Sex differences in the inflammatory mediator-induced sensitization of dural afferents

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CONSERVATIVE ESTIMATES INDICATE that over 20% of the adult population suffers from migraine. Interestingly, this debilitating pain disorder is far more common in women than in men, with a prevalence roughly three times higher in women than in men [18% vs. 6% (Stewart et al. 1992)] and an incidence more than two times higher [43% vs. 18% (Stewart et al. 2008)]. The underlying mechanisms of this sex difference have yet to be identified.

While there is still debate over the mechanisms underlying the initiation of a migraine attack, compelling evidence indicates that activation of primary afferents innervating the dural vasculature mediates migraine pain. Electrical stimulation of the dura and associated vasculature is associated with migraine-like pain (Wolff 1946). The majority of dural afferents are nociceptive in that they are sensitized by inflammatory mediators (IM) and are activated by algogenic substances (Levy and Strassman 2002; Oshinsky and Luo 2006; Strassman et al. 1991; McIlvried et al. 2010; O’Connor and van der Kooy 1988), a transmitter implicated in migraine pain based on its appearance in the jugular venous blood during the ictal phase of migraine (Moreno et al. 2002; Sarchielli et al. 2000) and the apparent efficacy of CGRP receptor antagonists for the treatment of migraine (Villalon and Olesen 2009). Importantly, the sensitization and subsequent activation of dural afferents is thought to be responsible not only for the initiation of migraine pain but for driving the central sensitization that serves to amplify afferent input as well as mediate the emergence of allodynia in cutaneous dermatomes (Burstein et al. 2004; Oshinsky and Luo 2006).

On the basis of the compelling evidence in support of a role for dural afferents in migraine, we hypothesized that the sex difference in the manifestation of migraine may be due, at least in part, to differences in the passive and active electrophysiological properties of dural afferents and/or their response to IM. To begin to test this hypothesis, whole cell patch-clamp techniques were used to study retrogradely labeled dural afferents from male and female rats. Results indicate that the magnitude of excitability once sensitized by IM is comparable in both sexes. However, sex differences were detected in the proportion of neurons sensitized by IM and in the underlying mechanisms of sensitization.

MATERIALS AND METHODS

Intact adult female and male Sprague-Dawley rats (Harlan, 200–290 g) were used for all experiments. Animals were housed one per cage in a temperature- and humidity-controlled animal facility on a 12:12-h light-dark schedule with food and water freely available. Prior to all procedures, animals were deeply anesthetized with an intraperitoneal injection containing ketamine (55 mg/kg), xylazine (5.5 mg/kg), and acepromazine (1.1 mg/kg). All procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health guidelines for the use of laboratory animals in research.

Afferents innervating the dura were identified after application of 1,1′dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) to the dura as previously described (Harriott and Gold 2008). After at least 10 days post afferent labeling, the trigeminal ganglia from intact male and female rats were removed bilaterally, enzymatically treated, and mechanically dissociated as previously described (Harriott et al. 2006). Intact adult male and female rats were used in the present study based on the recommendations detailed in the consensus statement on the study of sex differences in pain and analgesia (Greenspan et al. 2007) in which the authors weighed the strengths and weaknesses of
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initiating such a study with intact animals versus one in which gonadal hormones were surgically or chemically manipulated. In light of further recommendations in the same document, and our previous results with neurons from intact female rats, in which ~100% of dural afferents were sensitized by IM (Harriott and Gold 2009), we did not attempt to determine the stage of the female rats studied in their estrous cycle. Neurons were studied within 8 h after plating.

Electrophysiology: voltage and current clamp. All whole cell patch-clamp experiments were carried out with a HEKA EPC9 amplifier (HEKA Elektronik, Lambrecht/Rheinland-Pfalz, Germany). Glass electrodes (1–4 MΩ) were filled with (in mM) 110 K-methanesulfonate, 30 KCl, 5 NaCl, 1 CaCl2, 2 MgCl2, 10 HEPES, 11 EGTA, 2 Mg-ATP, and 1 Li-GTP, and the pH was adjusted with Tris-base to 7.2. Osmolality was adjusted to 320 mosmol/kgH2O with sucrose. The bath solution contained (in mM) 3 KCl, 130 NaCl, 2.5 CaCl2, 0.6 MgCl2, 10 HEPES, and 10 glucose. The pH was adjusted with Tris-base to 7.4, and osmolality was adjusted with sucrose to 320 mosmol/kgH2O.

