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Corneal afferents differentially target thalamic- and parabrachial-projecting neurons in trigeminal subnucleus 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 subnucleus 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.

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AUTHOR CONTRIBUTIONS

Study concept and design: SAA, SMH, DMH. Acquisition of data: SAA, SMH. Analysis and interpretation of data: SMH, DMH. Drafting of the manuscript: SAA, SMH, DMH. Critical revision of the manuscript for important intellectual content: SAA, DMH. Statistical analysis: SAA, SMH. Obtained funding: SAA. Administrative, technical, and material support: SMH, SAA. Study supervision: SAA. All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors have approved the final article.

Conflict of Interest Statement

The authors have no known conflicts of interest.

Keywords

Confocal microscopy; immunocytochemistry; retrograde tracing; FluoroGold; cholera toxin B subunit

1. 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; Mitchell et al., 2004) and neurons that project to the ipsilateral parabrachial nucleus and contribute to the autonomic and affective components of pain (Cechetti 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.

2. EXPERIMENTAL PROCEDURES

2.1. 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) were housed in pairs on a 12/12 light/dark cycle and were given access to food and water *ad libitum*.

2.1.1. Cholera toxin B subunit—(CTb; 1% solution in 0.1 M phosphate buffer; List Biological Laboratories, Inc., Campbell, CA) 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); 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) was affixed to the eye with petroleum jelly. The outer epithelial layer of the cornea within the steel ring was abraded with a 1 minute application of 1-heptanol (99%; Alfa Aesar, Ward Hill, MA) followed by saline rinses (Felipeco et al., 1999; Hegarty et al., 2010). The eye was dried again, the steel ring replaced around the abraded surface, and CTb (6 – 15 μ l) 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.

2.1.2. FluoroGold—(FG, 2% in saline; Fluorochrome, LLC, Denver, CO) 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) and FG was pressure injected (50–250 nl) using a picospritzer (Picospritzer® III, Parker Hannifin, Cleveland, OH) into thalamic or parabrachial nuclei using single-barrel glass micropipettes (Figure 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 minutes before removal. Incisions were closed with 3-0 monocril suture (Ethicon, Cornelia, GA), 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.

2.2. Immunocytochemistry and tissue preparation for confocal microscopy

2.2.1. 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 phosphate buffer (PB), pH 7.4). The brain regions of interest were placed in 2% paraformaldehyde for 30 minutes then placed in 0.1 M PB. Tissue was sectioned coronally (40 μ m) 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 minutes. Correct placement of FG injections was verified in tissue sections that contained thalamic and parabrachial nuclei (Figure 2) using an Olympus BX51 light and epifluorescent microscope interfaced with a DP71 digital camera and associated software.

2.2.2. Immunofluorescent Immunocytochemistry—For triple-labeling studies (Aicher et al., 2003; Winkler et al., 2006; Bailey et al., 2006), tissue sections from the brainstem were incubated for 48 hours at 4°C in a cocktail of primary antibodies: polyclonal goat anti-Cholera toxin B subunit 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 Nuclei, clone A60, 1:2,000, EMD Millipore, Billerica, MA). The primary antibody cocktail contained 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and 0.1% bovine serum albumin (BSA, Sigma-Aldrich) in 0.1 M Tris-Saline. The secondary antibody cocktail contained Alexa Fluor® 488 donkey anti-rabbit IgG (1:800, Life Technologies, Grand Island, NY), Alexa Fluor® 546 donkey anti-goat IgG (1:800, Life Technologies) and Alexa Fluor® 647 donkey anti-mouse IgG (1:800, Life Technologies). Tissue sections were protected from light and incubated for 2 hours at room temperature in secondary antibody cocktail. Sections were rinsed and then mounted on gelatin-coated slides, coverslipped with Prolong Gold™ Antifade reagent (Life Technologies) and stored at –20°C.

