Selective Inhibition of Trigemino-vascular Neurons by Fremanezumab: A Humanized Monoclonal Anti-CGRP Antibody

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A large body of evidence supports an important role for calcitonin gene-related peptide (CGRP) in migraine pathophysiology. This evidence gave rise to a global effort to develop a new generation of therapeutics that inhibit the interaction of CGRP with its receptor in migraineurs. Recently, a new class of such drugs, humanized anti-CGRP monoclonal antibodies (CGRP-mAbs), were found to be effective in reducing the frequency of migraine. The purpose of this study was to better understand how the CGRP-mAb fremanezumab (TEV-48125) modulates meningeal sensory pathways. To answer this question, we used single-unit recording to determine the effects of fremanezumab (30 mg/kg, IV) and its isotype control Ab on spontaneous and evoked activity in naive and cortical spreading depression (CSD)-sensitized trigemino-vascular neurons in the spinal trigeminal nucleus of anesthetized male and female rats. The study demonstrates that, in both sexes, fremanezumab inhibited naive high-threshold (HT) neurons, but not wide-dynamic range trigemino-vascular neurons, and that the inhibitory effects on the neurons were limited to their activation from the intracranial dura but not facial skin or cornea. In addition, when given sufficient time, fremanezumab prevents the activation and sensitization of HT neurons by CSD. Mechanistically, these findings suggest that HT neurons play a critical role in the initiation of the perception of headache and the development of cutaneous allodynia and central sensitization. Clinically, the findings may help to explain the therapeutic benefit of CGRP-mAb in reducing headaches of intracranial origin such as migraine with aura and why this therapeutic approach may not be effective for every migraine patient.

Key words: allodynia; cortical spreading depression; dorsal horn; headache; migraine; sensitization

Significance Statement

Calcitonin gene-related peptide (CGRP) monoclonal antibodies (CGRP-mAbs) are capable of preventing migraine. However, their mechanism of action is unknown. In the current study, we show that, if given enough time, a CGRP-mAb can prevent the activation and sensitization of high-threshold (central) trigemino-vascular neurons by cortical spreading depression, but not their activation from the skin or cornea, suggesting a potential explanation for selectivity to migraine headache, but not other pains, and a predominantly peripheral site of action.

Introduction

Calcitonin gene-related peptide (CGRP) is expressed in primary afferent neurons that provide sensory innervation to superficial

and deep tissues (Kruger et al., 1989; Silverman and Kruger, 1989). This peptidergic population consists primarily of Aδ- and C-fiber neurons (McCarthy and Lawson, 1990; Lawson et al., 1993; Lawson et al., 1996; Lawson et al., 2002; Ruscheweyh et al., 2007) and is predominantly nociceptive (Lawson et al., 2002).

A large body of evidence supports an important role for CGRP in the pathophysiology of migraine (Hansen and Ashina, 2014; Karsan and Goadsby, 2015; Russo, 2015). First, CGRP is present

Received March 1, 2017; revised April 14, 2017; accepted May 18, 2017.

Author contributions: A.M.-C., R. Nosedá, R. Nir, A.S., J.S., A.M.S., and R.B. designed research; A.M.-C. and R.B. performed research; A.M.-C., R. Nosedá, R. Nir, A.S., and R.B. analyzed data; A.M.-C., A.M.S., and R.B. wrote the paper.

This study was supported by Teva Pharmaceutical Industries and the National Institutes of Health (Grants R37-NS079678, R01 NS069847, and R01 NS094198 to R.B.). A.S. was supported by a grant from R. Chemers Neustein.

J.S. is an employee of and R.B. is a consultant to TEVA Pharmaceutical, which holds the patent for treating episodic and chronic migraine with fremanezumab and funded parts of the study. The remaining authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.0576-17.2017

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in the peripheral sensory innervation of the cranial meninges (O'Connor and van der Kooy, 1988; Tsai et al., 1988; Uddman et al., 1989; Keller and Marfurt, 1991; Edvinsson et al., 1998; Edvinsson et al., 2001), the neurons at the origin of a sensory pathway that has been critically implicated in the generation of migraine headache (Moskowitz, 1984; Strassman et al., 1996; Burstein et al., 2015). Second, levels of CGRP are increased in the blood and saliva during migraine attacks (Goadsby et al., 1990; Bellamy et al., 2006b; Cady et al., 2009) and exogenous CGRP administration triggers a delayed migraine-like headache in migraineurs (Hansen et al., 2010). Third, mice with increased expression of the CGRP receptor exhibit photophobia (Recober et al., 2009; Russo et al., 2009; Recober et al., 2010; Kaiser et al., 2012), a characteristic feature of migraine. Fourth, among the most well documented biological effects of anti-migraine agents such as sumatriptan is a suppression of CGRP release, which has been demonstrated in cultured trigeminal ganglion neurons (Durham and Russo, 1999), in meningeal tissue *in vitro* (Eltorp et al., 2000), in blood measurements during meningeal stimulation *in vivo* (Buzzi et al., 1991; Goadsby and Edvinsson, 1994), and in saliva measurements during migraine (Bellamy et al., 2006b; Cady et al., 2009). Triptans and other 5HT_{1D} agonists also block the increase in CGRP gene promoter activity in trigeminal ganglion neurons evoked by nitric oxide donors, which are potent headache-triggering agents, as well as other stimuli (Durham et al., 1997; Durham and Russo, 1998, 2003; Durham et al., 2004; Bellamy et al., 2006a).

The evidence supporting the importance of CGRP in migraine gave rise to a worldwide effort to develop a new generation of drugs that reduce the availability of CGRP in migraineurs. Recently, humanized CGRP monoclonal antibodies (CGRP-mAbs) were found to be effective in reducing the frequency of chronic migraine (Dodick et al., 2014a; Dodick et al., 2014b; Bigal et al., 2015a; Bigal et al., 2015b; Sun et al., 2016). However, the mechanisms by which these drugs produce their therapeutic effect and, more broadly, the mechanisms by which CGRP contributes to trigeminal neuron activation and migraine, are not fully understood. To better understand the mechanism of action of anti-CGRP antibodies in migraine, we examined their effects on spontaneous and evoked activity of high-threshold (HT) and wide-dynamic range (WDR) trigeminovascular neurons in the medullary and upper cervical dorsal horn in anesthetized male and female rats.

