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CORNEAL AFFERENTS DIFFERENTIALLY TARGET THALAMIC- AND PARABRACHIAL-PROJECTING NEURONS IN SPINAL TRIGEMINAL NUCLEUS CAUDALIS

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Abstract—Dorsal horn neurons send ascending projections to both thalamic nuclei and parabrachial nuclei; these pathways are thought to be critical pathways for central processing of nociceptive information. Afferents from the corneal surface of the eye mediate nociception from this tissue which is susceptible to clinically important pain syndromes. This study examined corneal afferents to the trigeminal dorsal horn and compared inputs to thalamic- and parabrachial-projecting neurons. We used anterograde tracing with cholera toxin B subunit to identify corneal afferent projections to trigeminal dorsal horn, and the retrograde tracer FluoroGold to identify projection neurons. Studies were conducted in adult male Sprague–Dawley rats. Our analysis was conducted at two distinct levels of the trigeminal nucleus caudalis (Vc) which receive corneal afferent projections. We found that corneal afferents project more densely to the rostral pole of Vc than the caudal pole. We also quantified the number of thalamic- and parabrachial-projecting neurons in the regions of Vc that receive corneal afferents. Corneal afferent inputs to both groups of projection neurons were also more abundant in the rostral pole of Vc. Finally, by comparing the frequency of corneal afferent appositions to thalamic- versus parabrachial-projecting neurons, we found that corneal afferents preferentially target parabrachial-projecting neurons in trigeminal dorsal horn. These results suggest that nociceptive pain from the cornea may be primarily mediated by a non-thalamic ascending pathway.

Abbreviations: BSA, bovine serum albumin, CVLM, caudal ventrolateral medulla, C1, cervical spinal cord segment 1, CTb, cholera toxin B subunit, FG, FluoroGold, -ir, -immunoreactive, LPB, lateral parabrachial nucleus, MPB, medial parabrachial nucleus, NTS, nucleus of the solitary tract, PVN, paraventricular nucleus of the hypothalamus, PB, phosphate buffer, PHA-L, *Phaseolus vulgaris*-leucoagglutinin, Po, posterior nucleus, Vc, spinal trigeminal nucleus caudalis, Vi, spinal trigeminal nucleus interpolaris, VPL, ventral posterolateral nucleus, VPM, ventral posteromedial nucleus.

Key words: confocal microscopy, immunocytochemistry, retrograde tracing, FluoroGold, cholera toxin B subunit.

INTRODUCTION

Corneal pain is an important public health issue since clinical conditions such as dry eye and uveitis are becoming more prominent. These conditions are sometimes due to increased contact lens use, increased prevalence of refractive lens surgery such as laser-assisted *in situ* keratomileusis (LASIK) and photorefractive keratectomy (PRK) (Belmonte et al., 2004; Wakefield and Chang, 2005; Gallar et al., 2007), and also due to the aging of the population. An understanding of the neural pathways that mediate corneal pain should enhance treatment for the clinical conditions.

The cornea is innervated by thinly myelinated A-delta and unmyelinated C fibers that respond to nociceptive mechanical, thermal, and chemical stimuli and send afferents via the ophthalmic branch of the trigeminal nerve to the trigeminal brainstem (Marfurt and Del Toro, 1987; Belmonte et al., 2004). Corneal afferent fibers project to superficial laminae of the ventrolateral spinal trigeminal nucleus caudalis (Vc) in two distinct regions: (1) the caudal transition zone with the cervical spinal cord (C1) and (2) the rostral transition zone with spinal trigeminal nucleus interpolaris (Vi) (Marfurt and Del Toro, 1987; Hegarty et al., 2010). A previous anatomical study from this laboratory demonstrated neurochemical differences in the peptide and glutamate content of central terminals of corneal afferents projecting to Vc/C1 and Vi/Vc (Hegarty et al., 2010). Electrophysiologically, the neurons in the rostral and caudal regions of Vc have different receptive fields (Meng et al., 1997), different responses to nociceptive stimuli, and differential modulation by ligands of opioid, glutamate, and neurokinin receptors (Bereiter and Bereiter, 1996; Meng et al., 1998; Hirata et al., 2000). These studies suggest that the neurons in Vc/C1 and Vi/Vc differentially process nociceptive input transduced by corneal afferents. Dissecting the connectivity of corneal-responsive neurons to supraspinal substrates of trigeminal nociception would help to better define the specific roles of Vc/C1 and Vi/Vc neurons in corneal nociception.