2.2.3. Primary antibodies—The goat anti-CTb antibody was raised against purified cholera toxin beta subunit (manufacturer's technical specifications). A previous study found that preadsorption with 1 μ g/ml and 5 μ g/ml of CTb abolished all immunostaining (Llewellyn-Smith et al., 1995). CTb labeling with the goat anti-CTb antibody was found only in the ventrolateral Vc/C1 and Vi/Vc regions ipsilateral to cornea that received application of the CTb tracer. The pattern of CTb labeling matched our previous study using CTb (Hegarty et al., 2010) and another study using horseradish peroxidase (HRP) to label corneal afferents (Marfurt and Del Toro, 1987). Based on the discrete labeling pattern, it is unlikely that the goat anti-CTb antibody bound nonspecifically to endogenous epitopes in Vc. The rabbit anti-FG antibody recognizes FluoroGold, also known as hydroxystilbamidine (manufacturer's technical specifications). A previous study found that preadsorption of 0.1 ml of the rabbit anti-FG antibody with 1 μ l of FG abolished all immunostaining (Lee et al., 2009). We also found that the pattern of immunocytochemical labeling matches the natural fluorescence of FG emitted under ultraviolet light; thus it is unlikely that the rabbit anti-FG antibody bound nonspecifically to endogenous epitopes in Vc. The mouse anti-NeuN

antibody recognizes the neuron-specific protein NeuN and was raised against purified cell nuclei from mouse brain (Mullen et al., 1992). Immunoblots on nuclear proteins from mouse brain, but not other mouse tissues such as heart, liver and thymus, demonstrated that the antibody recognizes three bands in the 46 – 48 kDa range (Mullen et al., 1992). Previous immunocytochemical studies confirmed that the mouse anti-NeuN antibody recognizes the nuclei from most neurons, except for Purkinje cells and photoreceptor cells, among others, and does not label glia (Mullen et al., 1992; Bottger et al., 2011).

2.3. Imaging and Analytic Methodology

2.3.1. Rostrocaudal Mapping—We captured darkfield images for ten sequential equally spaced rostrocaudal levels of brainstem beginning at the transition from cervical spinal cord to brainstem (Vc/C1) and moving rostrally to the trigeminal subnucleus interpolaris (Vi) (Figure 3). Every sixth section was chosen, resulting in approximately 240 μ m between sections. Sections from each animal at each of the ten levels were matched to a rat brain atlas diagram where possible (Paxinos and Watson, 1998) and images were compared across animals to ensure that comparable rostrocaudal levels were being chosen. If a matching tissue section for any of the ten rostrocaudal levels was damaged during immunocytochemical processing for an individual animal, that particular level was not included in the analysis for that animal.

Once sections were chosen and verified for each animal, low magnification epifluorescent images of CTb and FG immunoreactivity were captured on each of these sections. An ordinal categorical scale ranging from 0 to V, with 0 representing no labeling, and V representing the highest density of labeling seen within that case was utilized for qualitative assessment of CTb immunoreactivity throughout the ventrolateral trigeminal dorsal horn. Quantitative assessment of FG-immunoreactive (-ir) neurons was performed by first outlining the ventrolateral aspect of the trigeminal dorsal horn on darkfield images (Figure 4), and then counting the number of FG-ir cells within the region of interest on overlaid epifluorescent images. FG-ir cell counts were verified by an independent observer. The median CTb score (irrespective of FG injection site), as well as the average and standard error of the mean of FG-ir neurons from parabrachial-injected rats and thalamic-injected rats were reported (Figure 3). In order to be included in the study, an animal had to have (1) appropriately located injection sites within the thalamus or parabrachial nucleus (Figure 2, shaded regions) and (2) at least five retrograde neurons within the ventrolateral aspect of the trigeminal dorsal horn in at least two of the ten rostrocaudal levels ($n = 4$ parabrachial-injected rats; $n = 6$ thalamic-injected rats). These criteria resulted in the exclusion of 2 parabrachial-injected animals and 2 thalamic-injected animals whose injection sites were not appropriately located (Figure 2A, E, F, open shapes). All cases included in the analysis also met the criteria for anterograde labeling with CTb that we have previously established for corneal afferents (Hegarty et al., 2010), namely the presence of at least 25 labeled varicosities in a single vibratome section within Vc.

2.3.2. Confocal Imaging—Images were collected as Z stacks bounded by the vertical extent of immunoreactivity for CTb, FG and NeuN in the tissue on a Zeiss LSM510 META confocal microscope with a 40 $\times$ /1.3 NA EC Plan-NEOFLUAR oil objective using the single

pass, multi-tracking format. Sections for analysis were chosen based on the prior darkfield mapping described above and included the areas of CTb-ir corneal afferent input in the ventrolateral regions of the Vc/C1 and Vi/Vc transition zones. Confocal micrographs used for publication are projections of several optical sections that were adjusted for optimal brightness and contrast using Zeiss ZEN software.

