American Pain Society
RESEARCH
EDUCATION
TREATMENT
ADVOCACY
PUBLISHED BY ELSEVIER

The Journal of Pain, Vol ■■, No ■■ (■■), 2018: pp ■■-■■
Available online at www.jpain.org and www.sciencedirect.com

# Increased HCN Channel Activity in the Gasserian Ganglion Contributes to Trigeminal Neuropathic Pain

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**Abstract:** **Orofacial neuropathic pain caused by trigeminal nerve injury is a debilitating condition with limited therapeutic options. Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels regulate neuronal excitability and are involved in the development and maintenance of chronic pain. However, the effect of HCN channel activity in the Gasserian ganglion on trigeminal neuropathic pain has not been examined. We evaluated nociceptive behaviors after microinjection of the HCN channel blockers ZD7288 or ivabradine into the Gasserian ganglion in rats with trigeminal nerve injury. Both blockers dose-dependently ameliorated evoked and spontaneous nociceptive behavior in rats with trigeminal neuropathic pain. Moreover, the clinically available HCN channel blocker ivabradine showed a prolonged antinociceptive effect. In the Gasserian ganglion, HCN1 and HCN2 are major HCN isoforms. After trigeminal nerve injury, the counts of HCN1 as well as HCN2 immuno-positive punctae were increased in the ipsilateral Gasserian ganglions. These results indicate that the increased HCN channel activity in the Gasserian ganglion directly contributes to neuropathic pain resulting from trigeminal nerve injury.**

***Perspective:*** ***Trigeminal nerve damage-induced orofacial pain is severe and more resistant to standard pharmacological treatment than other types of neuropathic pain. Our study suggests that targeting HCN channel activities in the Gasserian ganglion may provide an alternative treatment of trigeminal neuropathy including trigeminal neuralgia.***



***Key words:*** *Trigeminal neuropathic pain, hyperpolarization-activated cyclic nucleotide-gated channel, ZD7288, ivabradine, Gasserian ganglion.*

Orofacial pain is estimated to affect up to 7% of the general population.[36] Chronic pain caused by trigeminal nerve damage is severe and more debilitating than other types of neuropathic pain.[4] Furthermore, neuropathic pain associated with the trigeminal nerve system is more resistant to treatments.[25,36] Studies have shown that pain caused by facial nerve injury has different physiological and pathological characteristics compared with pain induced by spinal nerve injury,[2,20,21] possibly because of the anatomical and molecular differences between facial and body sensory pathways.[3,31] Indeed, comprehensive RNA-Seq expression analysis reveals differences in the expression of ion channels and G-protein coupled receptors between the Gasserian ganglion (GG) and the dorsal root ganglion (DRG).[13,19]

Hyperpolarization-activated cyclic nucleotide-gated (HCN) cation channels (HCN1–4 isoforms) are widely expressed in peripheral sensory neurons in the DRG[15,22] and GG.[8,9] In DRG neurons, inward current ($I_h$) generated by hyperpolarization-activated cyclic nucleotide–gated channels contributes to nociceptor sensitization and pain by facilitating ectopic firing and hyperexcitability.[7,15,33,34] Upregulation of $I_h$ as well as

Received September 28, 2017; Revised December 15, 2017; Accepted January 3, 2018.
W.D., Z.Y., and S.S. contributed equally to this work.
This work was supported by National Institutes of Health grants R01 DE022901 and R01 DE018214 (J.M.) and National Natural Science Foundation of China 81771194 (S.Z.).
The authors have no conflicts of interest to declare.
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1526-5900/$36.00

https://doi.org/10.1016/j.jpain.2018.01.003

changes in HCN protein expression has been observed in neurons in the DRG[1] and spinal cord[29] of rodents with peripheral nerve injury. Inhibition of HCN channel activity alleviates neuropathic and inflammatory pain.[24,29,34] To date, it remains unclear whether HCN activity in the GG would affect nociceptive behavior and whether HCN protein expression in the GG would change after trigeminal nerve injury.

In this study, we used an improved trigeminal nerve injury model, in which the distal infraorbital nerve (IoN) was subjected to chronic constriction injury (dIoN-CCI),[11] to investigate the role of HCN channels in trigeminal neuropathic pain. The results of behavioral study indicated that microinjection of HCN channel blockers into the GG ameliorated evoked as well as non-evoked nociceptive behavior in dIoN-CCI rats and that the clinically available HCN channel blocker ivabradine had a better antinociceptive effect than ZD7288. Immunohistochemical analysis showed that HCN1 and HCN2 immunopositive puncta counts were increased in the GG of rats with dIoN-CCI.

