Anatomic Evidence for Information Exchange between Primary Afferent Sensory Neurons Innervating the Anterior Eye Chamber and the Dura Mater in Rat

Haixia Liu,[1] Xutao Zhu,[2,3] Yun Ling,[1] Xiaobin He,[2] Lei Pei,[4] Zhidan Zhang,[1] Fang Yang,[1] and Fuqiang Xu[2]

[1]Department of Ophthalmology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[2]Center for Brain Science, Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences, Wuhan, China
[3]University of Chinese Academy of Sciences, Beijing, China
[4]The Institute for Brain Research, Collaborative Innovation Center for Brain Science, Huazhong University of Science and Technology, Wuhan, China

Correspondence: Haixia Liu, Department of Ophthalmology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China; haixia72@aliyun.com.

HL and XZ are joint first authors.

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Purpose. Our previous studies suggested that mechanosensitive trigeminal ganglion (TG) nerve endings innervating the inner wall of the anterior eye chamber (IWAEC) might play a role in baroreception of the IOP. It has been reported that mechanosensitive TG nerve endings also innervate the dura mater. An acute IOP elevation evokes eye pain accompanied by an ipsilateral headache, suggesting that information exchange may occur between the primary afferent neurons (PANs) innervating the IWAEC and the dura mater. To verify the information exchange between PANs of the two locations, we investigated the anatomic connection between them.

Methods. Non–trans-synaptic tracers, 1,1′-dilinoleyl-3,3,3′,3′-tetramethylindo-carbocyanine, 4-chlorobenzenesulfonate (FAST DiI) and cholera toxin subunit-B with a 488-nm fluorescent tag (CTB-488), were applied to the dura of the anterior cranial fossa (DACF) and the anterior eye chamber (AEC) to label the PANs. A trans-synaptic tracer, GFP-expressing pseudorabies virus (PRV152), was injected into the AEC while FAST DiI was applied to the DACF to explore the connection between PANs. Fluorescent localization in the TG was studied with a confocal fluorescent microscope.

Results. Nine days after rats were treated with CTB-488 in the AEC and FAST DiI on the DACF, FAST DiI-labeled (red), and CTB-488-labeled (green) TG neurons were observed in the medial part of the TG, while double-labeled neurons were absent. If PRV152 was used to substitute CTB-488, then FAST DiI (red) and PRV152 (green) double-labeled TG neurons and axons were observed 3 days later.

Conclusions. Our results indicate that synapses exist between PANs of the IWAEC and the DACF, providing anatomic evidence for information exchange between them.

Keywords: IOP, trigeminal ganglion, dura mater, anterior cranial fossa, anterior eye chamber, primary afferent neuron

The lamina cribrosa forms the interface between the intraocular compartment and the retrobulbar compartment. Because the optic nerve is part of the central nervous system, it is surrounded by meninges and cerebrospinal fluid (CSF).[1,2] The translaminar pressure difference (TPD) is defined as the difference between the IOP and the intracranial pressure (ICP).[1,2] Any change in one of them can be associated with a disturbance of homeostasis of the optic nerve head, such as papilledema or glaucomatous optic neuropathy.

The IOP and ICP systems are relatively independent pressure systems, which keep themselves in a relatively stable state through aqueous and CSF circulations.[1–3] Significant correlations between the IOP and ICP have been widely reported. However, mechanisms by which the organism maintains the balance between the IOP and ICP have so far remained elusive.[1,3,4] In a recent experimental study, chemical stimulation of neurons in the dorsomedial and perifornical hypothalamus (DMH/PeF) regions evoked reproducible increases in the IOP, ICP, and TPD.[5] It was suggested that the ICP and IOP might be coregulated in part by neurons located in the DMH/PeF region of the hypothalamus.[5]

