Neuroscience 256 (2014) 23–35

GLUTAMATE DYSREGULATION IN THE TRIGEMINAL GANGLION: A NOVEL MECHANISM FOR PERIPHERAL SENSITIZATION OF THE CRANIOFACIAL REGION

J. C. LAURSEN,[a] B. E. CAIRNS,[a,b] X. D. DONG,[b,c] U. KUMAR,[b] R. K. SOMVANSHI,[b] L. ARENDT-NIELSEN[a] AND P. GAZERANI[a]*

[a] *Center for Sensory-Motor Interaction, Department of Health Science and Technology, Faculty of Medicine, Aalborg University, Fredrik Bajers Vej 7D3, DK-9220 Aalborg East, Denmark*

[b] *Faculty of Pharmaceutical Sciences, The University of British Columbia, 2405 Wesbrook Mall, Vancouver, BC V6T 1Z3, Canada*

[c] *College of Stomatology, Tianjin Medical University, Tianjin 300071, China*

Abstract—In the trigeminal ganglion (TG), satellite glial cells (SGCs) form a functional unit with neurons. It has been proposed that SGCs participate in regulating extracellular glutamate levels and that dysfunction of this SGC capacity can impact nociceptive transmission in craniofacial pain conditions. This study investigated whether SGCs release glutamate and whether elevation of TG glutamate concentration alters response properties of trigeminal afferent fibers. Immunohistochemistry was used to assess glutamate content and the expression of excitatory amino acid transporter (EAAT)1 and EAAT2 in TG sections. SGCs contained glutamate and expressed EAAT1 and EAAT2. Potassium chloride (10 mM) was used to evoke glutamate release from cultured rat SGCs treated with the EAAT1/2 inhibitor (3S)-3-[[3-[[4-(trifluoromethyl)ben zoyl]amino]phenyl]methoxy]-L-aspartic acid (TFB-TBOA) or control. Treatment with TFB-TBOA (1 and 10 μM) significantly reduced the glutamate concentration from 10.6 ± 1.1 to 5.8 ± 1.4 μM and 3.0 ± 0.8 μM, respectively ($p < 0.05$). Electrophysiology experiments were conducted in anaesthetized rats to determine the effect of intraganglionic injections of glutamate on the response properties of ganglion neurons that innervated either the temporalis or masseter muscle. Intraganglionic injection of glutamate (500 mM, 3 μl) evoked afferent discharge and significantly reduced muscle afferent mechanical threshold. Glutamate-evoked discharge was attenuated by the *N*-methyl-D-aspartate receptor antagonist 2-amino-5-phosphonovalerate (APV) and increased by TFB-TBOA, whereas mechanical sensitization was only sensitive to APV. Antidromic invasion of muscle afferent fibers by electrical stimulation of the caudal brainstem (10 Hz) or local anesthesia of the brainstem with lidocaine did not alter glutamate-induced mechanical sensitization. These findings provide a novel mechanism whereby dysfunctional trigeminal SGCs could contribute to cranial muscle tenderness in craniofacial pain conditions such as migraine headache.
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Key words: glutamate, trigeminal ganglion, craniofacial pain, satellite glial cells, excitatory amino acid transporters.

INTRODUCTION

Glutamate is the principal excitatory transmitter in the nervous system and is synthesized from glutamine by neurons (Platt, 2007). Extracellular glutamate levels in the CNS are continuously maintained at 1–2 μM by way of glutamate uptake through excitatory amino acid transporter 1 (EAAT1) and EAAT2, which are expressed by glial cells, mainly astrocytes (Anderson and Swanson, 2000; Danbolt, 2001). However, in spite of their vital role in maintaining low extracellular glutamate levels, EAATs can also serve as a glutamate release pathway under conditions such as elevated extracellular potassium levels (Szatkowski et al., 1990; Longuemare and Swanson, 1995), which may contribute to local elevation of extracellular glutamate levels.