## Methods

### *Animals*

Adult male Sprague Dawley rats weighing 270 to 300 g were purchased from Charles River Laboratories (Wilmington, MA). The experimental protocols were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

### *Surgical Procedures*

#### dIoN-CCI and Sham Surgery

The facial surface between the eye and whisker pad of a rat was gently shaved without damaging the whiskers. A .5-cm incision parallel to the midline was made starting at the caudal end of the third row of whisker lines toward the ipsilateral orbit. The superficial fascia was bluntly separated to expose the IoN trunk at its distal segment outside the orbital cavity. Two chromic catgut ligatures (4–0) were loosely tied around the distal part of the IoN (2 mm apart).[11]

Rats in sham groups underwent the same surgical procedure including skin incision and the IoN nerve dissection except for the actual nerve ligation.

#### Implantation of a Guide Cannula for Intra-GG Microinjection

Drug or vehicle was microinjected into the GG of rats.[16,18] Rats were anesthetized with intraperitoneal pentobarbital and placed in a Stoelting stereotaxic instrument (Wood Dale, IL). A guide cannula (C315G with an infusion cannula C315I; Plastics One, Roanoke, VA) was implanted next to the GG ipsilateral to the injury side. The implantation of the guide cannula was performed according to the following coordinates from the rat brain atlas[26]: 3.6 mm posterior to the Bregma, 2.8 mm lateral to the midline, and 11.2 mm ventral to the skull surface. The guide cannula was fixed to the skull using dental acrylic and jeweler's screws. A dummy cannula (33-gauge stainless steel wire) was inserted into the guide cannula to reduce the incidence of occlusion. Saline and drug solutions were microinjected into the GG site using a microinjection unit (33-gauge cannula) that extended .5 mm beyond the tip of the guide cannula. The microinjection unit was attached to a Hamilton microsyringe (Hamilton Robotics, Reno, NV) via polyethylene tubing (PE-10), and an infusion pump was programmed to deliver a volume of 1 μL over a period of 1 minute into the GG site. The needle was held for 1 minute before retraction, and the injection site was confirmed by visual examination of the GG upon completion of each experiment. ZD7288 and ivabradine were purchased from Selleckchem (Houston, TX) and dissolved in sterile saline.

### *Behavioral Tests*

All behavioral experiments were carried out with the investigators blinded to treatment conditions. Animals were habituated to the test environment for 2 consecutive days (30 minutes per day) before baseline testing.

#### Mechanical Allodynia

Orofacial sensitivity to mechanical stimulation was tested using von Frey filaments.[11,32] The stimuli were applied within the IoN territory, near the center of the vibrissal pad. This area was stimulated on both sides of the face after surgery (ie, ipsilateral and contralateral to the side where surgery had been performed). Stimuli were applied in an ascending order of intensity. The ipsilateral and contralateral sides were stimulated in a randomized order for each rat. The following criteria were used to determine a positive response to a filament: an immediate withdrawal reaction, attacking the filament by biting and grabbling, escaping by moving away from the filament, or asymmetric face stroke to the stimulated facial area. A threshold force of response (in grams) was defined as the first filament that evoked at least 2 reactions of 5 applications.[30]

#### Face-Grooming

The rat was placed into the Plexiglas enclosure and video recorded. Face-grooming episodes were counted over a period of 10 minutes after the rat was placed into the enclosure. A face-grooming episode was defined as an uninterrupted sequence of face-grooming action.[11,32]

### *Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction*

Total RNA was isolated from brain tissue using TRIzol (Life Technologies, Carlsbad, CA). cDNA was synthesized using ProtoScript II cDNA synthesis kit (New England Biolabs, Ipswich, MA). Quantitative polymerase chain reaction (PCR) was performed using QuantStudio 3 using the following Taqman probes from Applied Biosystems (Waltham, MA): HCN1 (Rn00670384_m1 and Rn01490048_m1), HCN2 (Rn01408575_gH and

Rn01408575_gH), HCN3 (Rn00586666_m1), HCN4 (Rn00572232_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Rn99999916_s1).

## *Immunohistochemistry and Image Analysis*

Immunostaining was carried out as previously reported[37] using the following primary and secondary antibodies: mouse anti-HCN1 antibody (1:800; ab84816, Abcam, Cambridge, MA), mouse anti-HCN2 antibody (1:1,000; ab84817, Abcam), and fluorescein isothiocyanate (FITC) conjugated goat anti-mouse antibody (1:300; Jackson ImmunoResearch Laboratories Inc, West Grove, PA). Primary antibody omission was used as a control. Two GG sections from each rat (4 sham rats and 4 dIoN-CCI rats) were stained and 2 micrographs from each GG section were captured with an Olympus (Tokyo, Japan) fluorescence microscope. Micrographs taken with 40× objective were used for adequate resolution and saved in 16-bit format. ImageJ from the National Institutes of Health (Bethesda, MD) was used for puncta counting. To make the counting more robust to random noise, all images were smoothed first (using a $3 \times 3$ mean filter). Because there were only a small number of neurons in each field, only the first 4 large neurons with clear morphology were