The trigeminal ganglion (TG) nerve was considered to be the primary afferent nerve in neural regulation of the IOP.[6–8] Our previous studies proved that mechanosensitive TG nerve endings innervate the inner wall of the anterior eye chamber (IWAEC), where aqueous circulation takes place, and that the transient receptor potential ankyrin-1 (TRPA1) is an essential mechanosensitive channel in the membranes of the TG nerve endings.[9–11] These results gave a possible answer as to how IOP variation triggers action potentials of the TG nerve. We proposed that mechanosensitive TG nerve endings might act as IOP baroreceptors in neural regulation of the IOP.[9–11]

Interestingly, as the IWAEC, where aqueous circulation takes place, TG nerve endings also innervate the dura mater, where CSF circulation takes place.[12–14] Studies show that dural afferents are mechanically sensitive.[12–14] It is well known that an acute IOP elevation evokes serious eye pain with an incidental, ipsilateral headache. Although a specific mechanism for this clinical phenomenon of glaucoma has not been elucidated, it suggests that information exchange may occur between the primary afferent neurons (PANs) innervating the IWAEC and the dura mater.

Based on the abovementioned studies and clinical phenomena, we hypothesized that the mechanosensitive TG nerve endings act as baroreceptors of the IOP and the ICP, that information exchange exists between the primary afferent sensory neurons innervating the IWAEC and the dura mater, and that a neural regulation mechanism coregulates the IOP and ICP to maintain a proper TPD.

To validate the hypotheses, we have done some preliminary exploration in the present study. We determined the connection between PANs innervating the IWAEC and the dura of the anterior cranial fossa (DACF) using two kinds of fluorescent tracers in the same rat.

Materials and Methods

Reagents

Cholera toxin subunit-B with a 488-nm fluorescent tag (CTB-488) and FAST Dil were purchased from Invitrogen (Carlsbad, CA, USA). GFP-expressing pseudorabies virus (PRV152) was obtained from BrainVTA (BrainVTA Co., Ltd., Wuhan, China). Rabbit anti-GFP polyclonal antibodies were obtained from Abcam (Cambridge, MA, USA). Goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibody was from Jackson Laboratory (Bar Harbor, ME, USA). 2-(4-Amidinophenyl)-6-indolecarbamidinedihydrochloride (DAPI) was from Biotechnology (Haimen, China). All the reagents were prepared as stock solutions and stored at −20°C or 4°C, according to the manufacturers' instructions.

Animals

Adult Sprague-Dawley rats (180–200 g) were individually housed in plastic cages with free access to food and water and maintained in climate- (23 ± 1°C) and light-controlled (12/12-hour dark/light cycle with light on at 8 AM) protected units for at least 10 days before the experiments. The Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology approved the experimental protocols. The handling, treatment, and procedures on animals were carried out according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research guidance. In total, 32 rats were used in this study.

FAST Dil Retrograde Labeling of TG Neurons Innervating the DACF

FAST Dil retrograde labeling was performed to identify the cell bodies of TG neurons innervating the DACF, similar to the methods described in previous literature.[15–17]

The rats were deeply anesthetized with 10% chloral hydrate (4 mL/kg) and placed in a stereotaxic apparatus (RWD 68030; RWD Life Science Co., Ltd., Shenzhen, China). The rats were topically anesthetized with 2% lidocaine (0.5 mL), applied to the skin. A longitudinal skin incision was made to expose the cranium, and a 5 mm in diameter craniectomy (5-mm right of midline, 5-mm anterior to bregma) was made using a cooled dental drill (90+102; STRONG, Guangdong, China) in the skull overlying the DACF, leaving the underlying dura exposed but intact. The borosilicate glass pipette was connected to a 10-μL Hamilton syringe (Hamilton, Reno, NV, USA). A small piece of gelatin sponge (~5 mm in diameter) was put on the DACF via the craniectomy. Two microliters of FAST Dil solution (2.5 mg/mL in dimethyl sulfoxide [DMSO]) was injected into the gelatin sponge, to avoid spreading of the dye. The vehicle was used as negative control. A mixture of dental cement powder was used to seal the craniectomy. The skin incision was closed using a silk suture.