IM-induced changes in excitability were assessed in current-clamp mode by five distinct measures: the emergence of spontaneous activity, action potential (AP) threshold, rheobase, current threshold, and accommodation. Spontaneous activity was assessed at resting membrane potential (Vr) for 30 s before and up to 90 s after the application of IM. The second two measures were determined with a 750-ms depolarizing square-pulse current injection. AP threshold was defined as the greatest depolarization reached before spike generation in response to depolarizing current injections. Rheobase was defined as the smallest amount of current needed to evoke a single AP. Because rheobase is positively correlated with cell size, values were normalized with respect to membrane capacitance to facilitate comparisons between neurons. A ramp and hold protocol consisting of a 250-ms ramp followed by 500-ms sustained current injection was used to monitor the impact of IM on accommodation. Accommodation was determined by counting the number of APs evoked during this protocol. The magnitude of current injection was adjusted so as to evoke an AP during the ramp phase of the stimulation protocol. This protocol was then used to stimulate neurons every 30 s before and after application of IM, where at least three stimuli were used to establish the stability of neuronal excitability prior to the application of IM. IM were applied for 90 s in the majority of experiments based on our previous results (Gold et al. 1996a) as well as those of others (Hargreaves et al. 1994; Momin and McNaughton 2009) indicating that the effects of a wide variety of IM on isolated neurons are rapid, with peak responses detected in milliseconds to seconds. To assess the presence of more slowly developing changes in excitability, IM were applied for up to 10 min to a subpopulation of dural afferents from male rats. As no spontaneous activity was detected in any neuron studied, a neuron was considered “sensitized” if the application of IM resulted in a significant increase in the number of APs evoked during the ramp and hold protocol, relative to baseline. To facilitate comparisons between neurons, IM-induced changes were analyzed as a percent change from baseline.

Passive and active electrophysiological properties were assessed for each neuron studied. Passive properties included input resistance (Ri), capacitance, and Vrest. After whole cell access was gained, Ri and capacitance were determined in voltage-clamp mode with amplifier circuitry. Ri was determined before and 90 s after application of IM and/or niflumic acid (NFA). Active electrophysiological properties consisted of parameters describing the AP waveform and included magnitude of AP overshoot, AP duration, magnitude of afterhyperpolarization (AHP), and AHP decay. Single APs were evoked with a 4-ms depolarizing current injection. The magnitude of AP overshoot was measured from 0 mV to the peak depolarization. AP duration was measured at 0 mV. The magnitude of AHP was measured from the Vrest to the maximum point of hyperpolarization. The decay of the AHP was estimated with a single exponential function fitted to the decay phase of the waveform.

The electrode solution used to record Na+ currents in relative isolation was composed of (in mM) 100 cesium methanesulfonate (CsMs), 5 NaCl, 40 TEA-Cl, 1 CaCl2, 2 MgCl2, 11 EGTA, 10 HEPES, 2 ATP-Mg, and 1 GTP-Li. The bath solution was composed of (in mM) 35 NaCl, 65 choline-Cl, 30 TEA-Cl, 0.1 CaCl2, 5 MgCl2, 10 HEPES, and 10 glucose. Voltage-clamp protocols were used to assess the impact of IM on the tetrodotoxin-resistant (TTX-R) Na+ current. A steady-state inactivation protocol consisting of 500-ms by 5-mV voltage steps from −110 mV was used to determine the prepulse potential to inactivate fast-inactivating tetrodotoxin-sensitive (TTX-S) Na+ current, leaving slowly activating TTX-R Na+ current in relative isolation. A voltage protocol was used to isolate TTX-R from TTX-S, to facilitate the assessment of changes in both currents in the same neuron. Importantly, we have previously demonstrated that the currents isolated with voltage steps are identical to those isolated with TTX (Gold et al. 2002). The impact of IM on Na+ current was monitored by evoking Na+ current with a step to 0 mV every 5 s. IM was applied after confirmation of the stability of evoked current. A neuron was considered responsive to IM if peak current was increased by 20% of baseline.