Materials and Methods

Surgical preparation. Experiments were approved by the Beth Israel Deaconess Medical Center and Harvard Medical School standing committees on animal care and were in accordance with the U.S. National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. Male and female Sprague Dawley rats (250–350 g) were anesthetized with urethane (0.9–1.2 g/kg, i.p.). They were fitted with an intratracheal tube to allow artificial ventilation (0.1 L/min of O₂) and an intrafemoral vein cannula for later infusion of drugs. Rats were placed in a stereotaxic apparatus and core temperature was kept at 37°C using a heating blanket. End-tidal CO₂ was monitored continuously and kept within physiological range (3.5–4.5 pCO₂). Once stabilized, rats were paralyzed with rocuronium bromide (10 mg/ml, 1 ml/h continuous intravenous infusion) and ventilated. For stimulation of the cranial dura later in the experiment, a 5 × 5 mm opening was carefully carved in the parietal and occipital bones in front and behind the lambda suture directly above the left transverse sinus. The exposed dura was kept moist using a modified synthetic interstitial fluid containing the following (in mM): 135 NaCl, 5 KCl, 1 MgCl₂, 5 CaCl₂, 10 glucose, and 10 HEPES, pH 7.2. For single-unit recording in the spinal trigeminal nucleus, a segment of the spinal cord between the obex and C2 was uncovered from overlying tissues, stripped of the dura mater, and kept moist with mineral oil.

Neuronal identification and selection. To record neuronal activity, a tungsten microelectrode (impedance 3–4 MΩ) was lowered repeatedly into the spinal trigeminal nucleus in search of central trigeminovascular neurons receiving convergent input from the dura and facial skin. Trigeminovascular neurons were first identified based on their responses to electrical stimulation of the dura. They were selected for the study if they exhibited discrete firing bouts in response to ipsilateral electrical (0.1–3.0 mA, 0.5 ms, 0.5 Hz pulses) and mechanical (with a calibrated von Frey monofilaments) stimulation of the exposed cranial dura and to mechanical stimulation of the facial skin and cornea. Dural receptive fields were mapped by indenting the dura [with the 4.19 g von Frey hair (VFH) monofilament] at points separated by 1 mm mediolaterally and rostro-caudally. Points at which dural indentation produced a response in ≥50% of the trials were considered inside the neuron's receptive field. Cutaneous receptive fields were mapped by applying innocuous and noxious mechanical stimulation to all facial skin areas and the cornea as described previously (Burstein et al., 1998). An area was considered outside the receptive field if no stimulus produced a response in ≥50% of the trials. Responses to mechanical stimulation of the skin were determined by applying brief (10 s) innocuous and noxious stimuli to the most sensitive portion of the cutaneous receptive field. Innocuous stimuli consisted of slowly passing a soft bristled brush across the cutaneous receptive field (one 5 s brush stroke from caudal to rostral and one 5 s brush stroke from rostral to caudal) and pressure applied with a loose arterial clip. Noxious stimuli consisted of pinch with a strong arterial clip (Palcsek et al., 1992; Dado et al., 1994; Burstein et al., 1998). More intense or prolonged stimuli were not used to avoid inducing prolonged changes in spontaneous neuronal discharge or response properties. Responses to mechanical stimulation of the cornea consisted of gentle and slow brushing strokes with a thin paintbrush (~10 hair follicles). Two classes of neurons were thus identified: WDR neurons (incrementally responsive to brush, pressure, and pinch) and HT neurons (unresponsive to brush). A real-time waveform discriminator was used to create and store a template for the action potential evoked in the neuron under study by electrical pulses on the dura; spikes of activity matching the template waveform were acquired and analyzed online and offline using Spike 2 software (CED).

Induction and recording of cortical spreading depression. Cortical spreading depression (CSD) was induced mechanically by inserting a glass micropipette (tip diameter 25 μm) ~1 mm into the visual cortex for 10 s. At a propagation rate of 3–5 mm/min, a single wave of CSD was expected to enter the neuronal receptive field within 1–2 min of cortical stimulation. For verification of CSD, cortical activity was recorded (ECG) with a glass micropipette (0.9% saline, ~1 MΩ, 7 μm tip) placed just below the surface of the cerebral cortex (~100 μm). The ECG electrode was positioned ~6 mm anterior to the visual cortex.

Treatment with the monoclonal anti-CGRP antibody fremanezumab (TEV-48125). Fremanezumab (formerly TEV-48125/LBR-101/RN-307; TEVA Pharmaceutical Industries) is a humanized CGRP-mAb. It was diluted in saline to a final dose of 30 mg/kg and administered intravenously (bolus injection, total volume 0.6–0.7 ml). A corresponding human IgG2 isotype control antibody (isotype-conAb) was also diluted in saline to a final dose of 30 mg/kg and administered intravenously (bolus injection, total volume 1.6–2.0 ml).

Experimental protocol. The experimental protocol included two parts. The first part was designed to compare CGRP-mAb versus isotype-conAb effects on spontaneous and induced activity of naive trigeminovascular neurons and the second part was designed to test CGRP-mAb versus isotype-conAb effects on the activation and sensitization of trigeminovascular neurons by CSD. Both parts included sampling of WDR and HT neurons in male and female rats. In the first part, the baseline neuronal profile was established by mapping the dural, cutaneous, and corneal receptive field; measuring responses (mean spikes/s) to mechanical stimulation of the dura (with a fixed force), skin (brush, pressure, pinch), and cornea (brush); and measuring spontaneous firing rate (recorded over 30 min before treatment). Once the baseline was established, CGRP-mAb or isotype-conAb were administered and receptive fields were remapped; neuronal responses to stimulation of the dura, skin, and cornea were reexamined; and the spontaneous activity rate was resampled.

