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Intrathecal carbenoxolone inhibits neuropathic pain and spinal wide-dynamic range neuronal activity in rats after an L5 spinal nerve injury

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HIGHLIGHTS

- Intrathecal carbenoxolone (CBX) inhibited neuropathic pain manifestations in rats.
- Neuropathic rats did not develop tachyphylaxis to repetitive CBX treatments.
- CBX attenuated response of wide-dynamic range neurons (WDR) to mechanical stimuli.
- CBX also inhibited windup, a short-form of neuronal sensitization, in WDR neurons.

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ABSTRACT

Spinal glial gap junctions may play an important role in dorsal horn neuronal sensitization and neuropathic pain. In rats after an L5 spinal nerve ligation (SNL), we examined the effects of intrathecal injection of carbenoxolone (CBX), a gap junction decoupler, on neuropathic pain manifestations and on wide-dynamic range (WDR) neuronal activity *in vivo*. Intrathecal injection of CBX dose-dependently (0.1–50 μg, 10 μl) inhibited mechanical hypersensitivity in rats at 2–3 weeks post-SNL. However, the same doses of glycyrrhizic acid (an analogue of CBX that does not affect gap junctions) and mefloquine hydrochloride (a selective neuronal gap junction decoupler) were ineffective. Intrathecal CBX (5 μg) also attenuated heat hypersensitivity in SNL rats. Further, rats did not develop tachyphylaxis to CBX-induced inhibition of mechanical hypersensitivity after repetitive drug treatments (25 μg/day) during days 14–16 post-SNL. Electrophysiological study in SNL rats showed that spinal topical application of CBX (100 μg, 50 μl), which mimics intrathecal drug administration, attenuated WDR neuronal responses to mechanical stimuli and to repetitive intracutaneous electrical stimuli (0.5 Hz) that induce windup, a short-form of activity-dependent neuronal sensitization. The current findings suggest that the inhibition of neuropathic pain manifestations by intrathecal injection of CBX in SNL rats may involve an inhibition of glial gap junctions and an attenuation of WDR neuronal activity in the dorsal horn.

1. Introduction

Peripheral nerve injury may lead to hypersensitivity to mechanical and thermal stimuli, and increase dorsal horn neuron excitability. The neighboring glial cells may also play an important role in exaggerated spinal nociceptive transmission after nerve injury [1]. A principal mechanism of communication between glial cells is through gap junctions [2], which allow second messengers (e.g., cAMP, inositol trisphosphate), ions, and small hydrophilic molecules (e.g., ATP) to pass freely between the cells [3,4]. Mounting evidence suggests that spinal glial gap junctions may play an important role in pathologic pain conditions [5,6]. Blocking gap junctions with carbenoxolone (CBX), a disodium salt of 3′-0-hydrogen succinate of glycyrrhetic acid, induces analgesia in various animal models of persistent pain [5,7,8]. However, one caveat of previous studies is that the specificity of CBX toward glial gap junctions is uncertain, as CBX is a nonselective gap junction

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decoupler and may also directly affect neuron membrane properties [9]. Further, the mechanisms and the downstream events (e.g., changes in neuronal activity) that contribute to CBX-induced analgesia are not fully clear.

We sought to answer these questions in rats after an L5 spinal nerve ligation (SNL), which is a standardized and highly repeatable neuropathic pain model that has been widely studied [10,11]. We first examined whether intrathecal administration of CBX attenuates behavioral mechanical and heat hypersensitivity in SNL rats. We then attempted to use pharmacological tools to evaluate whether CBX-induced analgesia occurs largely by inhibiting glial gap junctions. Wide-dynamic range (WDR) neurons represent an important component in the network of spinal nociceptive transmission and function as an essential cellular mediator of the central neuronal hyperexcitability underlying chronic pain [12,13]. WDR neurons display an action potential windup phenomenon to repetitive noxious stimuli, which reflects a short-term increase in neuronal excitability [12,14]. Finally, we conducted *in vivo* electrophysiological recording in SNL rats to examine whether spinal topical application of CBX, which mimics intrathecal drug administration, attenuates the responses of WDR neurons to mechanical stimuli and to repetitive intracutaneous electrical stimuli (0.5 Hz) that induce windup.

2. Materials and methods

2.1. Animals

Experiments were performed on adult male Sprague-Dawley rats (250–350 g, Harlan Laboratories, Inc., Indianapolis, IN). All experimental procedures were conducted in strict accordance with the guidelines established by the Johns Hopkins University Animal Care and Use Committee as consistent with the National Institutes of Health Guide for the Use of Experimental Animals. All animals were euthanized with sodium pentobarbital (100–300 mg, i.p.) at the end of the experiment.