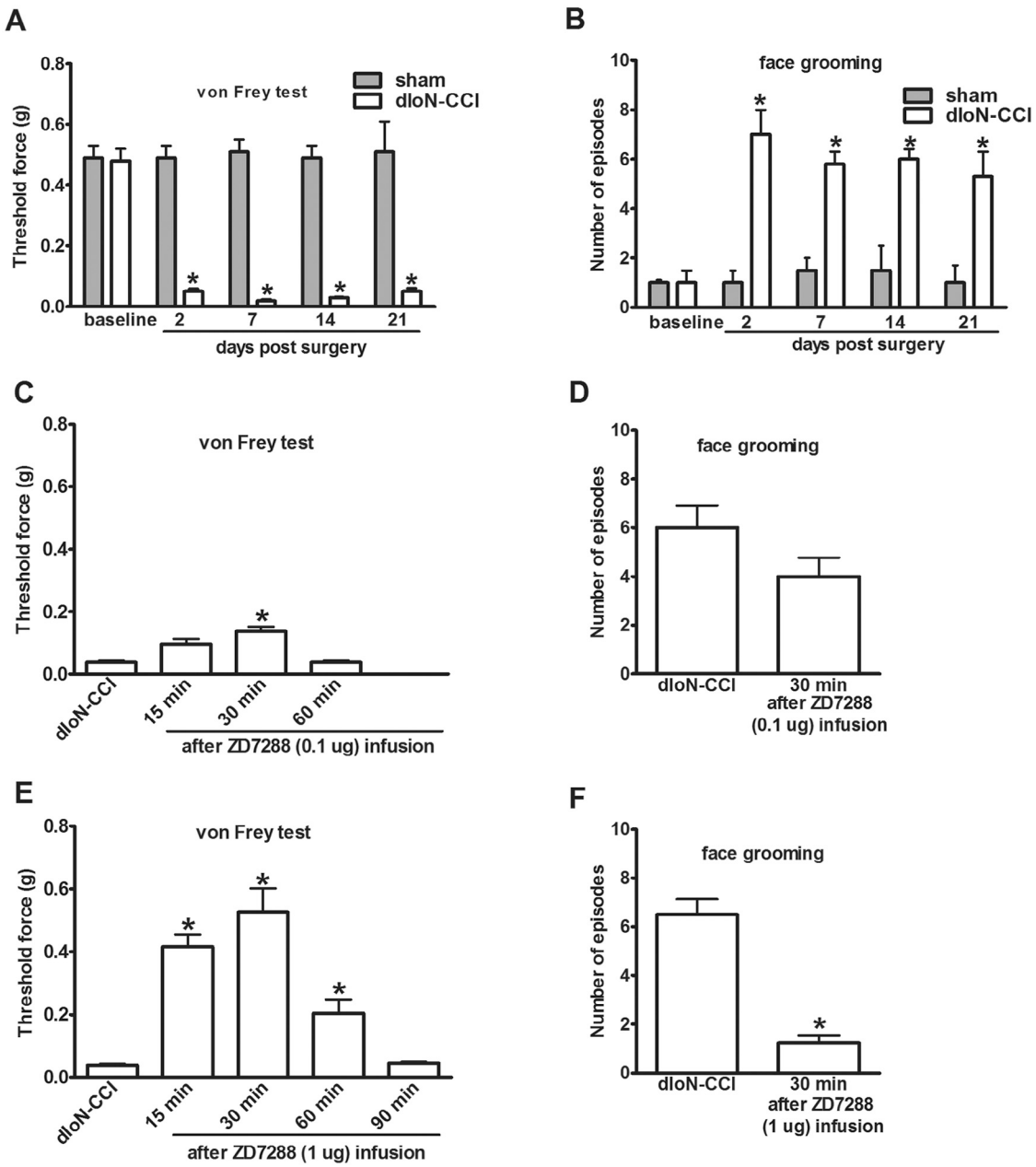


**Figure 1.** GG microinjection of ZD7288 reduced nociceptive behavior in dIoN-CCI rats. (**A**, **B**) dIoN-CCI induced mechanical allodynia and intense unilateral facial grooming, which lasted for weeks in rats (sham vs dIoN-CCI, $*P < .05$, n = 6). (**C**, **D**) Infusion of .1 μg of ZD7288 to the GG site ipsilateral to the dIoN-CCI side transiently reduced mechanical allodynia measured at 30 minutes after drug administration. (**E**, **F**) Microinjection of 1 μg of ZD7288 to the ipsilateral side of the GG reduced mechanical allodynia for at least 60 minutes. Facial grooming was also significantly reduced in dIoN-CCI rats (before vs after ZD7288 infusion, $* P < .05$, n = 6).

selected and measured individually. To get a count, ImageJ's FindMaxima command was used with a tolerance of 700.