There are two major groups of neurons in the superficial trigeminal dorsal horn that mediate nociception: neurons that project to the contralateral thalamus and mediate the sensory-discriminative component of pain (Fukushima and Kerr, 1979; Iwata et al., 1992; Sessle, 1999; Gauriau and Bernard, 2002;

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Mitchell et al., 2004) and neurons that project to the ipsilateral parabrachial nucleus and contribute to the autonomic and affective components of pain (Cechetto et al., 1985; Hylden et al., 1989; Slugg and Light, 1994; Feil and Herbert, 1995; Gauriau and Bernard, 2002; Mitchell et al., 2004). The thalamus is considered to be the main relay structure for the transmission of nociceptive stimuli from the periphery to the cortex (Millan, 1999). It integrates and encodes the quality, intensity, modality, and topography of the nociceptive stimuli and transmits this information to the cortex (Millan, 1999). A large percentage of neurons in the parabrachial nuclei that receive input from second-order neurons in the superficial spinal and trigeminal dorsal horns have been shown to send projections to the amygdala, hypothalamus, periaqueductal gray and ventrolateral medulla (Gauriau and Bernard, 2002). These areas are thought to contribute to the aversive emotional responses, behaviors and homeostatic changes that occur in the wake of a painful stimulus (Gauriau and Bernard, 2002). In the present anatomical study, we sought to determine if corneal afferents directly contact thalamic- or parabrachial-projecting second-order neurons and whether there are differences in connectivity in Vc/C1 versus Vi/Vc.

EXPERIMENTAL PROCEDURES

Experimental animals and tract tracing techniques

All protocols were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University. Male Sprague–Dawley rats (n = 14; 230–550 g; Charles River Laboratories, Wilmington, MA, USA) were housed in pairs on a 12/12 light/dark cycle and were given access to food and water *ad libitum*.

Cholera toxin B subunit. Cholera toxin B subunit (CTb; 1% solution in 0.1 M phosphate buffer (PB); List Biological Laboratories, Inc., Campbell, CA, USA) was used as an anterograde tracer to identify central projections of corneal afferents into the trigeminal dorsal horn (Todd et al., 2003; Hegarty et al., 2010). Each rat was anesthetized with vaporized isoflurane in oxygen (Isotex Tec3 (Datex-Ohmeda, Madison, WI, USA); 5% induction, 2–3% maintenance), then ophthalmic proparacaine hydrochloride (5% solution) drops were placed in the eye to reduce spontaneous eye movements. The eye was dried and a stainless steel metal retaining ring (7-gauge, Small Parts, Miramar, FL, USA) was affixed to the eye with petroleum jelly. The outer epithelial layer of the cornea within the steel ring was abraded with a 1-min application of 1-heptanol (99%; Alfa Aesar, Ward Hill, MA, USA) followed by saline rinses (Felipe et al., 1999; Hegarty et al., 2010). The eye was dried again, the steel ring replaced around the abraded surface, and CTb (6–15 μl) was placed inside the ring for 30 min before being rinsed off with saline. Rats received a subcutaneous injection of ketoprofen (2.5 mg/kg) to reduce discomfort.