2.3.3. Confocal analyses—Images were first analyzed for the number of NeuN-ir somata that received appositions from CTb-ir corneal afferent varicosities (Figure 5) which were operationally defined as approximately circular punctate enlargements that were present in at least two consecutive optical sections (Bailey et al., 2006; Hegarty et al., 2010). For appositional analyses, CTb-ir varicosities had to be directly adjacent to NeuN-ir somata in two consecutive 0.5 μ m thick optical sections (Figure 5, open arrowheads). All animals included in analyses had at least 25 identifiable varicosities from independent axonal sources in Vc/C1 and 50 in Vi/Vc to ensure a lower limit of acceptable corneal afferent labeling (Hegarty et al., 2010). We then determined whether the NeuN-ir somata were projection neurons. A NeuN-ir soma was considered to be a projection neuron if FG immunoreactivity was present in at least two consecutive optical sections through the NeuN-ir cell. The total number of FG-ir neurons in the field was also counted, regardless of appositions by CTb-ir varicosities. All analyses were verified by two independent blinded observers. Parabrachial-projecting neurons were evaluated at both the Vc/C1 and Vi/Vc transition regions (Figures 6 and 7), and thalamic-projecting neurons were only evaluated in the Vi/Vc transition region as thalamic-projecting neurons were not found in Vc/C1 (Figures 3 and 8).

2.3.4. Statistical analyses—A t-test was performed for each transition region, Vc/C1 and Vi/Vc, to compare the mean number of CTb-ir corneal afferent varicosities that form appositions with somata of NeuN-ir neurons in rats that received FG injections into either parabrachial or thalamic nuclei. A Chi-square test was performed to evaluate differences in the number of CTb-ir corneal afferent appositions onto FG-ir parabrachial-projecting neurons versus thalamic-projecting neurons in Vi/Vc. Average numbers of neurons or varicosities apposed to somata are reported with standard error of the mean (SigmaStat, Systat Software, Inc., San Jose, CA).

3. RESULTS

The present studies were designed to determine if corneal afferents contact neurons in the trigeminal dorsal horn that project to either thalamic or parabrachial nuclei. FluoroGold (FG) injections were made into either the parabrachial nucleus ($n = 4$) or the primary sensory nuclei of the thalamus ($n = 6$) to retrogradely label neurons in the trigeminal dorsal horn that send ascending projections to these regions. Each animal also received an application of cholera toxin B tracer (CTb) to the left cornea to identify trigeminal afferents that provide sensory input from the surface of the eye. Analyses were conducted in the ventrolateral trigeminal dorsal horn, subnucleus caudalis (Vc; Figure 1) at both its caudal (Vc/C1) and rostral (Vi/Vc) poles.

3.1. Injection sites

Thalamic injection sites were guided by previous studies from this laboratory and others that have demonstrated that injection of FluoroGold (FG) into the ventral posteromedial nucleus (VPM), the ventral posterolateral nucleus (VPL), and the posterior nucleus (Po) of thalamus resulted in contralateral labeling of projection neurons in the ventrolateral dorsal horn of Vc (Mitchell et al., 2004; Guy et al., 2005). Another study in which the anterograde tracer *Phaseolus vulgaris*-leucoagglutinin (PHA-L) was injected into the dorsal horn of Vc also found a dense projection to the VPM thalamic nucleus (Iwata et al., 1992). In the present study, successful thalamic FG injections (Figure 2) overlapped regions of the VPM, the VPL, and the Po (Figure 2B – D, shaded regions), whereas injections that were centered 0.5 mm rostral to these subnuclei failed to produce retrograde labeled neurons in the ventrolateral trigeminal dorsal horn (Figure 2A, open shapes).

Injections into the parabrachial nuclei were similarly guided by previous studies from this laboratory in which FG injected into the left medial (MPB) and lateral (LPB) parabrachial nuclei retrogradely labeled projection neurons in ipsilateral ventrolateral dorsal horn of Vc (Mitchell et al., 2004). Previous anatomical studies using other tracers have found similar projections from Vc to the MPB and LPB, as well as the Kölliker-Fuse nucleus (Cechetto et al., 1985; Slugg and Light, 1994; Feil and Herbert, 1995). In the present study, successful injections into the parabrachial nuclei included medial and lateral subnuclei and typically extended into adjacent regions (Figure 2E – H, shaded regions). Two injections were centered in the inferior colliculus, dorsal to the parabrachial nuclei, and did not result in labeling in the trigeminal dorsal horn (Figure 2E, F, open shapes).