2.2. L5 SNL

The SNL model was produced as described previously [13]. In brief, the rats were anesthetized with isoflurane (2%, Abbott Laboratories, North Chicago, IL), and the left L5 spinal nerve was ligated with a 6-0 silk suture and cut distally under aseptic conditions.

2.3. Behavioral tests

Hypersensitivity to punctuate mechanical stimulation with von Frey filaments (0.38, 0.57, 1.23, 1.83, 3.66, 5.93, 9.13, 13.1 g) was determined with the up-down method [15]. Abrupt paw withdrawal, licking, and shaking were considered positive responses. The paw withdrawal threshold (PWT) was determined according to the method and formula provided by Dixon [16]. Paw withdrawal latency (PWL) to radiant heat stimuli (cut-off time: 20 s) was measured with a plantar stimulator analgesia meter (IITC model 390, Woodland Hills, CA) [17]. To minimize experimenter bias, the investigator who performed the behavioral tests was blinded to the drug treatment conditions.

2.4. Intrathecal injection

Drugs were administrated through an implanted intrathecal catheter. For catheter implantation, a small slit was cut on the atlanto-occipital membrane, into which a saline-filled PE-10 tubing (6–7 cm) was inserted. After completing the experiment, intrathecal drug delivery was confirmed by injecting lidocaine (400 μg/20 μl, Hospira, Lack Forest, IL), which resulted in a temporary motor paralysis of the lower limbs.

2.5. Dorsal horn recordings

Extracellular recordings of dorsal horn neuron activity were obtained with microelectrodes as described previously [13]. Briefly, WDR neurons located in deep laminae (400–1200 μm to the dorsal surface) of the lumbar spinal segment were recorded. WDR neurons were characterized by using mechanical stimuli with intensities that ranged from mild to noxious texture [13]. We examined WDR neurons with defined receptive fields (RF) in the plantar region of the hindpaw. For mechanical tests, we briefly mapped the RF with a von Frey monofilament (10 g), and a "sensitive site" in the RF was identified for application of von-Frey stimulation (0.615 g, 3 s). In a separate experiment, windup of C-fiber-mediated responses (i.e., C-component) was examined by delivering a train of 16 electrical pulses (supra-C-fiber threshold, 2.0 ms, 0.5 Hz) through a pair of fine stimulating electrodes inserted subcutaneously in the RF [13]. The test module was applied before and 30–60 min after the drug treatment.

2.6. Drugs

Carbenoxolone disodium salt (CBX), glycyrrhizic acid (GCA), and mefloquine hydrochloride (MFQ) were purchased from Sigma–Aldrich. CBX was dissolved in saline (0.9%); GCA and MFQ were dissolved in DMSO initially and then further diluted to the final concentration with saline.

2.7. Data analysis

To establish the dose–response functions for CBX, we calculated percent maximum possible effect (%MPE) values with the equation: %MPE = [(post-drug PWT) − (pre-drug PWT)]/[(cut-off) − (pre-drug PWT)] × 100, where cutoff PWT = 21.5 g. The methods for statistical comparisons in each study are given in the figure legends. STATISTICA 6.0 software (StatSoft, Inc., Tulsa, OK) was used to conduct all statistical analyses. The Tukey honestly significant difference (HSD) post-hoc test was used to compare specific data points in ANOVA. Data are expressed as mean ± SEM; $P < 0.05$ was considered significant in all tests.

3. Results

3.1. Intrathecal injection of CBX inhibits mechanical and heat hypersensitivity in SNL rats

Mechanical hypersensitivity is an important manifestation of neuropathic pain. Intrathecal injection of CBX (0.1 μg: $n = 5$; 0.5 μg: $n = 6$; 5 μg: $n = 7$; 25 μg: $n = 8$; 50 μg: $n = 5$, 10 μl) dose-dependently increased PWT in the ipsilateral (left) hind paw of rats at 2–3 weeks post-SNL (Fig. 1A). The %MPEs of CBX at 1, 2, and 4 h after injection were calculated, and the averaged %MPEs were used to plot the dose–response function (Fig. 1B, ED_{50} = 3.9 μg/10 μl). The %MPEs of CBX at doses of 0.5, 5, and 50 μg were significantly greater than that of vehicle ($n = 6$). Because CBX may exert actions other than inhibiting gap junctions, we next tested whether intrathecal GCA (an analogue of CBX that shares similar structure and multiple properties of CBX but does not block gap junctions) [5,18–20], and MFQ (a selective neuronal gap junction decoupler) also alleviate mechanical hypersensitivity. At 0.81 and 8.1 nmol doses (10 μl), CBX significantly increased %MPE from the vehicle-treated group. However, at the same doses, neither GCA nor MFQ significantly increased %MPE (Fig. 1C). Although %MPE of GCA at the 81 nmol dose was higher than that of vehicle, rats exhibited severe signs