### Statistical Analysis of Behavioral Data

Behavioral data were analyzed using 2-way analysis of variance[37] as appropriate. One-way analysis of variance was used to examine real-time quantitative reverse transcription-PCR and image analysis data. Post hoc Waller–Duncan K-ratio t-test was performed to determine the source(s) of differences. GraphPad Prism 5 software (GraphPad Software, La Jolla, CA) was used for the statistical analyses. All data were expressed as mean ± standard error of the mean and the statistically significant level was set at $P < .05$.

## Results

### GG Microinjection of the HCN Channel Blocker ZD7288 Improved Nociceptive Behavior in dIoN-CCI Rats

Unilateral ligation of the IoN induced nociceptive behavior such as mechanical allodynia and intense unilateral facial grooming, which lasted for weeks (Figs 1A and 1B). To determine the effect of $I_h$ inhibition in the GG on trigeminal neuropathic pain, nociceptive behavior was

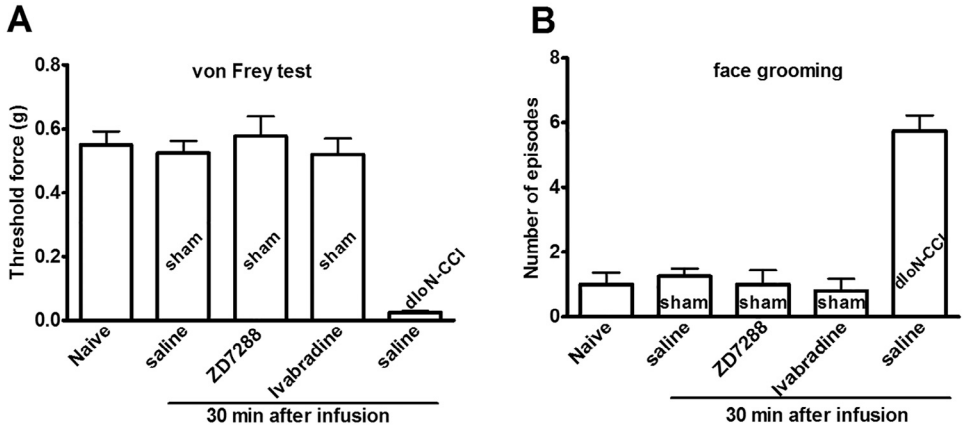


**Figure 2.** HCN blockers did not alter baseline nociception in sham rats when administered to the GG site. Saline, ZD7288 (1 μg), or ivabradine (1 μg) did not alter mechanical threshold force (**A**) or facial grooming (**B**) in sham rats measured at 30 minutes after being injected into the ipsilateral side of the GG. Naive versus sham groups, $P > .05$, n = 5. Microinjection of saline to the GG site did not change nociceptive behavior in dIoN-CCI (day 14) rats. Before versus after saline injection, $P > .05$, n = 5.