CTB-488 Retrograde Labeling of TG Neurons Innervating the IWAEC

CTB-488 retrograde labeling was performed to identify the cell bodies of TG neurons innervating the IWAEC in rats, as previously described.[9–11,18] Briefly, the rats were topically anesthetized with 0.4% oxybuprocaine hydrochloride eye drops after anesthesia with chloral hydrate and a borosilicate glass pipette with a 10- to 15-μm diameter tip was inserted through the cornea into the anterior chamber of the right eye. Caution was used to avoid injuring the iris and lens. The borosilicate glass pipette was connected to a 10-μL Hamilton syringe (Gastight; Hamilton). Two microliters of CTB-488 solution (0.2 mg/mL in PBS) was stereotaxically microinjected slowly into the anterior chamber (0.2 μL/min) to avoid induction of acute hypertension. The borosilicate glass pipette was kept in the cornea for 5 minutes after injection to avoid leakage of CTB-488. The vehicle was used as negative control.

PRV152 Retrograde Labeling of TG Neurons Innervating the IWAEC

The same method was used to inject 2 μL PRV152 (5×10^9 pfu/mL) into the right anterior eye chamber (AEC). The vehicle was used as negative control.

Rat Treatment After Fluorescent Tracer Application

The animals were declined access to water for 12 hours after revival. After recovery from anesthesia, the rats were housed individually in the animal facility for 3 (rats that had undergone PRV152 labeling) or 9 days to allow the transport of the tracer to the somata in the TG.

Tissue Preparation

Three or 9 days later, the rats were deeply anesthetized with 10% chloral hydrate and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) solution. The right TG ganglia were removed and post-fixed over night in 4% PFA before being sectioned into 40-μm slices (NX50; ThermoFisher, Waltham, MA, USA).

Immunofluorescence Staining to Amplify Signal of PRV152

The samples from the rats that had undergone FAST Dil and CTB-488 application were imaged directly using a TCS SP8 confocal laser-scanning microscope (TCS SP8; Leica, Wetzlar, Germany). Immunofluorescence staining was used to amplify the signal of PRV152 before being imaged.

After three rinses with PBS for 5 minutes each, the sections were treated with 10% goat serum albumin-PBS for 60 minutes at 37°C. The samples were then incubated with 1:1000 rabbit