It is crucial that glutamate levels are tightly regulated since excess levels can have deleterious effects such as neurotoxicity (Lau and Tymianski, 2010) and potentially also affect sensory transmission. Indeed, glutamate has been suggested to play a role in the pathogenesis of several craniofacial pain conditions including temporomandibular disorders and migraine headache (Vieira et al., 2007; Castrillon et al., 2010; Shimada et al., 2013). Moreover, it was demonstrated that systemically elevated glutamate levels not only give rise to spontaneous headache in humans but also lead to significant craniofacial muscle sensitization (Baad-Hansen et al., 2010; Shimada et al., 2013). These observations are translatable into rats, where it was shown that intravenous injections of monosodium glutamate lowered the mechanical threshold of afferents innervating the masseter muscle due to the accumulation of glutamate in the muscle, which lead to the activation of *N*-methyl-D-aspartate (NMDA) receptors

*Corresponding author. Tel: +45-9940-2412; fax: +45-9815-4008.
E-mail address: gazerani@hst.aau.dk (P. Gazerani).
Abbreviations: ANOVA, analysis of variance; APV, 2-amino-5-phosphonovalerate; CGRP, calcitonin gene-related peptide; CV, conduction velocity; DMEM, Dulbecco's modified minimum essential medium; DRG, dorsal root ganglion; EAAT, excitatory amino acid transporter; GS, glutamine synthetase; KCl, potassium chloride; NMDA, *N*-methyl-D-aspartate; PBS, Phosphate-buffered saline; PNS, peripheral nervous system; RT, room temperature; SGCs, satellite glial cells; TFB-TBOA, (3S)-3-[[3-[[4-(trifluoromethyl)ben zoyl]amino]phenyl]methoxy]-L-aspartic acid; TG, trigeminal ganglion; Vc, trigeminal nucleus caudalis.

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http://dx.doi.org/10.1016/j.neuroscience.2013.10.009

(Cairns et al., 2007). Hence, regulation of glutamate levels is not only crucial in the CNS but also in the peripheral nervous system (PNS) to prevent disturbances in sensory transmission. In spite of these observations, less is known about the regulation of glutamate levels in the PNS, compared to the CNS, and it is still not fully clarified whether peripheral glial cells contribute to this regulation.

Similar to the CNS, glial cells of the PNS are intimately associated with neurons and serve to regulate the microenvironment surrounding the neurons. In sensory ganglia of the PNS, such as the trigeminal ganglion (TG), satellite glial cells (SGCs) reside (Hanani, 2005; Pannese, 2010). These cells completely surround ganglion neurons and release substances that can act on adjacent neurons, which may alter the excitability of the neurons (Capuano et al., 2009). It is likely that SGCs also participate in regulating extracellular glutamate levels in sensory ganglia, since EAATs are expressed by SGCs in the dorsal root ganglion (DRG) (Berger and Hediger, 2000; Carozzi et al., 2008). Moreover, in mixed neuron-glial cultures it was recently demonstrated that depolarization of the soma of DRG neurons lead to accumulation of glutamate in the culture medium, which was more pronounced when EAATs on SGCs were blocked (Kung et al., 2013). This information implicates SGCs in the removal of extracellular glutamate and thus implies that they actively regulate intraganglionic glutamate levels. Dysfunction of this SGC capacity in the TG could potentially impact nociceptive transmission and contribute to the development of peripheral sensitization and craniofacial pain.

The aims of the current study are (1) to investigate if SGCs of rat TG express EAAT1, EAAT2, and contain glutamate, (2) to determine if SGCs isolated from the TG can be provoked to release glutamate and if this is mediated, in part, through EAATs, and (3) to characterize the *in vivo* response properties of TG neurons upon the injection of glutamate into the rat TG.