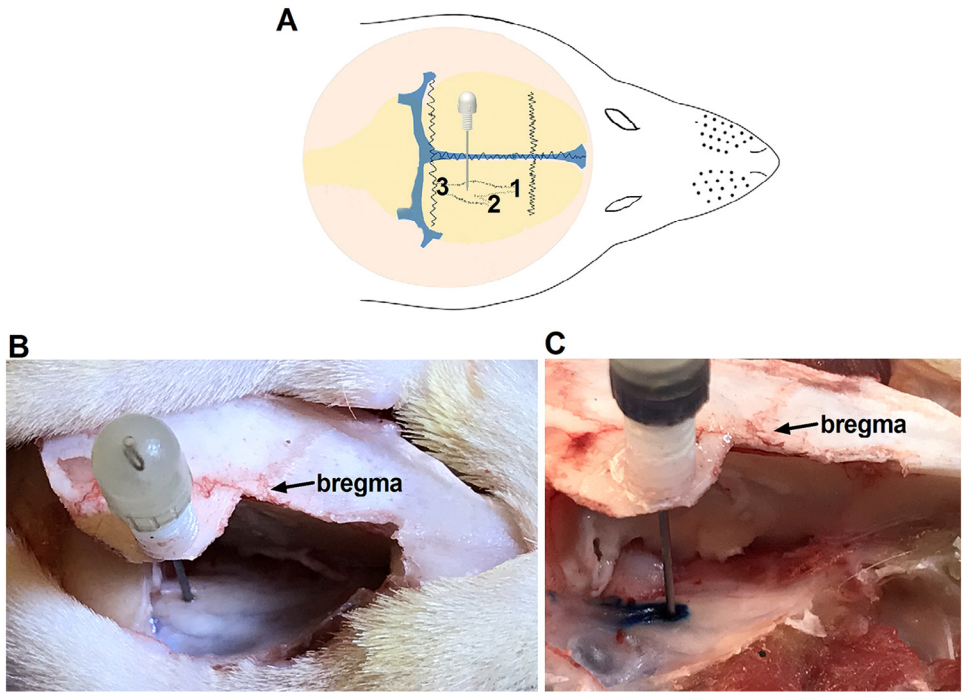


**Figure 3.** Schematic drawing and anatomical demonstration of the GG injection site. (**A**) Schematic drawing showing the site of the GG injection. The structure details: 1) V1/V2 (ophthalmic/maxillary), 2) V3 (mandibular), and 3) sensory root. (**B**) Anatomical position of the cannulation. (**C**) Methylene blue injection showing the drug injection site.

assessed in dIoN-CCI rats after microinjection of ZD7288 into the GG site ipsilateral to the dIoN-CCI side at 14 days after injury. Injections of .1 μg as well as 1 μg of ZD7288 improved mechanical allodynia and the effect of 1 μg of ZD7288 lasted for 90 minutes (Figs 1C and 1E). Measured at 30 minutes after 1 μg of ZD7288 injection, the threshold of dIoN-CCI rats to mechanical stimulus nearly returned to its preinjury (baseline) level. Asymmetry facial grooming was also reduced after 1 μg of ZD7288 infusion in the dIoN-CCI rats (Fig 1D). In contrast, the nociceptive behaviors in dIoN-CCI rats were not affected by saline (Fig 2). ZD7288 (1 μg) did not alter the baseline of either evoked or nonevoked nociceptive behavior in sham rats (Figs 2). The injection site was confirmed using visual examination or methylene injection of the GG site on completion of each experiment (Fig 3).

## *The Clinically Available HCN Blocker Ivabradine Had a Prolonged Antinociceptive Effect in dIoN-CCI Rats*

Ivabradine is a specific and selective HCN blocker currently being used to treat heart failure.[6] To further show that increased HCN channel activity in the GG contributes