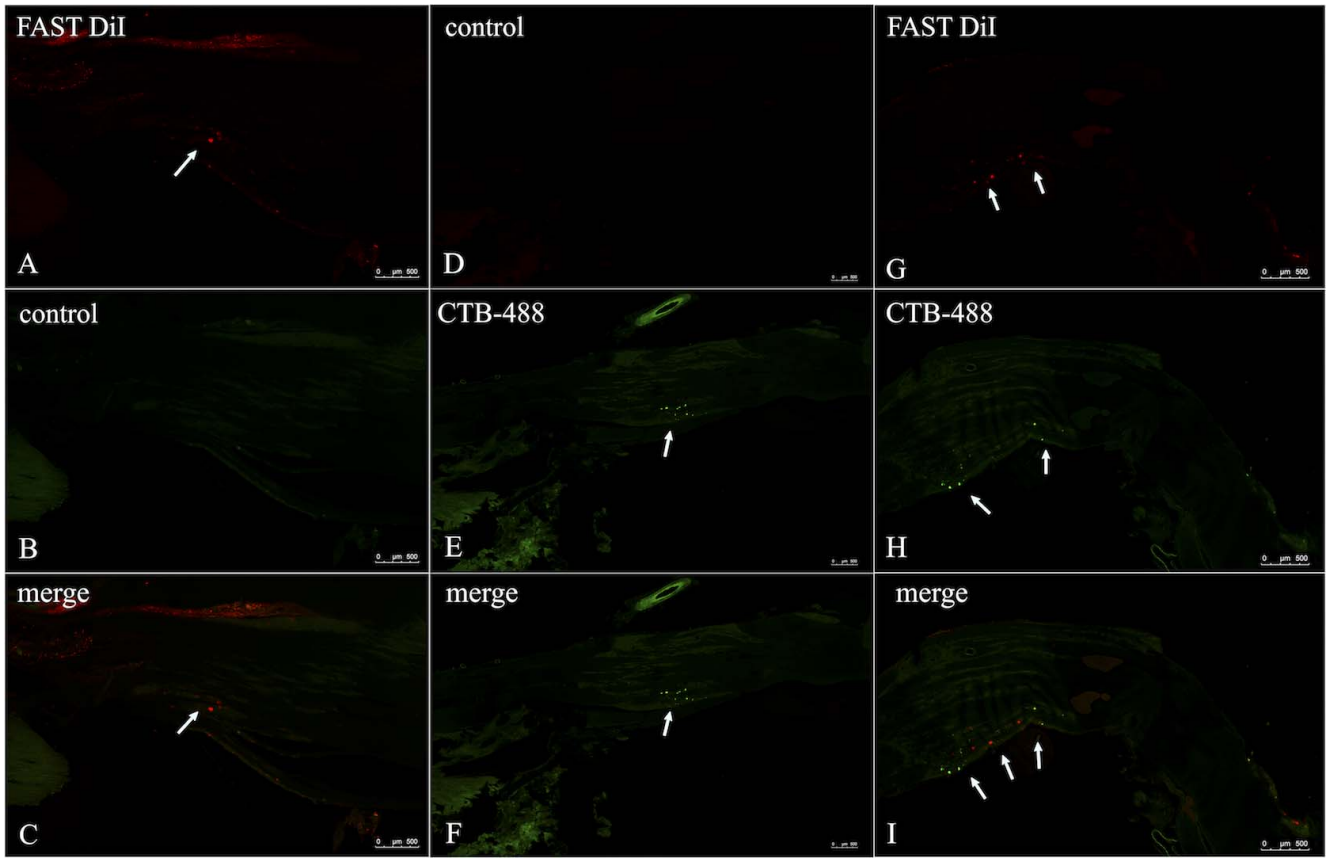

Figure 1. TG neurons innervating the DACF and the IWAEC, both distributed in the medial part of the TG. (**A–C**) Representative images of TG from five rats were applied with vehicle (IWAEC) and FAST DiI (DACF) and killed 9 days later. (**A**) *Arrows* indicate the FAST DiI (*red*)-labeled TG neurons distribute in the medial part of the TG. (**B**) Negative control. There was not any CTB-488 (*green*)-labeled TG neuron. (**C**) Only FAST DiI (*red*)-labeled TG neurons were found in the merged photo. (**D–F**) Representative images of TG from five rats treated with CTB-488 (IWAEC) and vehicle (DACF) and killed 9 days later. (**D**) Negative control. There was not any FAST DiI (*red*)-labeled neuron. (**E**) *Arrows* indicate the CTB-488 (*green*)-labeled TG neurons distribute in the medial part of the TG. (**F**) Only CTB-488 (*green*)-labeled TG neurons were found in the merged photo. (**G–I**) Representative images of TG from five rats treated with CTB-488 (IWAEC) and FAST DiI (DACF) and killed 9 days later. (**G**) *Arrows* indicate the FAST DiI (*red*)-labeled TG neurons. (**H**) *Arrows* indicate the CTB-488 (*green*)-labeled TG neurons. (**I**) FAST DiI (*red*)-labeled TG neurons innervating the DACF and CTB-488 (*green*)-labeled TG neurons innervating the IWAEC both distribute in the medial part of the TG. *Scale bars*: 500 μm.

anti-GFP antibody in PBS/0.3% Triton X-100/1% BSA/2% goat serum for 24 hours at 4°C. Following this, the samples were rinsed with PBS three times for 5 minutes each and then incubated with 1:200 goat anti-rabbit FITC-conjugated secondary antibody in PBS containing 0.3% Triton X-100, 2% goat serum, and 1% BSA for 1 hour at room temperature and then stained with DAPI for 10 minutes. After three washes with PBS for 5 minutes each, the samples were mounted with 70% glycerin and imaged.

