Neuroscience 228 (2013) 334–348

PRE- AND POST-SYNAPTIC SWITCHES OF GABA ACTIONS ASSOCIATED WITH CL^- HOMEOSTATIC CHANGES ARE INDUCED IN THE SPINAL NUCLEUS OF THE TRIGEMINAL NERVE IN A RAT MODEL OF TRIGEMINAL NEUROPATHIC PAIN

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Abstract—Although trigeminal neuropathic pain is one of the most common chronic pain syndromes, the etiology is still unknown. Here, a rat model was generated using chronic constrictive injury (CCI) with ligation of the infraorbital nerve to test the hypothesis that collapse of chloride homeostasis in trigeminal neurons causes impairment of γ-aminobutyric acid-ergic (GABAergic) inhibition and induces trigeminal allodynia. Rats showed a reduction and increase in pain threshold and pain response scores, respectively, to mechanical stimulation, 1 and 3 weeks after CCI. *In situ* hybridization and immunohistochemical analysis showed that inward-directed Na^+, K^+–$2Cl^-$ cotransporter (NKCC1) mRNA and protein were upregulated in the small-sized and large-sized primary neurons in the injured side of the trigeminal ganglion and in the peripherin-positive terminal, respectively, for the first 2 weeks, while outward-directed K^+–Cl^- cotransporter (KCC2) mRNA and protein were downregulated in secondary relay neurons on the injured side of the spinal trigeminal nucleus caudalis (Sp5C). Optical imaging of evoked synaptic responses using a voltage-sensitive dye revealed that pre- and post-synaptic GABA actions were disinhibited and excitatory in the injured side, respectively, but inhibited in the sham-operated side of the Sp5C. This downregulation of KCC2 in the Sp5C may result in an excitatory switch by impairing postsynaptic GABA inhibition. GABA-mediated presynaptic disinhibition was attenuated by bumetanide, suggesting that NKCC1 upregulation in primary neurons may facilitate pain transmission by presynaptic GABAergic depolarization. Such Cl^- homeostatic disruption resulting in perturbation of the inhibitory system possibly increases pain transmission, which may underlie the pathophysiology of trigeminal neuropathic pain.

Key words: trigeminal neuralgia, KCC2, NKCC1, trigeminal ganglion, allodynia, primary afferent depolarization.

INTRODUCTION

Trigeminal neuropathic pain is one of the most common chronic pain syndromes, of which the etiology is still unknown. Recently, cation–chloride cotransporters (CCC) have been implicated in the pathogenesis of neuropathic pain (De Koninck, 2007; Kahle et al., 2008; Price et al., 2005, 2009). The Na^+, K^+–$2Cl^-$ cotransporter (NKCC1), that normally accumulates Cl^-, and the K^+-Cl^- cotransporter (KCC2), that normally extrudes Cl^-, mutually control neuronal Cl^- homeostasis and modulate GABAergic function (Rivera et al., 1999; Delpire, 2000; Payne et al., 2003; Yamada et al., 2004; Blaesse et al., 2009). The trans-synaptic shift in Cl^- gradient by reduction of KCC2 in spinal lamina I neurons as a mechanism of neuropathic pain predicts that a loss of inhibition (disinhibition) in the dorsal horn of the spinal cord is a crucial substrate for chronic pain syndromes (Coull et al., 2003; Kahle et al., 2008; Price et al., 2005, 2009). However, the mechanisms that underlie such disinhibition remain to be elucidated. In addition, painful stimuli induce *in vivo* phosphorylation and membrane mobilization of NKCC1, which plays a critical role in neuronal excitability in the mouse spinal cord (Galan and Cervero, 2005). This suggests that it can contribute to hyperalgesic states by modulating the Cl^- concentration inside nociceptive neurons.

The rat trigeminal nervous system is anatomically composed of four nuclei (mesencephalic trigeminal nucleus, motor trigeminal nucleus, principal trigeminal nucleus, and spinal trigeminal nucleus) and the trigeminal ganglion (TG). The spinal trigeminal nucleus caudalis (Sp5C) is a critical relay site for processing nociceptive afferent input from the orofacial area and for its modulation

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Abbreviations: ACSF, artificial cerebrospinal fluid; BIC, bicuculline methiodide; CCC, cation–chloride cotransporters; CCD, charge-coupled-device; CCI, chronic constrictive injury; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; D-AP5, D-(−)-2-amino-5-phosphonopentanoic acid; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate; DRG, dorsal root ganglion; GABA, γ-aminobutyric acid; GABAergic, γ-aminobutyric acid-ergic; GST, glutathione *S*-transferase; HN, hypoglossal nucleus; ION, infraorbital nerve; KCC2, K^+–Cl^- cotransporter; NKCC1, Na^+, K^+–$2Cl^-$ cotransporter; PAD, primary afferent depolarization; PB, phosphate buffer; PFA, paraformaldehyde; ROI, region of interest; RT, room temperature; siRNA, short interfering RNA; Sp5C, spinal trigeminal nucleus caudalis; TG, trigeminal ganglion; TTX, tetrodotoxin.

0306-4522/12 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2012.10.043

by neuroplastic changes (Sessle, 2000). Although the trigeminal nervous system utilizes γ-aminobutyric acid (GABA) as a neurotransmitter in the processing of sensory and motor information, and KCC2 and NKCC1 are expressed in the Sp5C (Toyoda et al., 2005), little is known about the detailed mechanism of GABAergic function for nociception in these nuclei.