Figure 1. Recording sites (**A, F**), facial receptive fields (**B, D, G, I**), and dural receptive fields (**C, E, H, J**) of each of the 63 trigeminovascular neurons tested for effects of the CGRP-mAb (**A–C, F–H**, $n = 36$) or the isotype-conAb (**D, E, I, J**, $n = 27$) in male and female rats. **A, F**, Recording sites plotted on a representative transverse section through the first cervical segment. Black and grey circles represent HT and WDR neurons, respectively. **B, D, G, I**, Most sensitive regions of cutaneous (i.e., where brush, pressure, and pinch were applied) and corneal receptive fields. **C, E, H, J**, Mechanically sensitive receptive fields on the dura, which were all on or around the transverse sinus. The portion of the dura shown in the receptive field drawings is outlined by the dashed line in the inset in **H**. All dural and facial receptive fields were ipsilateral to the recorded neuron.

applied at 1, 2, 3, and 4 h after treatment. The resulting values for each measure were then compared with the respective baseline values obtained before treatment. In the second part, CSD was induced 4 h after administration of CGRP-mAb or isotype-conAb and, 2 h later (i.e., 6 h after treatment), receptive field size, spontaneous activity rate, and response magnitude to stimulation of the dura, skin, and cornea were measured again. The resulting post-CSD values for each measure were then compared with the respective pre-CSD values obtained at the 4 h posttreatment time. This part was initiated only in cases in which the physiological condition of the rats (heart rate, blood pressure, respira-

tion, end-tidal CO_2) and the neuronal isolation signal (signal-to-noise ratio $\geq 1:3$) were stable at the 4 h posttreatment time point.

At the conclusion of each experiment, a small lesion was produced at the recording site (anodal DC of $15 \mu\text{A}$ for 15 s) and its localization in the dorsal horn was determined postmortem using histological analysis as described previously (Zhang et al., 2011). Only one neuron was studied in each animal.

Data analysis. To calculate the response magnitude to each stimulus, the mean firing frequency occurring before the onset of the first stimulus (30 min for spontaneous activity, 10 s for mechanical stimulation of the

Figure 2. Effect of CGRP-mAb (**A–D**) and isotype-conAb (**E–G**) on spontaneous activity of trigeminovascular neurons in male and female rats. **A, D, E**, Plots of spontaneous discharge rate recorded at baseline (BL) and at 1–4 h after CGRP-mAb (**A, D**) or isotype-conAb (**E**) administration to HT neurons. Numbers in parentheses show the mean discharge rate for the 15 min sampling period at each time point. Bin width = 1 s. **B, C**, Histograms showing mean ($\pm$ SE) spontaneous discharge of HT and WDR neurons recorded at baseline and 1–4 h after CGRP-mAb administration in male (**B**) and female (**C**) rats. **F, G**, Histograms showing mean ($\pm$ SE) spontaneous discharge of HT and WDR neurons recorded at baseline and 1–4 h after isotype-conAb administration in male (**F**) and female (**G**) rats. * $p < 0.05$ compared with baseline. Numbers in parentheses in **B, C, F**, and **G** depict the number of neurons in each group. Note that the CGRP-mAb reduced baseline spontaneous activity in HT but not WDR neurons (male only).

Figure 3. Effect of CGRP-mAb (**A–C**) and isotype-conAb (**D–F**) on response of trigeminovascular neurons to dural indentation in male and female rats. **A, D**, Responses to indentation of the dura with a VFH (4.19 g) at baseline (BL) and at 1–4 h after CGRP-mAb (**A**) or isotype-conAb (**D**) administration to HT neurons. Numbers in parentheses show the mean discharge rate during the stimulus. Bin width = 1 s. **B, C**, Mean ($\pm$ SE) discharge rates in response to dural stimulation at baseline and 1–4 h after drug administration for the entire sample of neurons that received CGRP-mAb in male (**B**) and female (**C**) rats. **E, F**, Mean ($\pm$ SE) discharge rates in response to dural stimulation at baseline and 1–4 h after drug administration for the entire sample of neurons that received isotype-conAb in male (**E**) and female (**F**) rats. * $p < 0.05$ compared with baseline. Numbers in parentheses in **B, C, E**, and **F** depict the number of neurons in each group. Note that, in both sexes, CGRP-mAb reduced responsiveness to mechanical stimulation of the dura in HT but not WDR neurons.

dura, skin, and cornea) was subtracted from the mean firing frequency that occurred throughout the duration of each stimulus. In the first part of the study, corresponding values for each measure (determined at 1, 2, 3, and 4 h after treatment) were compared with the respective baseline values obtained before CGRP-mAb or isotype-conAb administration. In the second part of the study, resulting values for each measure (determined 2 h after CSD induction) were compared with the respective values obtained before CSD induction in the two treatment groups (CGRP-mAb and isotype-conAb). A neuron was considered activated when its mean firing rate after CSD exceeded its mean baseline activity by 2 SDs of that mean for a period >10 min, which translated to $\geq 33\%$ increase in activity. A neuron was considered sensitized if, 2 h after occurrence of CSD, it exhibited enhanced responses to at least 3 of the following 5 stimuli: dural indentation, brushing, pressuring or pinching the skin, and brushing the cornea. Mean firing rates of respective values were compared using non-parametric statistics (Wilcoxon signed-ranks test). Two-tailed level of significance was set at 0.05.

Results

The database for testing CGRP-mAb versus isotype-conAb effects on spontaneous and induced activity of naive trigemino-vascular neurons consists of 63 neurons. Of these, 31 were classified as WDR and 32 as HT. Of the 31 WDR neurons, 18 (11 in males, 7 in females) were tested before and after administration of the CGRP-mAb and 13 (7 in males, 6 in females) were tested before and after administration of the isotype-conAb. Of the 32 HT neurons, 18 (11 in males, 7 in female) were tested before and after administration of the CGRP-mAb and 14 (8 in males, 6 in females) were tested before and after administration of the isotype-conAb.