Results

The PANs of the IWAEC and the DACF Both Aggregate in the Ophthalmic Region of TG

The trigeminal nuclei are organized somatotopically. In rats, the TG has three lobes, an ophthalmic, a maxillary, and a mandibular. Each lobe innervates a specific region of the face. The most medial part of TG was considered to be the ophthalmic region in rats.[19] Our previous study in rats found that PANs of the IWAEC aggregate in the dorsomedial part of the ipsilateral TG, within the ophthalmic region.[18] In the present study, we injected another non–trans-synaptic tracer, CTB-488, into the AEC and got the same result as before.[18] The green CTB-488–labeled neurons were observed in the medial part of the ipsilateral TG (Fig. 1E) and could not be found in the negative control (Fig. 1D).

The innervation of cranial dura mater was explored thoroughly; the DACF and the tentorium cerebelli were innervated by nerve fibers of the ophthalmic trigeminal division while the middle cranial fossa was innervated by nerve fibers of the mandibular and maxillary divisions.[15,20] If a connection between the PANs innervating the IWAEC and the dura mater really exists, it is most likely constituted by neurons in the same ophthalmic region, so we investigated the somatotopic organization of the DACF in rats first. Nine days after application of FAST DiI on the DACF, the red FAST DiI-labeled neurons could be observed in the medial part of the ipsilateral TG (Fig. 1A) and could not be found in the negative control sample (Fig. 1D).

Single TG Neurons do not Project to the IWAEC and the DACF Concurrently

To investigate whether one TG neuron can project not only to the AEC but also to the DACF, we applied CTB-488 into the right AEC and FAST DiI on the ipsilateral DACF concurrently.

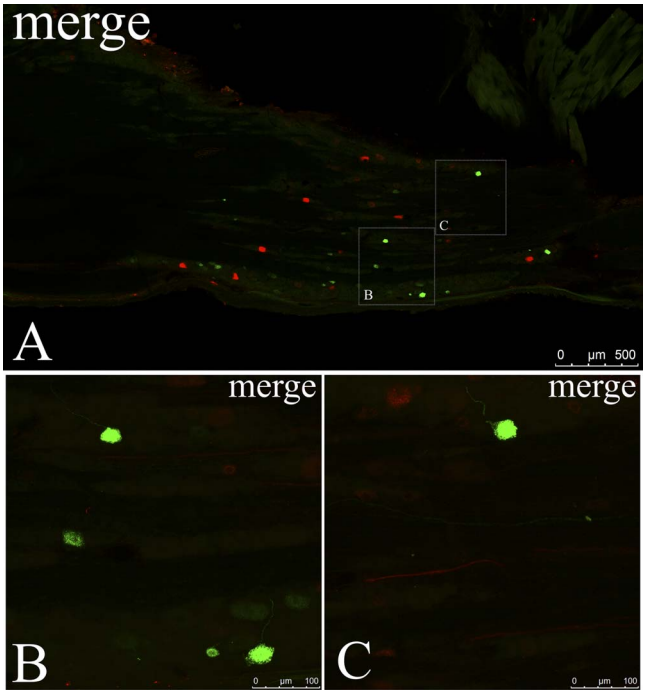

Figure 2. Single TG neurons did not project to the IWAEC and the DACF concurrently. (**A**) Representative images of TG ganglia from five rats treated with CTB-488 (IWAEC) and FAST DiI (DACF) and killed 9 days later. *Arrows* indicate the FAST DiI (*red*)-labeled TG neurons innervating the DACF and CTB-488 (*green*)-labeled TG neurons innervating the IWAEC. Double-labeled TG neurons and axons were not found. *Scale bars*: 500 μm. (**B**, **C**) There was not any double-labeled TG neuron or axon in the amplified rectangle areas of the top panel. *Scale bars*: 500 μm.