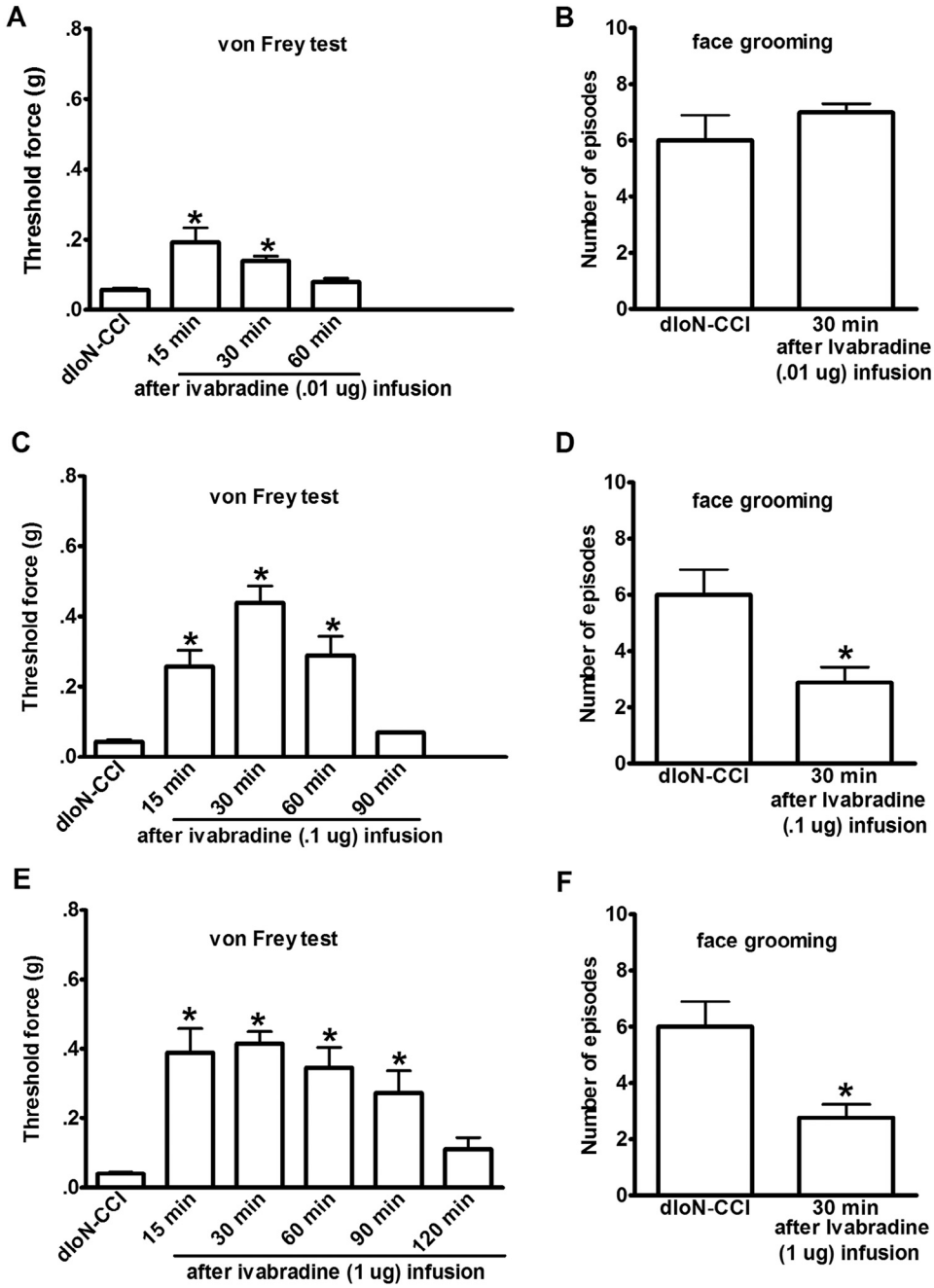


**Figure 4.** GG microinjection of ivabradine produced a prolonged analgesic effect in dIoN-CCI rats. (**A**, **B**) Microinjection of .01 μg of ivabradine to the GG site ipsilateral to dIoN-CCI slightly reduced mechanical allodynia measured at 15 and 30 minutes, but facial grooming was not improved when examined at 30 minutes. (**C**–**F**) Microinjection of .1 μg or 1 μg of ivabradine to the ipsilateral GG site reduced mechanical allodynia and facial grooming in dIoN-CCI rats. Before versus after ivabradine injection, $*P < .05$, n = 6.

to trigeminal neuropathic pain, we examined the antinociceptive effect of ivabradine. At 14 days after dIoN-CCI, microinjection of .01 μg of ivabradine into the ipsilateral GG only slightly improved mechanical allodynia after 15 minutes of infusion (Fig 4A). The nociceptive threshold was significantly increased at 15 minutes after microinjection of .1 μg or 1 μg of ivabradine, which lasted for 90 minutes or 120 minutes respectively (Figs 4C and 4E). Asymmetry facial grooming was also reduced after ivabradine (.1 or 1 μg) infusion in the dIoN-CCI rats (Figs 4D and 4F). Ivabradine (1 μg) infusion into the GG did not alter the baseline of either evoked or nonevoked nociceptive behavior in sham rats (Fig 2).

### *HCN1 and HCN2 Immunopositive Puncta Counts Were Increased in the GG of dIoN-CCI Rats*

Previous studies suggest that 4 HCN isoforms are expressed in the DRG[17] and GG.[8] HCN1 and HCN2 isoforms are considered to be relevant to pain signaling in peripheral and central nerve systems.[1,10,37] We analyzed mRNA levels of HCN isoforms in the GG using quantitative PCR. We found that the mRNA levels of HCN1 and HCN2 were much higher than HCN3 and HCN4 in the GG (Fig 5). Immunohistochemistry also showed a high expression of HCN1 as well as HCN2 protein in the GG of a sham rat (Fig 6A and 6D). Furthermore, at 14 days after dIoN-CCI, the counts of HCN1 and HCN2 immunopositive punctae were increased in the ipsilateral GG of dIoN-CCI rats compared with sham rats (Fig 6).

## Discussion

Damage to the trigeminal nerve causes debilitating orofacial pain, which is known to be more resistant to treatment than other types of neuropathic pain. We report that microinjection of the HCN blocker ZD7288 or ivabradine into the GG site ameliorated evoked as well as nonevoked nociceptive behaviors in dIoN-CCI rats with trigeminal neuropathic pain. Our behavioral data suggested that the clinically available HCN channel blocker ivabradine had a strong antinociceptive effect. Among 4 HCN isoforms, we found that expression levels of HCN1 and HCN2 isoforms were higher than those of HCN3 and HCN4 isoforms in the GG. We also observed increase in HCN1 and HCN2 immunopositive puncta counts in the ipsilateral GG of dIoN-CCI rats.