The database for testing CGRP-mAb versus isotype-conAb effects on the activation and sensitization of the neurons by CSD consists of 50 neurons. Of these, 23 were classified as WDR and 27 as HT. Of the 23 WDR neurons, 13 (7 in males, 6 in females) were tested in the CGRP-mAb-treated animals and 10 (5 in males, 5 in females) in the isotype-conAb-treated animals. Of the 27 HT neurons, 14 (8 in males, 6 in female) were tested in the CGRP-mAb-treated animals and 13 (7 in males, 6 in females) in the isotype-conAb-treated animals.

Recording sites, receptive fields, and neuronal classes

Recording site, maps of dural and cutaneous receptive fields, and cell types did not differ between neurons tested for CGRP-

Figure 4. Effect of CGRP-mAb (A–D) and isotype-conAb (E–H) on response of central trigeminovascular neurons to innocuous and noxious mechanical stimulation of cutaneous receptive fields of male and female rats. **A, B, E, F,** Responses to mechanical stimulation of the cutaneous receptive fields of HT (**A, E**) and WDR (**B, F**) with brush, pressure, and pinch at baseline (BL) and at 1–4 h after CGRP-mAb or isotype-conAb administration. Numbers in parentheses show the mean discharge rate during each stimulus. Bin width = 1 s. **C, D,** Mean ($\pm$ SE) discharge rates in response to cutaneous stimulation at baseline and 1–4 h after drug administration for the entire sample of neurons that received CGRP-mAb in male (**C**) and female (**D**) rats. **G, H,** Mean ($\pm$ SE) discharge rates in response to cutaneous stimulation at baseline and 1–4 h after drug administration for the entire sample of neurons that received isotype-conAb in male (**G**) and female (**H**) rats. Numbers in parentheses in **C, D, G,** and **H** depict the number of neurons in each group. Note that CGRP-mAb did not reduce responsiveness to innocuous and noxious mechanical stimulation of the skin in either sex or class of neurons.

Figure 5. Effect of CGRP-mAb (**A–C**) and isotype-conAb (**D–F**) on response of central trigeminovascular neurons to mechanical stimulation of the cornea in male and female rats. **A, D**, Responses to mechanical stimulation of the cornea by gentle brushing at baseline (BL) and at 1–4 h after CGRP-mAb (**A**) or isotype-conAb (**D**) administration to HT neurons. Numbers in parentheses show the mean discharge rate during each stimulus. Bin width = 1 s. **B, C**, Mean ($\pm$ SE) discharge rates in response to cornea stimulation at baseline and 1–4 h after drug administration for the entire sample of neurons that received CGRP-mAb in male (**B**) and female (**C**) rats. **E, F**, Mean ($\pm$ SE) discharge rates in response to cornea stimulation at baseline and 1–4 h after drug administration for the entire sample of neurons that received isotype-conAb in male (**E**) and female (**F**) rats. Numbers in parentheses in **B, C, E**, and **F** depict the number of neurons in each group. Note that CGRP-mAb did not reduce responsiveness to mechanical stimulation of the cornea in either sex or class of neurons.

mAb and those tested for the isotype-conAb (Fig. 1). All identified recording sites were localized in laminae I–II and IV–V of the first cervical segment of the spinal cord and the caudal part of nucleus caudalis. In all cases, the most sensitive area of the dural receptive field was along the transverse sinus and the most sensitive area of the cutaneous receptive field was around the eye, involving the cornea in >90% of the cases.

Spontaneous activity of naive central trigeminovascular neurons

In male rats, intravenous administration of the CGRP-mAb reduced the spontaneous activity of the HT but not the WDR neurons (Fig. 2*A, B*). In the HT group, neuronal firing decreased within 3–4 h by 90% ($p = 0.040$). Occasionally, the firing rate of some HT neurons decreased within 1–2 h after the intravenous administration of the CGRP-mAb (Fig. 2*D*). In contrast, intravenous administration of the isotype-conAb did not alter the spontaneous activity of either group of neurons (Fig. 2*E, F*).

In females, unlike in males, intravenous administration of the CGRP-mAb did not reduce the spontaneous activity of HT or WDR neurons (Fig. 2*C*). Similarly, intravenous administration of the isotype-conAb did not alter the spontaneous activity of either group of neurons (Fig. 2*G*). Critically, the baseline (i.e., before any treatment) spontaneous firing rate of HT and WDR neurons did not differ between the male and the female rats ($p = 0.14$). For the HT neurons, mean spikes/s before any treatment was 1.7 ± 1.1 in the male versus 1.9 ± 1.0 in the female ($p = 0.55$). For the WDR neurons, mean spikes/s before any treatment was 0.3 ± 0.6 in the male versus 2.2 ± 1.1 in the female ($p = 0.16$).

Sensitivity of naive central trigeminovascular neurons to dural indentation

In both male and female rats, intravenous administration of the CGRP-mAb reduced the sensitivity to mechanical stimulation of the dura in the HT but not the WDR neurons (Fig. 3*A–C*). In males, the firing of HT neurons decreased by 75% ($p = 0.047$), whereas, in females, it decreased by 61% ($p = 0.017$). Regardless of the sex, intravenous administration of the isotype-conAb did not alter the sensitivity to dural stimulation in either group of neurons (Fig. 3*D–F*).

