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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).