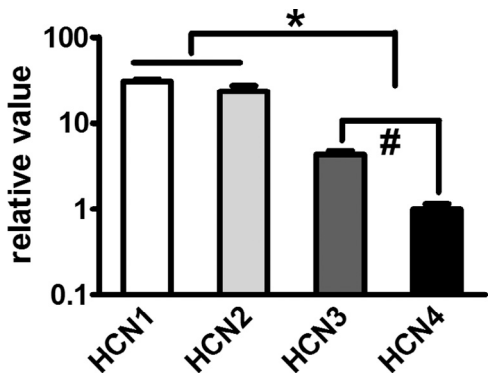


**Figure 5.** HCN1 and HCN2 isoforms were predominately expressed in the rat GG. Quantitative reverse transcription-PCR analysis of HCN1 to 4 mRNA levels in the rat GG. HCN1 and 2 versus HCN3 and 4, *$P < .05$, n = 5; HCN3 versus HCN4, #$P < .05$, n = 5.

Spinal nerve injury or inflammation induced HCN channel dysfunction, which has been associated with spontaneous ectopic firing of DRG sensory neurons contributing to the development and maintenance of pain.[7,12,27,33] Tissue specific deletion of HCN2 in DRG sensory neurons reduced mechanical hyperalgesia in mice with complete Freund's adjuvant (CFA)-induced inflammatory pain.[27] Despite the known presence of HCN channels in the GG,[8] the role of HCN channel activity in the GG in trigeminal neuropathic pain condition has not been studied. Using microinjection of HCN channel blockers into the GG, our study provides evidence that increased HCN channel activity in the GG contributes to the behavioral manifestation of chronic pain after trigeminal nerve injury.

All 4 HCN channel subunits (HCN1–4) have been detected in the sensory neurons in the DRG[9,17,23] and GG.[8,9] In DRG neurons, HCN1and HCN2 are the dominant isoforms[17,23] related to pain after nerve injury.[7,27] In this study, we found that the expression of HCN1 and HCN2 mRNA was much higher than that of HCN3 and HCN4 in the rat GG. To quantify the observed visual difference in HCN1 and HCN2 immunostaining of the GG sections between sham and dIoN-CCI rats, we performed HCN1 and HCN2 immunopositive puncta counting for the statistical analysis. To get a count, the ImageJ (National Institutes of Health) FindMaxima command was used with a tolerance of 700. A tolerance or threshold of 700 was found to be less sensitive to variation in the final intensity of acquired immunostaining micrographs in our case. Compared with sham, dIoN-CCI neurons had significant higher counts of HCN1 or HCN2 immune-positive puntae (both $P < .001$). The puncta counting is considered more suitable for quantifying the immunostaining to reflect nerve injury-induced changes in HCN channels in the GG in this study. First, most of the punctae of HCN1 and HCN2 immunostaining signal were of similar size and intensity, suggesting that each puncta represents a uniform complex. In a report by Fox et al,[14] a $K^+$ channel similar to HCN, was expressed in human embryonic kidney (HEK) cells and the punctae of immunostain were found to represent single channels. Therefore, our puncta counts may represent the quantity of the active form of the channel more appropriately than a simple intensity metric. Second, the lack of a proper internal control, which should be consistent and reliable, prevents an accurate normalization of the immunofluorescence signal. Without a good normalization, staining efficiency and photobleaching-associated variation in immunosignal rendered the integrated pixel intensity method far less sensitive than desirable.

We compared the antinociceptive effect of 2 HCN channel blockers ZD7288 and ivabradine. ZD7288 has been widely used as a selective HCN blocker to study the role of HCN channel activity in heart tissue and neurons. The analgesic effect of ZD7288 is consistent with a role of HCN