Figure 6. Effect of isotype-conAb (**A–C**) and CGRP-mAb (**D–F**) on activation of trigeminovascular neurons by CSD. CSD was induced 4 h after drug treatment. **A, D**, Discharge of trigeminovascular neurons before CSD induction (top), during CSD induction (middle), and 2 h after CSD (bottom) in HT neurons that received isotype-conAb (**A**) or CGRP-mAb (**D**) 4 h before CSD induction. Bin width = 1 s. **B, C**, Mean ($\pm$ SE) discharge rates for the entire sample of HT and WDR trigeminovascular neurons that were tested for CSD responses after isotype-conAb administration in male (**B**) and female (**C**) rats. **E, F**, Mean ($\pm$ SE) discharge rates for the entire sample of HT and WDR trigeminovascular neurons that were tested for CSD responses after CGRP-mAb administration in male (**E**) and female (**F**) rats. Discharge is shown at baseline (4 h after drug treatment, before CSD induction) and 2 h after CSD. * $p < 0.05$ compared with baseline. Numbers in parentheses in **B, C, E**, and **F** depict the number of neurons in each group. Note that, in both sexes, CGRP-mAb prevented the activation of HT trigeminovascular neurons by CSD.

Figure 7. Expansion of dural and cutaneous receptive fields after occurrence of CSD in male and female rats. Blue and pink colors illustrate dural and cutaneous receptive fields before and 2 h after CSD induction in isotype-conAb- (**A**) and CGRP-mAb (**B**)-treated rats. Note that CGRP-mAb reduced the incidence of receptive field expansion.

Sensitivity of naive central trigeminovascular neurons to mechanical stimulation of the periorbital skin and the cornea

Intravenous administration of the CGRP-mAb (Fig. 4*A–D*) or the isotype-conAb (Fig. 4*E–H*) did not alter the responses of HT or WDR neurons to innocuous (brush, pressure) or noxious (pinch) mechanical stimulation of the skin or the cornea (Fig. 5*A–F*) in male or female rats.

Cortical spreading depression

Effects of CGRP-mAb ($n = 27$) or isotype-conAb ($n = 23$) on activation of central trigeminovascular neurons by CSD was tested in 50 neurons in which baseline firing rate (i.e., mean spikes/s before induction of CSD) was reliable and consistent over hours. At baseline (i.e., before CSD), the spontaneous firing rate of HT and WDR neurons did not differ between the male and the female rats ($p = 0.14$). For the HT neurons, mean spikes/s before induction of CSD was 1.2 ± 0.6 in the male versus 3.3 ± 1.7 in the female ($p = 0.29$). For the WDR neurons, mean spikes/s before induction of CSD was 1.5 ± 0.6 in the male versus 3.5 ± 2.2 in the female ($p = 0.37$).

CSD-induced activity in central trigeminovascular neurons

In male rats, 2 h after induction of CSD and 6 h after isotype-conAb administration, the mean firing rate of the 7 HT neurons increased from 1.1 ± 0.8 spikes/s before CSD to 10.2 ± 2.1 after CSD ($p = 0.019$), whereas the mean firing rate of the 5 WDR neurons did not increase (0.5 ± 0.3 spikes/s before CSD versus 1.6 ± 0.5 after CSD; $p = 0.14$) (Fig. 6*A,B*). In contrast, in the CGRP-mAb-treated rats, the response magnitude of the 8 HT neurons remained unchanged 2 h after induction of CSD and 6 h after CGRP-mAb administration (1.2 ± 0.6 spikes/s before CSD vs 1.9 ± 1.5 after CSD, $p = 0.29$; Fig. 6*D,E*). In other words, the expected CSD-induced activation of the HT neurons was prevented by the CGRP-mAb treatment.

In female rats, 2 h after induction of CSD and 6 h after isotype-conAb administration, the mean firing rate of the 6 HT neurons increased from 1.9 ± 1.0 spikes/s before CSD to 10.0 ± 4.5 after

CSD ($p = 0.027$), whereas the mean firing rate of the 5 WDR neurons remained unchanged (2.6 ± 1.2 spikes/s before CSD vs 2.2 ± 0.9 after CSD, $p = 0.73$) (Fig. 6*C*). In contrast, in the CGRP-mAb-treated rats, the response magnitude of the 6 HT neurons remained unchanged 2 h after induction of CSD and 6 h after CGRP-mAb administration (3.3 ± 1.7 spikes/s before CSD vs 5.0 ± 3.4 after CSD, $p = 0.45$; Fig. 6*F*). As in the male, the expected CSD-induced activation of the HT neurons was prevented by the CGRP-mAb treatment.

To further examine CGRP-mAb effects on the activation of WDR and HT neurons by CSD, we also performed a case-by-case analysis. Of all CGRP-mAb- and isotype-conAb-treated WDR neurons, 5/13 and 4/10, respectively, were activated by CSD, a mere 2% difference. In contrast, of all CGRP-mAb and isotype-conAb-treated HT neurons, 2/14 and 13/13, respectively, were activated by CSD, an 86% difference.

CSD-induced sensitization

Regardless of activation by CSD, 11/13 HT and none of the WDR neurons fulfilled our criteria for the development of sensitization (defined in the data analysis section). Therefore, CGRP-mAb ability to interfere with the development of sensitization after CSD is presented for HT but not WDR neurons.

Expansion of dural receptive fields and enhanced responses to mechanical stimulation of the dura after CSD

In the isotype-conAb-treated group, dural receptive fields expanded in 5/7 HT neurons in males and 6/6 HT neurons in females (Fig. 7*A*). Two hours after induction of CSD (6 h after isotype-conAb administration), neuronal responses to dural indentation with VFH increased in all 7 HT neurons in the males (12.8 ± 3.9 spikes/s before CSD vs 22.0 ± 3.7 after CSD; $p = 0.026$) and all 6 HT neurons in the females (8.5 ± 1.7 before CSD vs 21.6 ± 5.1 after CSD, $p = 0.047$) (Fig. 8*A–C*).

In contrast, in the CGRP-mAb-treated group, expansion of dural receptive fields, which was smaller when it occurred, was recorded in only 2/8 HT neurons in the male and 0/6 in the female (Fig. 7*B*). Two hours after induction of CSD (6 h after CGRP-mAb administration), neuronal responses to dural indentation with VFH remained unchanged in all HT neurons in both the males (1.8 ± 0.6 before CSD vs 1.9 ± 1.5 after CSD, $p = 0.83$) and the females (10.5 ± 1.6 before CSD vs 8.1 ± 6.4 after CSD, $p = 0.72$; Fig. 8*D–F*), which is indicative of prevention of sensitization.