Experimental neuropathy in the rat produced by tying four loosely constrictive ligatures (chronic constriction injury, CCI) around one sciatic nerve has been widely used as a model of hyperalgesia and allodynia (Bennett and Xie, 1988; Costigan et al., 2009). It has also been clinically documented that trigeminal nerve injury also leads to allodynia and hyperalgesia (Gregg, 1990; Robinson et al., 2004). The infraorbital nerve (ION), which is made up solely of sensory fibers, forms almost the entire maxillary division of the trigeminal nerve in the rat. CCI of the ION (CCI-ION) can produce an experimental rat model of trigeminal neuropathic pain (Imamura et al., 1997; Kitagawa et al., 2006; Vos et al., 1994). Here, we used this model to test the hypothesis that collapse of Cl^- homeostasis in trigeminal relay and/or primary neurons causes reduction of GABAergic inhibition, which may induce allodynia.

EXPERIMENTAL PROCEDURES

All experimental procedures were approved by the committee for animal use at the Hamamatsu University School of Medicine in accordance with guidelines for animal experimentation and ethical use of animals issued by the Government of Japan and the NIH. All efforts were made to minimize the number of animals used and their suffering, and followed the guidelines of the Committee for Research and Ethical Issues of IASP published in PAIN, 16 (1983).

Surgery

We used adult male Wistar rats weighing approximately 130 g (Japan SLC, Shizuoka, Japan). Rats were delivered to the animal house at least 7 days before experimentation, and were housed singly with free access to food and tap water. Rats received CCI to the right ION and a sham operation on the other side. All surgery was performed under general anesthesia with 50 mg/kg (intraperitoneal) of pentobarbital sodium. All incisions were made intraorally, which allowed the hair on the snout and the vibrissae to remain intact. An incision approximately 1 cm long was made along the gingivobuccal margin. The incision was made just proximal to the first molar. Approximately 0.5 cm of the ION was freed of adhering tissue, and two 5.0 polyglycolic acid ligatures were tied loosely around it. The incision was sutured at two points using 4.0 silk (Imamura et al., 1997). The sham operation was identical except that the nerve was not ligated.

Mechanical stimulation

For mechanical stimulation, a graded series of Von Frey hairs (Pressure Aesthesiometer, Stoelting Co., Chicago, IL, USA) were used. After handling, including a week of habituation to daily stimuli, rats were tested 1, 2, 3 and 4 weeks after the operation ($n = 5$ at each time point). Each rat was placed in a Plexiglas box (length 42 cm; width 25 cm; height 21 cm) in a dimly lit room for 1 h before behavioral testing to acclimatize animals. To detect the pain threshold, testing was started with the finest filaments, gradually increasing the intensities until rats exhibited withdrawal reactions, in which they turned their head slowly away or pulled it briskly backward, sometimes a single face wipe of the receiving area occurred. In addition, we used the 0.4 g Von Frey hair as touch stimuli to record behavioral response scores. The behavioral responses evoked by these stimuli were scored as follows: 0 = no response; 1 = detection or approach the stimulus object; 2 = withdrawal reaction; 3 = escape and or attack action; 4 = asymmetric face grooming (details described by Vos et al. (1994). The interval of the stimuli between the two sides was at least 1 min. The above mechanical stimulus was made every day and the data were collected on the last day of the week, when animals were used for behavioral tests (e.g., 1 week refers to the 7th day of stimulation after the operation and therefore, animals have been stimulated 14 times in total including the habituation period).

We also performed habituation tests on naïve rats (data not shown). Pain thresholds and behavioral scores were studied using the Friedman test ($n = 8$), which showed there were no significant differences in responses after the end of week 1 of mechanical stimulation (i.e. habituation period) and up to 5 weeks.

Tissue preparation

At various time points after the operation, rats that showed behavioral signs of allodynia were anesthetized with pentobarbital sodium, deeply anesthetized with ether and then sacrificed on the last day of the week. Brains were quickly removed and immediately frozen on powdered dry ice. Serial sections of Sp5C and TG (20 μm thick) were cut using a cryostat, thaw-mounted onto poly-L-lysine (PLL)-coated slides and stored at −80 °C.

In situ hybridization histochemistry

The *in situ* hybridization histochemical technique used for KCC2 and NKCC1 is described in detail elsewhere (Toyoda et al., 2003, 2005). Briefly, hybridization was performed by incubating paraformaldehyde (PFA)-fixed sections for 24 h at 42 °C in a buffer of the following composition: 0.6 M NaCl and 0.06 M sodium citrate, 50% (v/v) deionized formamide, 0.12 M phosphate buffer (PB), 2.5% (v/v) tRNA, and 10% (v/v) dextran sulfate in Denhardt's solution containing [^{35}S]dATP (PerkinElmer Life and analytical Sciences, Boston, MA, USA) -labeled probes ($1–2 \times 10^7$ dpm/mL, 0.2 mL/slide). The sections were coated with Kodak NBT-2 emulsion, kept at 4 °C for 2–3 weeks, and then developed in a D-19 developer. We used the exposure times of 2 weeks for KCC2 and 3 weeks for NKCC1 for emulsion autoradiography. Sections were counterstained with thionin solution to allow morphological identification.

The specificity of the probes has already been confirmed (Kanaka et al., 2001). The sequences of the probes are as follows:

KCC2, TGGCTTCTCCTCGTTGTCACAAGCTGTCTCTTC-GGG;

NKCC1, ACATCCTTGGTACCAGGTGACTTTTCTTGTGA-TGAC.

Antibody production

Anti-NKCC1 antibodies against 951–1000 or 1108–1141 amino acid residues of mouse NKCC1 (NM_009194) were produced in the present study. Glutathione *S*-transferase (GST) fusion proteins were used for antigens by subcloning the cDNA fragments into the BamHI/EcoRI site of the pGEX4T-2 plasmid (GE Healthcare Biosciences, Piscataway, NJ, USA). The fusion protein was emulsified with Freund's complete adjuvant in the