Drugs. All salts and test compounds were obtained from Sigma-Aldrich (St. Louis, MO). The IM solution consisted of bradykinin (10 μM), histamine (1 μM), and prostaglandin (PG)Et2 (1 μM). Bradykinin was dissolved in 1% acetic acid, PG was dissolved in 100% EtOH, and histamine was dissolved in water at stock concentrations of 100 mM, 100 mM, and 10 mM, respectively; stocks were divided into aliquots and stored at −20°C until the day of use. A normal bath solution containing final concentrations of 0.01% EtOH and 0.001% acetic acid was used as the vehicle control. NFA was dissolved in 100% EtOH, which was then diluted to 0.2%, yielding a final NFA concentration of 10 μM. All drugs were administered through a gravity-fed perfusion system.

Data analysis. Data were analyzed with PulseFit (HEKA), SigmaPlot, and Prism software. Differences in baseline, rheobase, and threshold between neurons from males and females were compared with an unpaired Student’s t-test. Changes in excitability and passive and active properties, before and after IM, were determined with a paired t-test. Data are expressed as means ± SE. P < 0.05 was considered statistically significant.

RESULTS

Data were collected from 120 dural afferents acutely dissociated from 38 (16 female, 22 male) Sprague-Dawley rats. The size distribution of these neurons was similar to that of our previous study (Vaughn and Gold 2010), with a median cell body capacitance of 24.91 pF (with 20.06 pF and 27.79 pF as 25th and 75th percentiles, respectively). The size of dural afferents from male and female rats was comparable (P > 0.05) (Fig. 1).

Passive and active electrophysiological properties. Conventional whole cell patch configuration was used for the initial characterization of passive and active electrophysiological properties. These data were collected from dural afferents acutely dissociated from 12 female and 14 male rats. There were no significant differences between dural afferents from male and female rats with respect to baseline passive or active electrophysiological properties or baseline excitability with the whole cell patch-clamp technique (Table 1).

Males versus females: difference in the response to inflammatory mediators. To determine whether there is a sex difference in the magnitude of IM-induced sensitization (see materials and methods for definition) of dural afferents, excitability was assessed before and after application of an IM solution containing PGE2, bradykinin, and histamine in 21 female and
37 male dural afferents. Neither male nor female afferents displayed spontaneous activity in the absence or presence of IM. Consistent with previous observations, almost all dural afferents (20/21) from females were sensitized after the application of IM. In contrast, nearly half of the dural afferents (18/37) from males demonstrated no change in excitability following 90-s IM application and thus were considered unresponsive (Fig. 1). The difference in proportion of dural afferents from male and female rats sensitized by IM was significant ($P < 0.01$, Fisher’s exact test). To determine whether the smaller fraction of dural afferents from male rats sensitized by IM could be due to the presence of slower second messenger signaling processes, a subgroup (17) of the afferents from male rats were studied with a 10-min IM application. An increase in excitability was detected in seven of these neurons, and this increase was detectable within 90 s. In the remaining 10 neurons, no change in excitability was detected throughout the 10-min IM application period.

To begin to assess the basis for the sex difference in the proportion of dural afferents sensitized by IM, we assessed the impact of IM on TTX-R voltage-gated Na$^+$ currents ($I_{Na}$) in male dural afferents. This is a stable current shown to be a common target for the second messenger pathways initiated by an array of IM (Gold 1999) in a variety of different afferents including dural afferents (Vaughn and Gold 2010) and therefore serves as a sensitive bioassay for the presence of IM-induced receptor activation in primary afferents. TTX-R $I_{Na}$ was measured before and after application of IM to 14 dural afferents from 3 male rats. Thirteen of these demonstrated an IM-induced increase in TTX-R $I_{Na}$, indicating that functional IM receptors are present on a larger percentage of male dural afferents than were sensitized by the same combination of mediators ($P = 0.01$) (Fig. 2). Consistent with our previous results from female rats (Vaughn and Gold 2010), we detected no significant influence of IM on TTX-S currents in the same group of neurons: pre- and post-IM maximal conductance changes of TTX-R $I_{Na}$ were not significantly different ($P > 0.05$).
was detected in 13 of 14 afferents studied and was associated with a \( G \) application (neuron tested were normalized to the maximal conductance determined before \( G \)). Conductance-voltage (G-V) data from each neuron tested were fitted with a Boltzmann equation with values for potential for half-maximal activation (\( V_{1/2} \)) of \(-2.9 \pm 0.3\) and \(-5.8 \pm 1.3\) and slope of \( 4.5 \pm 0.6\) and \( 3.8 \pm 0.7\) for Pre- and Post-IM, respectively. Inset, typical TTX-R \( I_{\text{Na}} \) evoked in dural afferents from male rats before (Baseline) and 90 s after (IM) the IM application.