3.2. Distribution of corneal afferents and retrogradely labeled neurons throughout ventrolateral Vc

Although retrogradely labeled neurons were found in more dorsal regions of Vc, as well as within the reticular formation, we confined our analysis to the ventrolateral region of the trigeminal dorsal horn that receives corneal afferents (Marfurt and Del Toro, 1987; Hegarty et al., 2010) (Figure 4, boxes). Using epifluorescent and darkfield microscopy we examined the distributions of thalamic- and parabrachial-projecting neurons beginning at the caudal aspect of the trigeminal dorsal horn, at the transition between subnucleus caudalis and the cervical spinal cord (Vc/C1) and moved rostrally to subnucleus interpolaris (Vi; Figure 3). The distributions of thalamic- or parabrachial-projecting trigeminal neurons observed were quite distinct from each another (Figure 3). Thalamic-projecting neurons (Figure 3, gray bars) were absent from the ventrolateral aspect of Vc/C1 and began to appear in small numbers moving rostrally through Vc with a relative peak observed in Vi/Vc, followed by a small reduction in number moving rostrally into Vi. In contrast, parabrachial-projecting neurons (Figure 3, open bars) were found in much greater numbers throughout the rostrocaudal extent of the ventrolateral trigeminal dorsal horn. Moving rostrally through the brainstem, the average number of parabrachial-projecting neurons gradually increased to a peak at Vi/Vc and dropped off rather rapidly moving rostrally into Vi.

The pattern of CTb-ir corneal afferents (Figure 3, red dotted line) to trigeminal dorsal horn exhibited a topography similar to what we have previously observed (Hegarty et al., 2010).

Corneal afferents were detected throughout the rostrocaudal levels examined but exhibited two distinct peaks of immunoreactivity. A smaller peak was observed at Vc/C1 and a larger peak with more abundant labeling was observed at Vi/Vc (Figure 3, red circles).

Based on the observations above, we chose to use confocal microscopy to evaluate whether corneal afferents were apposed to trigeminal projection neurons in the transition regions at either end of subnucleus caudalis, Vc/C1 and Vi/Vc (Figure 3, green bars; Figure 4) where corneal afferents are most abundant and have been extensively studied. The caudal transition region, Vc/C1, corresponded to a relative peak of corneal afferent labeling (Figure 6B) as well as a fair number of parabrachial-projecting neurons (Figure 6A). Thalamic-projecting neurons were not present at this level and could therefore not be evaluated. The rostral transition region, Vi/Vc, corresponded to peak levels of corneal afferents (Figures 3, 7B, 8B) and projection neurons to each target (parabrachial: Figure 7A; thalamus: Figure 8A), and was therefore selected as a region for further analysis.

3.3. By confocal microscopy, corneal afferents contact neurons in caudal and rostral Vc

Before evaluating differences between thalamic- and parabrachial-injected animals, we wanted to ensure comparable CTb application across all animals, regardless of the FG injection site. In Vc/C1 and Vi/Vc we counted every CTb-ir corneal afferent varicosity (from one section per transition area per animal) from independent fibers that formed appositions with NeuN-ir somata (Figures 5, 6 – 8, panels B, C and D, open and closed arrowheads point to examples of appositions), regardless of the injection site. In Vc/C1 we found no difference in the average number of CTb-ir varicosities apposed to NeuN-ir somata in animals receiving FG injections into either thalamic (9 ± 2 appositions per animal) or parabrachial (12 ± 3 appositions per animal; t-test, $p = 0.43$) nuclei. Likewise, in Vi/Vc, no difference in the average number of CTb-ir varicosities apposed to all NeuN-ir somata was observed between rats receiving injections into thalamic (23 ± 4) or parabrachial nuclei (25 ± 5 appositions per animal; t-test, $P = 0.76$). These results suggest consistent CTb applications across animals and comparability of CTb-ir corneal afferent detection between injection groups. Therefore, any differences found in corneal afferent connectivity between populations of projection neurons were most likely due to differences between neuronal targets and not variations in the CTb application.

3.4. Corneal afferents preferentially contact parabrachial-projecting neurons in caudal and rostral Vc

By confocal microscopy, parabrachial-projecting neurons were reliably detected in Vc/C1 with an average of 16 ± 3 retrogradely labeled projection neurons observed per animal (Figure 4A). Approximately 13% of CTb-ir corneal varicosities (2 ± 0.3 varicosities per animal) that made an apposition onto a soma contacted a parabrachial-projecting neuron (Figure 6, closed arrowhead; Figure 9, Vc/C1, left panel, white bar). Unlike parabrachial-projecting neurons, thalamic-projecting neurons were absent from the ventrolateral trigeminal dorsal horn in Vc/C1 suggesting preferential connectivity between corneal afferents and neurons projecting to parabrachial nuclei (Figure 9, Vc/C1, left panel).