Nine days later the green CTB-488-labeled and the red FAST DiI-labeled neurons both presented in the medial part of the TG (Figs. 1G–I, 2A). However, no double-labeled TG neurons or fibers were found in any sections from the five rats (see Figs. 2A–C).

These results indicate that single TG neurons do not project to the IWAEC and the DACF concurrently.

Connecting Synapses Exist Between TG Neurons Innervating the IWAEC and the DACF

Different from FAST DiI and CTB-488, PRV152 is a trans-synaptic marker in the central nervous system, capable of retrograde trans-synaptic infection.[21] After infecting TG neurons from the nociceptive nerve ending, PRV152 can travel trans–multi-synaptically to infect the other neurons.[21] To determine whether connections exist between the PANs innervating the IWAEC and the DACF, we injected PRV152 into the AEC and applied FAST DiI on the DACF. Because rats usually do not survive more than 4 days after PRV152 injection, the rats were killed 3 days later. The green PRV152-labeled and the red FAST DiI-labeled neurons both could be observed in the medial part of the TG (see Figs. 3A–C). Furthermore, double-labeled (yellow) TG neurons and fibers could be identified in sections from all five rats (Figs. 3C, 4A–D). As shown in Fig. 4D, PRV152 (green) could cross synapses from a PRV152-labeled axon (green) to infect a FAST DiI-labeled (red) TG neuron.

These results demonstrate that synapses exist between PANs innervating the IWAEC and the DACF.

Discussion

Our present study demonstrates the existence of synapses between PANs innervating the IWAEC and the DACF in rat. Here, we suggest that information exchange occurs between them.

The anatomy of TG is not completely the same among different kinds of mammals. In rabbits, the TG has only two lobes, a mandibular and a maxillary-ophthalmic,[22] while in rats and humans, the TG has three lobes, an ophthalmic, a maxillary, and a mandibular.[23] Previous studies have found that there is a high homology between rats and humans in trigeminal innervation,[19,15,20] so rats are considered as a valid model to study the anatomic and functional characteristics of trigeminal innervation in humans.[15]

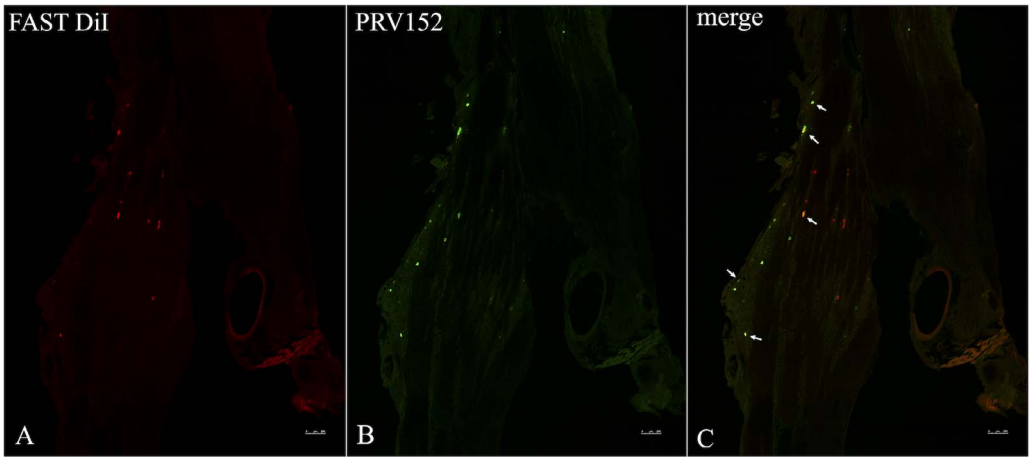

Figure 3. Double-labeled TG neurons of FAST DiI (*red*) and PRV152 (*green*) in the TG. (**A**–**C**) Representative images of TG ganglia from five rats treated with PRV152 (IWAEC) and FAST DiI (DACF) and killed 3 days later. (**A**) *Arrows* indicate the FAST DiI (*red*)-labeled TG neurons innervating the DACF. (**B**) PRV152 (*green*)-labeled TG neurons and axons including the primary afferent TG neurons innervating the IWAEC and TG neurons trans-synaptically infected by PRV152 from the primary afferent TG neurons. (**C**) Some FAST DiI (*red*)-labeled meningeal afferent TG neurons trans-synaptically infected by PRV152 were double-labeled (*yellow*). *Arrows* indicate the double-labeled TG neurons and axons. *Scale bars*: 75 μm.