Expansion of cutaneous receptive fields and enhanced responses to mechanical stimulation of the periorbital skin after CSD (i.e., central sensitization)

In the isotype-conAb-treated group, facial receptive fields expanded in 5/7 HT neurons in males and 6/6 HT neurons in females (Fig. 7*A*). Two hours after induction of CSD (6 h after isotype-conAb administration), responses to brush and pressure increased significantly in all 13 HT neurons (7 in males, 6 in females) (Fig. 9*A–C*). In males, responses to brush and pressure

Figure 8. Enhanced responses to mechanical stimulation of the dura after CSD are prevented by CGRP-mAb. **A, D**, Responses to indentation of the dura before CSD induction (BL) and 2 h after CSD in HT neurons that received treatment with isotype-conAb (**A**) or CGRP-mAb (**D**) 4 h before CSD induction. Numbers in parentheses show the mean discharge rate during each stimulus. Bin width = 1 s. **B, C, E, F**, Mean ($\pm$ SE) discharge in response to dural indentation before CSD induction (baseline) and 2 h after CSD in neurons that received treatment with isotype-conAb (**B, C**) or CGRP-mAb (**E, F**). Neurons recorded in males are shown in **B** and **E**; neurons recorded in females are shown in **C** and **F**. * $p < 0.05$ compared with baseline. Numbers in parentheses in **B, C, E**, and **F** depict the number of neurons in each group. Note that CGRP-mAb prevented the development of intracranial mechanical hypersensitivity in HT neurons in both sexes.

increased from 0.0 to 18.2 ± 9.1 spikes/s ($p = 0.046$) and from 16.6 ± 4.2 to 35.8 ± 9.1 spikes/s ($p = 0.045$), respectively (Fig. 9B). In females, responses to brush and pressure increased from 0.0 to 8 ± 6.5 spikes/s ($p = 0.027$) and from 9.3 ± 2.7 to 31.8 ± 13.6 spikes/s ($p = 0.016$), respectively (Fig. 9C). In contrast, responses to pinch increased significantly in all HT neurons in females (19.3 ± 5.0 spikes/s before CSD vs 45.8 ± 12.4 spikes/s after CSD, $n = 6$, $p = 0.027$), but not in the males (33.8 ± 7.1 spikes/s before CSD vs 52.4 ± 10.3 spikes/s after CSD, $n = 6$, $p = 0.068$) (Fig. 9B, C).

In the CGRP-mAb-treated rats, facial receptive fields expanded in only 2/8 HT neurons in males and 0/6 HT neurons in females. Two hours after induction of CSD (6 h after CGRP-mAb administration), neuronal responses to brush ($p = 0.35$), pres-

sure ($p = 0.63$), and pinch ($p = 0.78$) remained unchanged in all HT neurons in both males and females (Fig. 9D–F), suggesting that the CGRP-mAb prevented induction of sensitization.

Enhanced responses to corneal stimulation after CSD

In the isotype-conAb-treated rats, responses to corneal stimulation after CSD increased significantly in female (7.6 ± 1.9 spikes/s before CSD vs 21.0 ± 6.4 spikes/s after CSD, $n = 6$, $p = 0.044$), but not in male (11.0 ± 2.6 spikes/s before CSD vs 21.6 ± 8.7 spikes/s after CSD, $n = 7$, $p = 0.19$) HT neurons (Fig. 10A–C).

In the CGRP-mAb-treated female rats, response to brushing the cornea remained unchanged in the 6 HT neurons ($p = 0.51$), suggesting prevention of sensitization, and, as expected, it also remained unchanged in the 8 HT neurons in the males ($10.8 \pm$

Figure 9. Enhanced responses to cutaneous stimulation after CSD are prevented by CGRP-mAb. **A, D**, Responses to mechanical stimulation of the cutaneous receptive fields with brush, pressure, and pinch before CSD induction (baseline, BL) and 2 h after CSD for 2 HT neurons that received isotype-conAb (**A**) or CGRP-mAb (**D**) 4 h before CSD induction. Numbers in parentheses show the mean discharge rate during each stimulus. Bin width = 1 s. **B, C, E, F**, Mean ($\pm$ SE) discharge in response to cutaneous stimulation before (baseline) and 2 h after CSD induction in HT neurons that received treatment with isotype-conAb or CGRP-mAb 4 h before CSD induction. Neurons recorded in males are shown in **B** and **E**; neurons recorded in females are shown in **C** and **F**. * $p < 0.05$ compared with baseline. Note that CGRP-mAb prevented the development of cutaneous mechanical hypersensitivity in HT neurons in both sexes.

3.3 spikes/s before CSDs vs 9.4 ± 1.8 spikes/s after CSD, $p = 0.60$) (Fig. 10D–F).

Discussion

This study demonstrates that the humanized CGRP-mAb fremanezumab inhibits activation and sensitization of HT but not WDR trigeminovascular neurons (Fig. 11). In males, the CGRP-mAb inhibited the spontaneous activity of naive HT neurons and their responses to stimulation of the intracranial dura but not facial skin or cornea, whereas, in females, it only inhibited their responses to stimulation of the intracranial dura. When given sufficient time, however, the CGRP-mAb prevented in both sexes the activation and consequential sensitization of the HT neurons by CSD, but not the partial activation of WDR neurons. Mechanistically, these findings suggest that HT neurons play a critical role (not recognized before) in the initiation of the perception of headache and the development of allodynia and central sensitization. Clinically, the present findings may help to explain the therapeutic effectiveness of CGRP-mAb in preventing headaches

of intracranial origin such as migraine and why this therapeutic approach may not be effective for every migraine patient. Although our data show little effect on the processing of nociceptive signals that originate extracranially (skin, cornea), it may be interesting to determine whether CGRP-mAb can prevent headaches/migraine of extracranial origin.