Parabrachial-projecting neurons were observed in much greater numbers at the rostral pole of Vc than caudally. Three times as many parabrachial-projecting neurons per section were observed rostrally (47 ± 16) than caudally (16 ± 3). Approximately 25% of CTb-ir corneal varicosities (6 ± 2 CTb-ir varicosities per animal) that made an apposition onto a soma in Vi/Vc contacted a parabrachial-projecting neuron at this level of Vc (Figure 7, closed arrowheads; Figure 9, Vi/Vc, right panel, white bar).

Thalamic-projecting neurons (19 ± 2 neurons per section per animal) were not as numerous as parabrachial-projecting neurons (47 ± 16) in Vi/Vc. Approximately 8% of CTb-ir corneal varicosities (2 ± 0.5 CTb-ir varicosities per animal) that made an apposition onto a soma contacted a thalamic-projecting neuron (Figure 8, closed arrowhead; Figure 9, Vi/Vc, right panel, gray bar). Therefore, corneal afferents that contact somata differentially target the two populations of projection neurons in Vi/Vc (Figure 9, Vi/Vc, right panel, *Chi-square 11.64, $P < 0.001$).

These results suggest differential targeting by corneal afferents to parabrachial- and thalamic-projecting neurons. In Vc/C1, corneal afferents are more likely to contact parabrachial-projecting neurons since thalamic-projecting neurons are not present at this level of Vc (Figure 9, Vc/C1, left panel; Figure 10); and even in Vi/Vc where corneal afferents and both types of projection neurons are abundant, corneal afferents differentially contact parabrachial-projecting neurons (Figure 9, Vi/Vc, right panel; Figure 10).

It is noteworthy that corneal afferents often contacted neurons in Vc that did not contain any retrograde tracer (open arrows, Figures 6d, 7d, 8d). While the lack of retrograde tracer does not definitely prove that these neurons do not project to parabrachial or thalamus, it is likely that corneal afferents also contact neurons that do not project to these regions and are involved in other functions, including brainstem reflex pathways involved in ocular surface homeostasis.

4. DISCUSSION

The major finding of this study is that corneal afferents preferentially target parabrachial-projecting neurons in trigeminal dorsal horn, and only rarely send direct projections to thalamic-projecting neurons. These results suggest that corneal pain is mediated primarily through a non-thalamic network, although it is possible that polysynaptic pathways with thalamic-projecting neurons may exist. Our findings may explain why corneal pain is fairly poorly localized and is often referred to periocular regions.

Previous anatomical studies from this laboratory (Mitchell et al., 2004) and others (Cechetto et al., 1985; Iwata et al., 1992; Allen et al., 1996) examined connections between the trigeminal nuclear complex and supraspinal substrates of nociception, specifically the thalamus and the parabrachial nucleus, which are thought to be involved in the sensory-discriminative and autonomic-emotional aspects of pain, respectively (Saper, 1995; Sessle, 1999; Gauriau and Bernard, 2002). We found that after injections of the retrograde tracer FluoroGold (FG) into the right ventral posteromedial (VPM) / ventral posterolateral (VPL) thalamic nuclei, projection neurons were found contralaterally in the ventrolateral dorsal

horn of Vc. When FG was injected into the left medial (MPB) and lateral (LPB) parabrachial nuclei, retrogradely labeled projection neurons were found ipsilaterally in the ventrolateral dorsal horn of Vc (Mitchell et al., 2004). In the present study we also found abundant numbers of parabrachial-projecting neurons in Vc, although a more detailed topographic analysis demonstrated that these cells were more numerous at rostral compared to caudal levels of Vc. Our topographic analysis also demonstrated a pattern in which FG-labeled thalamic-projecting neurons were present in very low numbers in ventrolateral Vc/C1 and then became more abundant in ventrolateral Vi/Vc; this is in agreement with a previous study that also used FG to retrogradely label projection neurons from thalamus to Vc (Guy et al., 2005). The majority of the thalamic FG injection sites encompassed the lateral portion of the VPM (Figure 2A – D). Injection of FG into this area of thalamus preferentially labels projection neurons in the ventral half of Vc, but these neurons are very sparse (Guy et al., 2005).