Investigative Ophthalmology & Visual Science

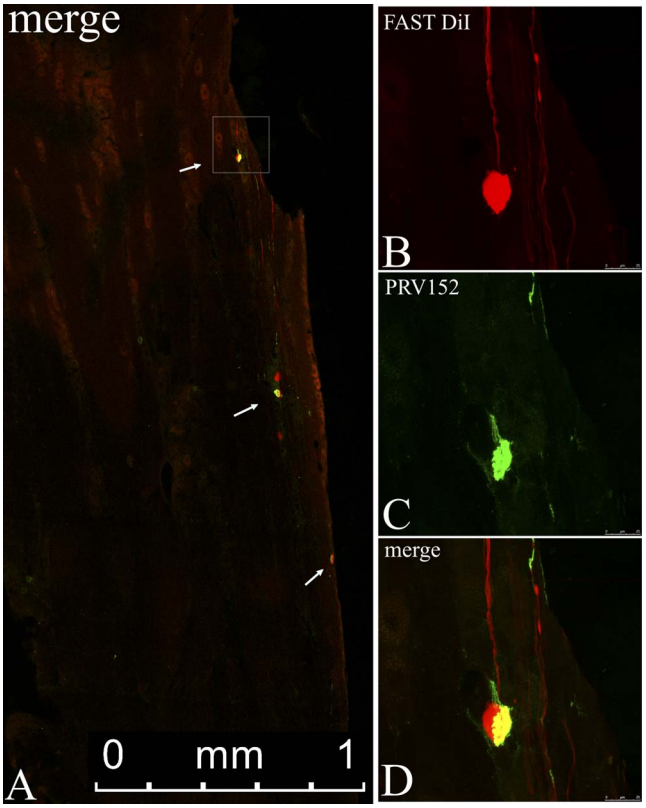

FIGURE 4. PRV152 crosses the synapse from a TG neuron innervating the IWAEC into another TG neuron innervating the DACF. (**A–D**) Representative images of TG ganglia from five rats treated with PRV152 (IWAEC) and FAST DiI (DACF) and killed 3 days later. *Arrows* indicate the double-labeled (*yellow*) TG neurons. *Scale bars*: 1 mm. The *rectangle area* of the *left panel* was amplified to (**B–D**). (**B**) A FAST DiI (*red*)-labeled TG neuron and axon innervating the DACF. (**C**, **D**) PRV152 (*green*) crossed a synapse via a PRV152 (*green*)-labeled axon to infect a FAST DiI (*red*)-labeled TG neuron, and more than half part of the neuron was double-labeled (*yellow*). *Scale bars*: 25 μm.

In this study, retrograde neuronal tracing revealed PANs innervating the DACF exclusively in the medial part of the TG. It was previously shown that the most medial part of the TG connects to the ophthalmic region in rats.[19] Other studies found the DACF and the tentorium cerebelli were innervated by nerve fibers of the ophthalmic division in humans and other primates.[15,20] Our result is consistent with previous studies on innervation of the dura mater.

When we used CTB-488 in the AEC and FAST DiI on the DACF to trace the PANs, CTB-488–labeled and FAST DiI-labeled cell bodies both presented in the medial part of the TG; colabeled neurons and fibers were absent. However, when we replaced CTB-488 with PRV152, colabeled neurons and fibers were observed. As opposed to CTB-488 and FAST DiI, PRV152 is a retrograde trans-synaptic marker.[21] After infecting TG neurons from the nociceptive nerve endings, PRV152 can travel trans–multi-synaptically to infect other neurons. Our results demonstrate that single TG neurons do not project to the IWAEC and the DACF concurrently, but connecting synapses exist between PANs of these areas. We provide anatomic evidence for information exchange between PANs innervating the IWAEC and the DACF in rats.