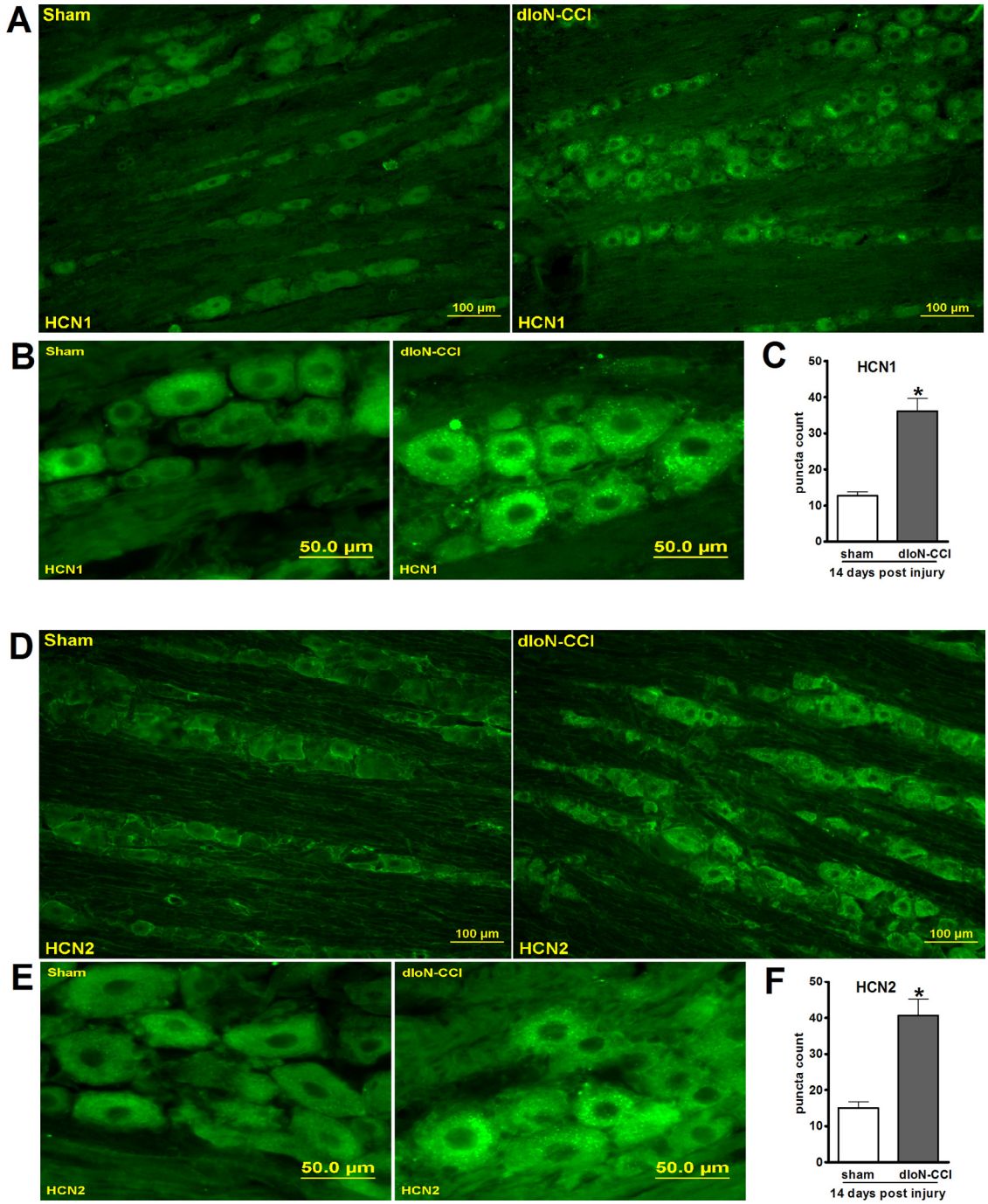


**Figure 6.** HCN1 and HCN2 immunostaining of the GG sections. Representative micrographs of the ipsilateral GG isolated from sham or dIoN-CCI (14 days) rats were stained with anti-HCN1 (**A** and **B**) or anti-HCN2 (**D** and **E**) antibody. The ipsilateral GG of dIoN-CCI rat showed and increase in HCN1 (**C**) and HCN2 (**F**) immunopositive puncta counts. $*P < .001$, $n = 4$.

activity in the pain condition. However, one study suggested that ZD7288 may also block $Na^+$ channels in the DRG (ex vivo) and in vitro,[35] suggesting that inhibition of $Na^+$ channels could partially contribute to the antinociceptive effect of ZD7288. Ivabradine is a clinical drug that selectively and specifically blocks HCH current of cardiac sinoatrial node,[6] which is mainly HCN4 current. Studies indicate that ivabradine is also very effective in blockage of HCN1 current.[6] Our data showed an antinociceptive effect of ZD7288 as well as ivabradine in dIoN-CCI rats when administered directly to the GG site, supporting the notion that increased HCN channel activity in the GG is an underlying mechanism of trigeminal neuropathic pain. Of interest is that ivabradine exhibited prolonged analgesic effects in dIoN-CCI rats. The differences in molecular mechanisms of HCN channel blockage between ZD7288[28] and ivabradine[6] may contribute to their differences in analgesic effects for neuropathic pain.

## Conclusions

All known HCN blockers cause bradycardia resulting from blocking the sinoatrial HCN4.[6] Thus, broad HCN channel blockers may not be useful when applied

systemically in neuropathic pain management. In contrast, administering a drug at the sites of peripheral pain transmission may avoid the side effects of systemic delivery. A recent study showed that under the guidance of computed tomography, a drug can be delivered precisely around the DRGs to decrease pain transmission in swine.[5] Our study suggests that microinjection of an HCN blocker around the GG site might provide an alternative treatment of trigeminal neuropathic pain because the GG is enclosed in the Meckel cave, which is readily accessible via needle access. This approach, coupled with the development of specific blockers of HCN1 and HCN2, would be clinically beneficial to patients with trigeminal neuropathic pain.

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