This is the first study to our knowledge to test the effects of CGRP-mAb on the responsiveness of different classes of central trigeminovascular neurons. Previously, Storer et al. (2004) showed that the CGRP-R antagonist BIBN4096BS inhibits naive central trigeminovascular neuron responses to electrical stimulation of the superior sagittal sinus and microiontophoretic administration of L-glutamate.

Fremanezumab effects on HT versus WDR

When given intravenously, CGRP-mAb reduced baseline spontaneous activity in HT but not WDR neurons. Considering current and previous evidence that WDR trigeminovascular neurons are activated by a variety of dural stimulation used to study the

Figure 10. Enhanced responses to mechanical stimulation of the cornea after CSD are prevented by CGRP-mAb (female only). **A, D**, Responses to corneal stimulation (gentle brush) before CSD induction (BL) and 2 h after CSD in 2 HT neurons that received treatment with isotype-conAb (**A**) or CGRP-mAb (**D**) 4 h before CSD induction. Bin width = 1 s. **B, C, E, F**, Mean ($\pm$ SE) discharge in response to corneal stimulation before CSD induction (baseline) and 2 h after CSD in neurons that received treatment with isotype-conAb (**B, C**) or CGRP-mAb (**E, F**) 4 h before CSD induction. Neurons recorded in males are shown in **B** and **E**; neurons recorded in females are shown in **C** and **F**. * $p < 0.05$ compared with baseline. Note that CGRP-mAb prevented the development of corneal hypersensitivity in HT neurons in female but not male rats.

pathophysiology of migraine (Davis and Dostrovsky, 1988; Burstein et al., 1998; Storer et al., 2004; Zhang et al., 2011), it is reasonable to conclude that activation of WDR alone is insufficient to induce the headache perception in episodic migraine patients whose headaches are completely or nearly completely prevented by CGRP-mAb therapy (Bigal et al., 2015a). Conversely, it is also reasonable to speculate that activation of WDR trigeminovascular neurons alone may be sufficient to induce the headache perception in those episodic migraine patients who do not benefit from CGRP-mAb therapy because the headache could be unaffected by elimination of the signals sent to the thalamus from HT trigeminovascular neurons.

Outside of migraine and the trigeminovascular system, HT and WDR neurons have also been thought to play different roles

in the processing of noxious stimuli and the perception of pain (Craig, 2002, 2003a, 2003b). Although most HT neurons exhibit small receptive fields and respond exclusively to noxious mechanical stimuli, most WDR neurons exhibit large receptive fields and respond to both mechanical and thermal noxious stimuli (Price et al., 1976; Price et al., 1978; Hoffman et al., 1981; Dubner and Bennett, 1983; Bushnell et al., 1984; Surmeier et al., 1986; Ferrington et al., 1987; Dubner et al., 1989; Maixner et al., 1989; Laird and Cervero, 1991). Based on these differences, it is generally believed that HT neurons make a greater contribution to the spatial encoding (size, location) of pain and a lesser contribution to the encoding of pain modalities, whereas WDR neurons make a greater contribution to the radiating qualities of the pain. It is also reasonable to hypothesize that those patients

	Naïve state				Post-CSD state			
	Male		Female		Male		Female	
Spontaneous Activity	HT	WDR	HT	WDR	HT	WDR	HT	WDR
CGRP-mAb	Inhibition	No inhibition	No inhibition	No inhibition	Prevention	No prevention	Prevention	No prevention
Isotype	No inhibition	No inhibition	No inhibition	No inhibition	Activation/sensitization	Partial activation	Activation/sensitization	Partial activation
Von Frey Hair (Dura)	HT	WDR	HT	WDR	HT	WDR	HT	WDR
CGRP-mAb	Inhibition	No inhibition	Inhibition	No inhibition	Prevention	No sensitization	Prevention	No sensitization
Isotype	No inhibition	No inhibition	No inhibition	No inhibition	Sensitization	No sensitization	Sensitization	No sensitization
Brush	HT	WDR	HT	WDR	HT	WDR	HT	WDR
CGRP-mAb	No inhibition	No inhibition	No inhibition	No inhibition	Prevention	No sensitization	Prevention	No sensitization
Isotype	No inhibition	No inhibition	No inhibition	No inhibition	Sensitization	No sensitization	Sensitization	No sensitization
Pressure	HT	WDR	HT	WDR	HT	WDR	HT	WDR
CGRP-mAb	No inhibition	No inhibition	No inhibition	No inhibition	Prevention	No sensitization	Prevention	No sensitization
Isotype	No inhibition	No inhibition	No inhibition	No inhibition	Sensitization	No sensitization	Sensitization	No sensitization
Pinch	HT	WDR	HT	WDR	HT	WDR	HT	WDR
CGRP-mAb	No inhibition	No inhibition	No inhibition	No inhibition	No sensitization	No sensitization	Prevention	No sensitization
Isotype	No inhibition	No inhibition	No inhibition	No inhibition	No sensitization	No sensitization	Sensitization	No sensitization
Cornea	HT	WDR	HT	WDR	HT	WDR	HT	WDR
CGRP-mAb	No inhibition	No inhibition	No inhibition	No inhibition	No sensitization	No sensitization	Prevention	No sensitization
Isotype	No inhibition	No inhibition	No inhibition	No inhibition	No sensitization	No sensitization	Sensitization	No sensitization

Figure 11. Summary of effects of Fremanezumab (CGRP-mAb) vs isotype on HT and WDR neurons in the naïve and post-CSD state. Naïve state. CGRP-mAb, but not isotype, inhibits baseline (naïve state) spontaneous activity and dural mechanosensitivity in males, and dural mechanosensitivity in females, of HT but not WDR neurons. Post-CSD state. CSD produces activation/sensitization (increased spontaneous activity) and sensitization of responses to dural mechanical stimulation and facial brush and pressure in male HT neurons, and, in addition to these effects, also produces sensitization to facial pinch and corneal mechanical stimulation in female HT neurons. CSD produces partial activation in male and female WDR neurons. CGRP-mAb, but not isotype, prevents the sensitizing effects of CSD in HT neurons, but not in WDR neurons.

unresponsive to fremanezumab are the ones whose headaches affect large areas of the head (i.e., frontal, temporal, occipital, bilateral), whereas the ones whose headaches are well localized to small and distinct areas will be among the responders.

