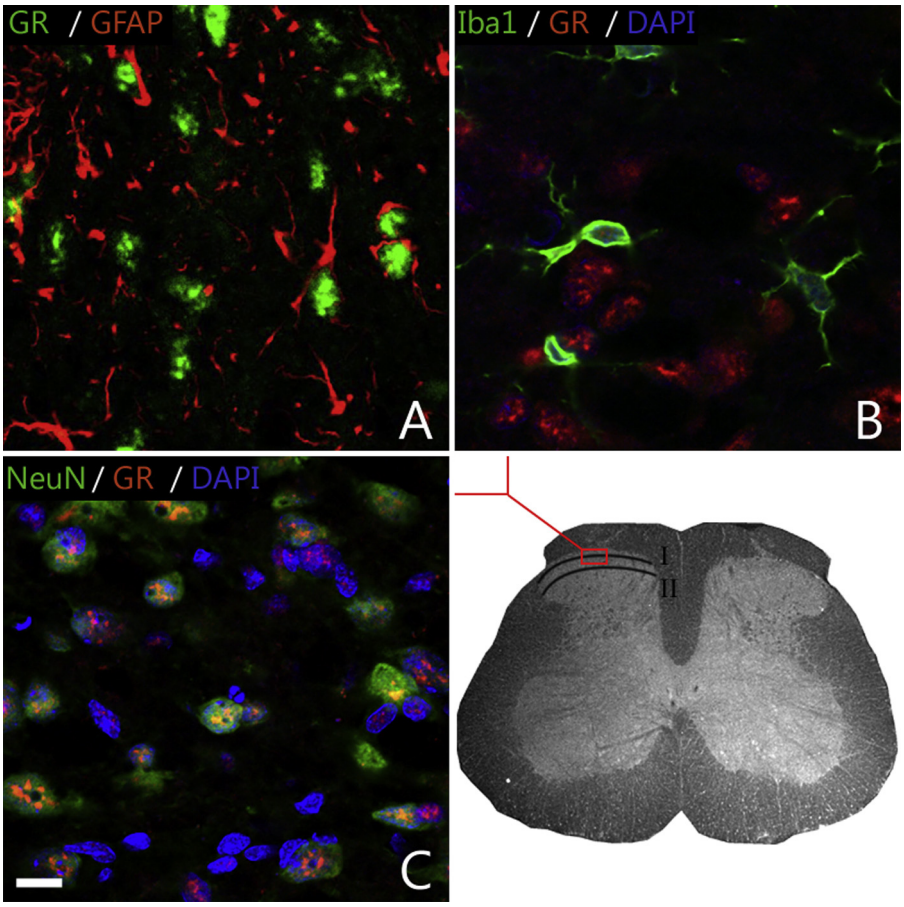

Fig. 6. Glucocorticoid (GR = green or red) co-localization with (A) astrocytes (GFAP = red), (B) microglia (Iba 1 = green), and (C) neurons (NeuN = green) in lamina I and II in the spinal dorsal horn indicated in the lower right. Nuclei staining with DAPI = blue. GR predominantly co-localized with the neuronal marker NeuN (C), and had only minor co-localization with the microglial marker Iba1 (B). No co-localization of GR with GFAP (A), the astrocyte marker, was observed.

5. Conclusion

In conclusion, our study shows that intrathecal MPA at the maximum usable dose employed, had no effect on the established tactile allodynia in a SNL model. tGR and pGR are upregulated in the spinal cord dorsal horn in the face of neuronal damage in both intrathecal MPA treated animals and saline controls. Intrathecal MPA treatment decreased *tGR* mRNA levels in the spinal cord dorsal horn after SNL, not lowering tGR and pGR immunoreactivity intensities, and not decreasing ligation-induced mechanical hypersensitivity in rats.

Conflicts of interest

None.

Authors' contribution

M. Rijsdijk performed the research, R. Ramachandran, N.M. Agalave and A. Baharpoor participated in data collection, M Rijsdijk, T.L. Yaksh and C.I. Svensson designed the research study, C.I. Svensson, C.J. Kalkman and T.L. Yaksh contributed essential reagents and tools, M. Rijsdijk, C.I. Svensson, A.J.M. van Wijck, C.J. Kalkman and T.L. Yaksh analyzed the data, M. Rijsdijk wrote the paper and C.I. Svensson, A.J.M. van Wijck, C.J. Kalkman, R. Ramachandran, N.M. Agalave and T.L. Yaksh revised the manuscript. All authors approved the final manuscript.

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