EXPERIMENTAL PROCEDURES

For all experiments, adult male Sprague–Dawley rats were used (Charles River, Canada). Experimental protocols were approved by the University of British Columbia Animal Care Committee (No. #A11-0279) and the study was performed in accordance with the guidelines established by the Canadian Council on Animal Care and the International Association for the Study of Pain.

Immunohistochemistry

To investigate the expression of glutamine synthetase (GS), EAAT1, EAAT2, and glutamate in SGCs of the TG, immunohistochemistry was performed ($n = 3$). Initially the rats were anaesthetized with 4% isoflurane (AErrane, Baxter Corporation, Mississauga, Ontario, Canada) and transcardially perfused first with cold (4 °C) heparinized isotonic saline and then with 4% paraformaldehyde to fix the tissue *in situ*. After extraction of both TG, the tissue was dehydrated for 2 days in 20% sucrose and then for an additional 2 days in 40% sucrose. Serial transverse sections of 10 µm were cut though the TG at −20 °C using a Minotome Plus cryostat (Triangle Biomedical Science, Durham, NC, USA) and transferred to poly-lysine-coated glass slides (Fisher Scientific Company, Ottawa, Ontario, Canada).

Tissue slices were then incubated at room temperature (RT) (20–22 °C) for 1 hour in 5% bovine serum albumin (BSA; Fisher Bioreagents, Ottawa, Ontario, Canada) containing 0.2% Triton X-100 (Sigma Aldrich, USA). Next, they were washed in phosphate-buffered saline (PBS) and primary antibodies including rabbit anti-GS (GS; 1:10,000; Sigma Aldrich, USA), goat anti-EAAT1 (1:250; Santa Cruz Biotechnology, Santa Cruz, USA), goat anti-EAAT2 (1:250; Santa Cruz Biotechnology, USA), and mouse anti-glutamate (1:2000; Thermo Scientific, USA) were applied and left for incubation overnight at 4 °C. The next day, slices were thoroughly washed in PBS before secondary antibodies (donkey anti-mouse, 1:700; Invitrogen, Canada; donkey anti-rabbit, 1:700; Invitrogen, Canada; donkey anti-goat 1:700; Invitrogen, Canada) were applied. After 1.5 h of incubation in the dark at RT, two final washes with PBS, and one with distilled water, were performed and the glass slides were mounted using AquaPerm mounting medium (Thermo Scientific, USA). As a control, primary antibodies were omitted from the staining procedures.

Five non-overlapping images were acquired at 400× magnification for each staining procedure with a confocal microscope (Leica DM 2500, Concord, Ontario, Canada) equipped with a digital camera (Leica DFC310 FX, Canada) using the Leica application suite software (Version 2.3.6, build 5381). Whole-image noise-to-signal ratio was adjusted with ImageJ (National Institutes of Health, USA).

In vitro glutamate release

Trigeminal SGC isolation. For *in vitro* studies SGCs were isolated from the TG of adult rats ($n = 10$) based on a previously described method (Capuano et al., 2009) with minor modifications introduced. Initially, rats were deeply anaesthetized with 4% isoflurane and the circulatory system was perfused with cold (4 °C) heparinized isotonic saline. Both TG were aseptically removed and care was taken to remove the fibrous capsule encapsulating the TG during the isolation to minimize the risk of fibroblast contamination. The tissue was then suspended in 5 mL ice-cold PBS without Ca^{2+} and Mg^{2+} (Sigma Aldrich, St. Louis, MO, USA) supplemented with 1% penicillin and streptomycin (Invitrogen, Burlington, ON, Canada) and glucose (6 g/L). The tissue was digested in a collagenase solution (5 mg/mL; Sigma Aldrich, USA) for 15 min at 37 °C, followed by further digestion for 10 min at 37 °C in 0.125% trypsin (Invitrogen, Canada) where DNAse (Ambion, Burlington, ON, Canada) was added for the last 5 min. Afterward the tissue was suspended in 5 mL

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