Connectivity has also been studied using antidromic activation of corneal-receptive neurons in Vc/C1 and Vi/Vc from thalamic and parabrachial nuclei (Meng et al., 1997; Meng et al., 1998; Hirata et al., 1999; Hirata et al., 2000). The parabrachial studies found that the majority of corneal-receptive Vc/C1 neurons could be antidromically activated from lateral parabrachial nucleus, including the Kölliker-Fuse nucleus which is in agreement with anatomical studies (Cechetto et al., 1985; Feil and Herbert, 1995). However, corneal-receptive neurons in the rostral Vi/Vc area could not be antidromically activated from the ipsilateral or contralateral parabrachial nucleus (Meng et al., 1997; Meng et al., 1998; Meng et al., 2000). In both Vc/C1 and Vi/Vc, we found contacts between corneal afferents and dorsal horn neurons that project to the ipsilateral parabrachial nucleus. It is possible that the confinement of our CTb tract-tracing to a small area in the middle of the cornea and the lack of modality-specific stimulation may account for some of the discrepancies with the antidromic studies. However, it is also possible that antidromic activation combined with stimulus-evoked responses may represent polysynaptic pathways that would not be detected in the current study which is focused on direct cellular pathways.

Antidromic activation was also used to examine corneal-receptive Vc/C1 and Vi/Vc neurons projecting to thalamic nuclei (Hirata et al., 1999; Hirata et al., 2000). Vc/C1 neurons reportedly project selectively to the posterior thalamic nuclear group (Po) but neither Vc/C1 nor Vi/Vc neurons could be activated by stimulation in the contralateral ventral posteromedial thalamic nucleus (VPM). The reported lack of connections between the caudal and rostral aspects of Vc and the contralateral VPM is not consistent with anatomical studies using both anterograde (*Phaseolus vulgaris*-leucoagglutinin, PHA-L) (Iwata et al., 1992) and retrograde (FG) (Mitchell et al., 2004; Guy et al., 2005) tracers. Anatomical studies suggest that both caudal and rostral Vc neurons project to contralateral Po and VPM (Iwata et al., 1992; Mitchell et al., 2004; Guy et al., 2005). Our present studies support that there are neurons in Vi/Vc that project to these regions of thalamus, but we found that they rarely receive direct contacts from corneal afferents. It is possible that the activation reported in previous electrophysiological studies is mediated by polysynaptic relays between the corneal afferents and the thalamic-projecting neurons in trigeminal dorsal horn, perhaps via interneurons (Fukushima and Kerr, 1979).

Interestingly, differential synaptic connectivity has been demonstrated in other brain areas, such as the case of projection neurons from the nucleus of the solitary tract (NTS) to the caudal ventrolateral medulla (CVLM) and paraventricular nucleus of the hypothalamus (PVN). Anatomical and electrophysiological evidence showed that cranial visceral afferents directly contact the somata and dendrites of neurons that project from the NTS to the CVLM indicating a monosynaptic pathway, while other NTS neurons projecting to the PVN demonstrated a lack of direct afferent input, suggesting a polysynaptic pathway (Bailey et al., 2006). We suggest that the trigeminal dorsal horn projection neurons may be similar in their synaptic connectivity to supraspinal substrates of pain, with preferentially monosynaptic connections with parabrachial-projecting neurons and potentially polysynaptic connections to thalamic-projecting neurons.

4.1. Methodological considerations

Our analyses were confined to CTb-ir corneal afferents that are thought to be lightly myelinated A-delta fibers (Todd et al., 2003). However, the pattern and density of anterograde tracing were similar to reports using other anterograde tracers, such as HRP (Marfurt and Del Toro, 1987). Our analyses were also confined to examining contacts with cell bodies of projection neurons; thus, afferent connections to distal dendrites of projection neurons may have gone undetected. However, since the analyses were conducted in similar fashion for both groups of projection neurons, our findings support a preferential network between corneal afferents and the parabrachial ascending pathway compared to the thalamic pathway. We did not seek to determine subtle differences in the topography of projections to thalamus, but even with large and extensive injections we were not able to detect a robust direct pathway for the corneal afferents to the thalamus.