FluoroGold. FluoroGold (FG, 2% in saline; Fluorochrome, LLC, Denver, CO, USA) was used as a retrograde tracer to identify neurons in the trigeminal dorsal horn that project to thalamic or parabrachial nuclei. Prior to, or following, CTb application to the cornea, rats were placed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) and FG was pressure injected (50–250 nl) using a picospritzer (Picospritzer® III, Parker Hannifin, Cleveland, OH, USA) into thalamic or parabrachial nuclei using single-barrel glass micropipettes (Fig. 1). Coordinates from Bregma for thalamic injections were: 2.8 mm caudal, 2.0 mm lateral, 6.0 mm ventral; and for parabrachial injections: 9.7 mm caudal, 2.1 mm lateral, 5.7 mm ventral. The bite bar settings for the injections were: −9 mm for the thalamic injections and −11 mm for the parabrachial injections. Following the microinjection into the brain, the pipette was left in place for 5 min before removal. Incisions were closed with 3-0 monocryl suture (Ethicon, Cornelia, GA, USA), covered in antiseptic ointment, and the rats were monitored during recovery. FG was selected as the retrograde tracer of choice because it produces only retrograde labeling (Van Bockstaele et al., 1994; Aicher et al., 1995) and it can be detected using immunocytochemical methods that are compatible with confocal microscopy.

Immunocytochemistry and tissue preparation for confocal microscopy

Transcardial perfusion. Five to ten days after surgery rats were overdosed with pentobarbital sodium (150 mg/kg) and perfused transcardially through the ascending aorta with the following sequence of solutions: (1) 10 ml heparinized saline (1000 units/ml), (2) 50 ml 3.8% acrolein in 2% paraformaldehyde, and (3) 200 ml 2% paraformaldehyde (in 0.1 M PB, pH 7.4). The brain regions of interest were placed in 2% paraformaldehyde for 30 min then placed in 0.1 M PB. Tissue was sectioned coronally (40 μm) on a vibrating microtome and processed for appropriate immunocytochemical procedures. Prior to immunocytochemical processing, sections were incubated in a 1% sodium borohydride solution for 30 min. Correct placement of FG injections was verified in tissue sections that contained thalamic and parabrachial nuclei (Fig. 2) using an Olympus BX51 light and epifluorescent microscope interfaced with a DP71 digital camera and associated software.

Immunofluorescent Immunocytochemistry. For triple-labeling studies (Aicher et al., 2003; Bailey et al., 2006; Winkler et al., 2006), tissue sections from the brainstem were incubated for 48 h at 4 °C in a cocktail of primary antibodies: polyclonal goat anti-CTb IgG (CTb, 1:25,000, List Biological Laboratories), polyclonal rabbit anti-FG IgG (1:15,000, Fluorochrome, LLC), and a monoclonal mouse anti-NeuN antibody IgG (NEUronal

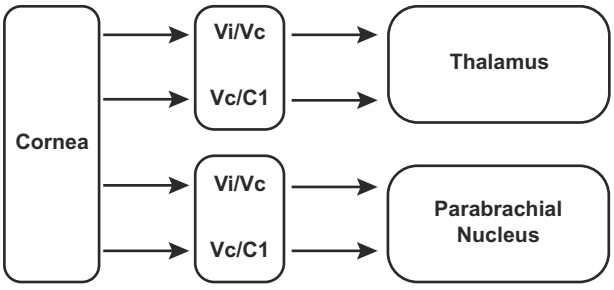

Fig. 1. Schematic representation of the peripheral and central substrates under study. Corneal afferents projecting to the ventrolateral aspect of the caudal (Vc/C1) and rostral (Vi/Vc) trigeminal dorsal horn were labeled with CTb. Concurrent injections of the retrograde tracer FluoroGold into either the thalamic or parabrachial nuclei were utilized to identify populations of projection neurons in the trigeminal dorsal horn. We assessed the connectivity between CTb-ir corneal afferents and FG-ir projection neurons in ventrolateral trigeminal dorsal horn at both the caudal (Vc/C1) and rostral (Vi/Vc) levels of the spinal trigeminal nucleus caudalis.