Specificity to headache

Fremanezumab reduced responsiveness to mechanical stimulation of the dura (both in males and females), but not to innocuous or noxious stimulation of the skin or cornea. This finding, together with the fact that the CGRP-mAb also prevented the activation of HT trigeminovascular neurons by CSD, provides a scientific basis for fremanezumab’s effectiveness in preventing headaches of intracranial origin. Conversely, lack of effects on modulating the processing of sensory and nociceptive signals that arise in the facial skin and cornea predicts that this class of drugs will have little therapeutic effect on treating prolonged trigeminal pain conditions such as dry eye and herpes-induced trigeminal neuralgia. Given that fremanezumab inhibited activation of central trigeminovascular neurons from the dura (mechanical, CSD), but not skin or cornea, and that the size of this molecule is too large to penetrate the blood–brain barrier readily, it is reasonable to suggest that the inhibitory effects described above were secondary to (primary) inhibition of responses to dural indentation and CSD in peripheral trigeminovascular neurons.

Given the wide distribution throughout the body of CGRP fibers (Kruger et al., 1988; Kruger et al., 1989; Silverman and Kruger, 1989) and their presence in multiple spinal cord segments (Hansen et al., 2016; Nees et al., 2016) and in multiple sensory dorsal root ganglia (Edvinsson et al., 1998; Edvinsson et al., 2001; Cho et al., 2015; Kestell et al., 2015; Spencer et al., 2016), it is surprising that the CGRP-mAb had little or no effect on the responses of the central neurons to noxious stimulation of the skin and cornea. If one accepts the notion that the CGRP-mAb

acts mainly in the periphery, it is also reasonable to propose that peripheral aspects of the sensory innervation of the meninges and the way that this innervation affects sensory transmission in the dorsal horn differ from those involved in the generation of cutaneous, corneal, or other (somatic) pains. Studies on fremanezumab’s effects in animal models of other pain conditions should allow us to interpret more accurately the difference between CGRP-mAb effects in the dura versus extracranial tissues not believed to have a distinct initiating role in migraine.

Inhibition of CSD-induced activation and sensitization

To the best of our knowledge, this is the first demonstration of sensitization of central trigeminovascular neurons by CSD. This sensitization, which we observed in HT but not WDR neurons in both males and females, was prevented by the CGRP-mAb administration. These novel findings raise the possibility that cutaneous allodynia in attacks preceded by aura (Burstein et al., 2000) is mediated by HT neurons that are unresponsive to innocuous mechanical stimulation of the skin at baseline (interictally in patients and before induction of CSD in animals), but becomes mechanically responsive to brush after the CSD. According to this scenario, one would predict that, among migraine aura patients, responders to the prophylactic treatment of CGRP-mAb would show no signs of cutaneous allodynia, a potential biomarker of treatment success and a readily testable hypothesis.

Male versus female

This is also the first study to test CGRP-mAb effects in both male and female rats. Although our overall analysis-by-sex (which we cannot compare to any previous study) suggests that the therapeutic benefit of this class of drugs should be similar in male and female migraineurs, it also shows that, in the naïve state, CGRP-mAb reduces the spontaneous activity in male but not female HT

neurons and that, after induction of sensitization by CSD, only HT neurons recorded in females exhibited signs of sensitization to noxious stimulation of the skin and cornea. Given that migraine is more common in women than men, it is tempting to interpret the differences as suggesting that hyperalgesia (rather than allodynia) is more likely to develop in women than in men during migraine with aura and that attempts to reduce neuronal excitability by CGRP-mAb in the interictal state (i.e., as a preventative) may also be more challenging in women than in men. Mechanistically, the three observed differences could be attributed to greater excitability of female HT neurons either due to these neurons' internal properties or to differences in the strength of inputs that they receive from peripheral nociceptors. Whereas no data exist to support the first option, it is possible that differences in the activation of dural immune cells and inflammatory molecules in females compared with males (McIlvried et al., 2015) can support the second option. Regarding fremanezumab's ability to reduce spontaneous activity in male but not female rats, one may take into consideration data showing that female rats express fewer CGRP receptors in the trigeminal ganglion and spinal trigeminal nucleus and higher levels of CGRP-encoding mRNA in the dorsal horn (Stucky et al., 2011).

Finally, whereas most HT neurons were inhibited by the fremanezumab within 3–4 h, a few were inhibited within 1–2 h. These relatively short times (hours rather than days), however, were achieved using intravenous rather than the subcutaneous administration mode reported in the clinical trials with fremanezumab (Bigal et al., 2015a; Bigal et al., 2015b). To date, no information is available to allow one to speculate on the mechanisms/reasons for the delayed (hours rather than seconds) inhibition of the neurons by the intravenous administration of the CGRP-mAb. In the absence of an immediate effect, it may be reasonable to speculate that CGRP-mAb inhibits the peripheral trigeminal neurons indirectly by altering meningeal immune and/or vascular functions.

We must state the caveat that the occurrence of CSD was verified by recording an ECG just anterior to the visual cortex using a glass micropipette. Therefore, the possibility that CSD was unintentionally triggered by the insertion of this micropipette was accounted for and ruled out by a continuous ECG recording. The small size of the tip (7 μm) and the short insertion distance (<100 μm) may account for the lack of CSD. Furthermore, the ECG recording electrode was placed in the cortex at least 2 h before beginning of neuronal recording and 6 h before final reading of neuronal activity.

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