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ABBREVIATIONS

BSA	bovine serum albumin
C1	cervical spinal cord segment 1
CTb	cholera toxin B subunit
FG	FluoroGold
-ir	-immunoreactive
LPB	lateral parabrachial nucleus
MPB	medial parabrachial nucleus

PB	phosphate buffer
PHA-L	<i>Phaseolus vulgaris</i> -leucoagglutinin
Po	posterior nucleus
Vc	trigeminal subnucleus caudalis
Vi	trigeminal subnucleus interpolaris
VPL	ventral posterolateral nucleus
VPM	ventral posteromedial nucleus

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HIGHLIGHTS

CTb was used to label corneal afferents to trigeminal subnucleus caudalis (Vc).
FluoroGold (FG) labeled thalamic- and parabrachial-projecting neurons in Vc.
CTb-labeled corneal afferents project more densely to rostral Vc than caudal Vc.
Thalamic- and parabrachial-projecting neurons are more abundant in rostral Vc.
Corneal afferents preferentially target parabrachial-projecting neurons in Vc.

Figure 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 subnucleus caudalis.

Figure 2.

FluoroGold (FG) injection sites into the thalamus (A – D) or parabrachial nuclei (E – H). The top panel indicates the rostrocaudal locations of the illustrations in panels A – H. The FG injections that resulted in successful retrograde labeling in the ventrolateral aspect of the trigeminal dorsal horn are shaded with gray and those that did not are represented as open shapes. The injection outlines include both the core of the injection site and the halo often seen with FG injections; thus the injection site depictions are larger than the core area from which neurons were successfully labeled. The two FG injections near thalamus that did not result in labeling in the trigeminal dorsal horn were located too rostrally, illustrated by the open shapes in panel A at –2.1 mm caudal to Bregma. In all successful thalamic cases, the

core of the FG injection site that produced retrograde labeling in Vc included Po, VPM, while some also included VPL. The two FG injections near parabrachial nuclei that did not result in labeling were too dorsal (open shapes, E and F). In all successful PB cases, the core of the FG injection site included the lateral PB (LPB). Scale bars = 1 mm. Representative diagrams are modified from the digital atlas of Paxinos and Watson (Paxinos and Watson, 1998) and are reproduced here with permission from the publisher.

Figure 3.

Rostrocaudal distribution of retrogradely labeled parabrachial- (white bars) or thalamic- (gray bars) projecting trigeminal neurons and CTb-ir corneal afferents (red circles) in the ventrolateral aspect of the trigeminal dorsal horn. Two relative peaks of CTb-ir corneal afferents were observed; the most caudal peak is at the Vc/C1 transition area (Vc/C1 in green bar), and a more robust peak is seen rostrally at the Vi/Vc transition area (Vi/Vc in green bar). The rostral peak of CTb-ir corneal afferents coincides with the peak of both the parabrachial-projecting and thalamic-projecting neurons. In contrast, a smaller number of parabrachial-projecting neurons were observed and no thalamic-projecting neurons were present in caudal Vc/C1. Regions corresponding to the rostral and caudal peaks of CTb-ir (green bars) were examined using confocal microscopy to look for appositions between CTb-ir corneal afferents and FG-ir projection neurons (green = areas sampled). Scale bar = 1 mm.

Figure 4. Darkfield micrographs of the ventrolateral trigeminal dorsal horn illustrating retrograde labeling from parabrachial nuclei in caudal (A) and rostral (B) brainstem, and retrograde labeling from the thalamus in rostral brainstem (C). Retrogradely labeled neurons were located primarily in the outer laminae of Vc and at the transition region between Vc and Vi. Retrogradely labeled neurons were also seen in the reticular formation (Rt) but this area was not included in the analysis. The open boxes approximate where cell counts were made as well as where appositions were quantified. The neurons projecting to parabrachial nuclei are

more numerous than neurons projecting to thalamic nuclei, both caudally (panel A versus no neurons projecting to thalamus) and rostrally (B versus C). Arrows indicate dorsal and medial directions for orientation on these coronal sections. spV = spinal trigeminal tract. Scale bar = 250 μ m.

Figure 5.

Corneal afferents form appositions with somata of neurons in the trigeminal dorsal horn. Confocal micrographs of consecutive optical sections in the Z-axis, separated by 0.5 μm , demonstrate a CTb-ir corneal afferent varicosity (white) apposed to a NeuN-ir somata (blue) for two consecutive sections (open arrowheads). Varicose fibers may have multiple contacts with a single neuron, but each varicose fiber was only counted once with respect to a neuronal target for this analysis. Scale bar = 10 μm .

Figure 6.