To the best of our knowledge, this study represents the first evidence suggesting that information exchange between the PANs innervating the IWAEC and the DACF takes place. However, what does it mean to our organism?

Prior preclinical studies have shown that dural afferents are mechanically sensitive.[12–14] Although receptors or structures on meningeal afferent endings have yet to be fully described, several recent studies demonstrated that TRPA1 is expressed[16] and functional[24] on dural afferents in rodents. TRPA1 is a member of the large TRP family of ion channels and functions as a Ca^{2+}-permeable nonselective cation channel.[25] Numerous studies have demonstrated that the TRPA1 channel protein affects the mechanosensation process in tissues.[25–27] There is accumulating evidence indicating that TRPA1 channels contribute to mechanical stimulus–evoked pain.[24,28,29] Previously, the hypothesis that activation of meningeal afferent nociceptors and TRPA1 channels on the membrane play a role in the pathophysiology of headache disorders was the most widely accepted.[30]

Intriguingly, our recent study indicated that some TG nerve endings innervating the IWAEC are also mechanically sensitive, and that TRPA1 is an important mechanotransducer in the membrane.[9–11] Accumulating evidence has shown that mechanosensitive TG nerve endings in the IWAEC may play a role in IOP sensing.[6–11,31,32] Previously, we did not know how an acute IOP elevation could evoke serious eye pain with an ipsilateral headache. Our results provide a reasonable explanation for this clinical phenomenon of glaucoma.

IOP, ICP, and blood pressure (BP) systems are relatively independent pressure systems in our organism. Evidence shows that under physiologic conditions, the pressures in all three fluid-filled compartments correlate positively with each other.[2,5,33–35] Although within this triangle of pressure relationships, BP, the highest one among the three pressures, may act as the driving force[2]; most efforts have been made to investigate the correlation between the IOP and ICP. Previously, it was hypothesized that the ICP influences the pressure in the superior ophthalmic vein and thus in the episcleral vein in a retrograde manner, and this pressure directly and linearly influences the IOP.[4] Recently, accumulating evidence has shown that there are many similarities between the ICP and IOP systems, and this two-pressure system might be coregulated in part by the nervous system. Clinically, high IOP and high ICP can both activate the vagus nerve and induce nausea or vomiting. Physiologically, the IOP and ICP are dynamic parameters with circadian variations (24 hours).[3] The response to changes in posture and intra-abdominal or intrathoracic pressure are similar.[36] Both aqueous fluid and CSF are produced by carbonic anhydrase-catalyzed reactions, and the chemical composition of these fluids is almost the same, with more proteins and less ascorbates in CSF.[3] Chemical stimulation of neurons in the DMH/PeF region evoked increases in both of these fluids.[5] Most interesting is the similar mechanosensitive afferent on the IWAEC, where the aqueous circulation takes place, and the dura mater, where CSF circulates. It is well known that arterial baroreceptors are mechanoreceptive nerve endings that innervate the adventitia of carotid sinuses and the aortic arch.[37] If mechanosensitive TG nerve endings act as IOP baroreceptors in neural regulation of the IOP, mechanosensitive TG nerve endings may act as ICP baroreceptors in neural regulation of the ICP too. Our present study shows the synapses of PANs innervating the IWAEC and the DACF provide new evidence for coregulation of the IOP and the ICP.

Our current study has one limitation. We were unable to do a comparative tracing study in humans. However, due to the high homology of the trigeminal innervation between rats and

humans, studies performed in rats are substantial in understanding the trigeminal innervation of humans.

In summary, our results provide anatomic evidence for information exchange between PANs innervating the IWAEC and the DACF. It presents a valuable clue to further explore the mechanism that coordinates the regulation of the IOP and the ICP in mammals.

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Investigative Ophthalmology & Visual Science

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