Corneal afferents contact parabrachial-projecting neurons in caudal trigeminal dorsal horn. Confocal micrographs illustrating parabrachial-projecting FG-ir neurons (A, red), CTb-ir corneal afferents (B, white), NeuN-ir somata (C, blue), and the overlay of all three (D) in ventrolateral Vc/C1. Open arrowheads indicate CTb-ir varicosities that are apposed to NeuN-ir only somata. The single closed arrowhead points toward a CTb-ir varicosity that is apposed to a neuron that is immunoreactive for both NeuN as well as FG and are thus

parabrachial-projecting. Image is a Z projection of 17 consecutive optical sections for a total thickness of 8.5 μm . Scale bar = 20 μm .

Figure 7.

Corneal afferents contact parabrachial-projecting neurons in rostral trigeminal dorsal horn. Confocal micrographs illustrating parabrachial-projecting FG-ir neurons (A, red), CTb-ir corneal afferents (B, white), NeuN-ir somata (C, blue), and the overlay of all three (D) in ventrolateral Vi/Vc. The open arrowhead indicates a CTb-ir varicosity that is apposed to a NeuN-ir only somata. The closed arrowheads indicate CTb-ir varicosities that are apposed to neurons that are immunoreactive for both NeuN as well as FG and are thus parabrachial-

projecting. Image is a Z projection of 17 consecutive optical sections for a total thickness of 8.5 μm . Scale bar = 20 μm .

Figure 8.

Corneal afferents contact thalamic-projecting neurons in rostral trigeminal dorsal horn. Confocal micrographs illustrating thalamic-projecting FG-ir neurons (A, red), CTb-ir corneal afferents (B, white), NeuN-ir somata (C, blue), and the overlay of all three (D) in the ventrolateral Vi/Vc. Open arrowheads indicate CTb-ir varicosities that are apposed to NeuN-ir only somata. The closed arrowhead indicates a CTb-ir varicosity that is apposed to a neuron that is immunoreactive for both NeuN as well as FG and is thus a thalamic-

projecting neuron. Image is a Z projection of 17 consecutive optical sections for a total thickness of 8.5 μm . Scale bar = 20 μm .

Figure 9.

The average number of appositions observed between CTb-ir afferent varicosities and somata of neurons at the caudal transition of subnucleus caudalis (Vc/C1; left panel) and the rostral transition of subnucleus caudalis (Vi/Vc; right panel) per animal. Appositions were counted between CTb-ir varicosities and all somata; which included somata displaying immunoreactivity only to the neuronal marker NeuN (black bars), as well as cells that were immunoreactive for both NeuN and FG, the retrograde tracer injected into either thalamic (Thalamic injected, gray bar) or parabrachial nuclei (Parabrachial injected, white bars). **Left panel.** In Vc/C1, a small number of appositions onto the somata of parabrachial-projecting neurons (Parabrachial Injected; white bar) were observed while no appositions between corneal afferents and thalamic-projecting neurons were observed because no thalamic-projecting neurons were present at this level of the trigeminal subnucleus caudalis (Thalamic Injected). No difference in the average number of CTb-ir afferent appositions onto all neurons (Thalamic Injected vs Parabrachial Injected) was observed (t-test, $p=0.43$). **Right panel.** In Vi/Vc, CTb-ir afferents were generally more abundant as compared to Vc/C1, thus there are more CTb-ir afferent appositions onto NeuN-ir only somata (black bars) rostrally (right panel) than caudally (left panel). In Vi/Vc, there were three times more appositions between corneal afferents and parabrachial-projecting neurons (Parabrachial Injected, white bar) than between corneal afferents and somata of thalamic-projecting neurons (Thalamic Injected, gray bar; * Chi-square test, number of CTb-ir varicosities that contact Vi/Vc somata in both sets of FG-injected animals, $p < 0.001$). No difference in the average number of CTb-ir afferent appositions onto all neurons (Thalamic Injected vs Parabrachial Injected) was observed (t-test, $p=0.76$).

Figure 10.

Schematic representation of the results of the study. More corneal afferents project to the Vi/Vc transition in brainstem (thick arrows), than the Vc/C1 transition area (dashed arrows). A strong direct connection was also observed between CTb-ir corneal afferents in the ventrolateral trigeminal dorsal horn and the somata of parabrachial-projecting neurons, with a more robust connection being seen at the Vi/Vc (thick arrow) transition than the Vc/C1 transition (thin arrow). A small projection was also observed between CTb-ir corneal afferents in the rostral transition zone, Vi/Vc, and the somata of thalamic-projecting nuclei (dashed arrow). There were no thalamic-projecting neurons in ventrolateral Vc/C1, thereby precluding analysis of CTb-ir corneal afferent connectivity to thalamic-projecting somata in this region.