(\( G_{\text{max}} \)) were 9.33 ± 2.52 nS and 8.26 ± 1.63 nS, respectively (\( P > 0.05\)).

Of the dural afferents sensitized by IM, the magnitude of change in all measures of excitability was comparable in males and females (Fig. 3). Interestingly, there were both similarities and differences between male and female dural afferents with respect to the changes in passive and active electrophysiological properties associated with the increase in excitability. A significant (\( P < 0.01\)) IM-induced decrease in \( R_f \) was detected in both groups of responsive neurons. This was associated with a significant (\( P < 0.01\)) depolarization of the \( V_{\text{rest}} \). An increase in the AP overshoot was also observed in afferents from both males and females. The magnitude of all three changes was comparable. In contrast, significant increases in AP duration and AHP magnitude were only observed for male dural afferents (Table 1).

Role of \( I_{\text{IM-Cl}} \) in IM-induced changes in passive and active electrophysiological properties. NFA, originally identified and subsequently used clinically as an inhibitor of COX-2, is commonly used experimentally as a nonselective \( Cl^- \) channel blocker (Danko et al. 2011; Forrest et al. 2010; Sagheddu et al. 2010). There are at least some data to suggest NFA has efficacy for the treatment of migraine (1975 U.S. Patent 4,024,279). We have previously demonstrated that the IM-induced activation of a NFA-sensitive \( Cl^- \) current (\( I_{\text{IM-Cl}} \)) plays a dominant role in the sensitization of female dural afferents (Vaughn and Gold 2010). Consistent with the suggestion that \( I_{\text{IM-Cl}} \) plays a critical role in the sensitization of male dural afferents, there was no decrease in \( R_f \) in neurons from male rats that did not demonstrate an IM-induced increase in excitability. Furthermore, \( I_{\text{IM-Cl}} \) activation appeared comparable in sensitized male and female dural afferents based on the magnitude of the IM-induced changes in \( R_f \) and \( V_{\text{rest}} \) (Table 1). Nevertheless, to confirm this prediction, we assessed the magnitude of the NFA-sensitive current in sensitized male and female dural afferents from six female and four male rats. The NFA-sensitive current in male and female dural afferents (2.01 ± 0.89 pA/pF and 2.44 ± 0.97 pA/pF, respectively) was comparable between groups (\( P > 0.05\); Fig. 4).

DISCUSSION

The purpose of this study was to begin to test the hypothesis that the sex difference in the manifestation of migraine is due to differences in the baseline excitability of dural afferents and/or the influence of IM on dural afferent excitability. While we observed no difference between afferents from male and female rats with respect to baseline excitability or the magnitude of IM-induced sensitization of dural afferents, our results suggest at least two major differences that may contribute to the sex difference in the manifestation of migraine. First, a significantly greater proportion of dural afferents (20/21) from female rats were sensitized after IM application than in males (19/37). This difference in the proportion of IM-sensitized neurons was present despite the observation that IM-induced activity was detectable in the majority (13/14) of dural afferents from male rats, as indicated by an IM-induced increase in TTX-R \( I_{\text{Na}} \). Second, changes in the active electrophysiological properties of afferents sensitized by IM were different in males and females (Fig. 3).
changes in gene expression may contribute to the manifestation of migraine, acute application of mediators precluded the engagement of such processes. Fourth, the use of depolarizing current injection to assess changes in excitability precluded our ability to detect changes mediated by transduction mechanisms. Finally, we studied dural afferents from rats. While several animal models of migraine have recently been developed, it has yet to be determined whether there is a sex difference in the behavioral changes associated with these models. Nevertheless, as with the use of rats in the present study, the use of such preclinical models of migraine is predicated on the assumption that even nonmigraineurs could become migraineurs under the right conditions.

There are at least three explanations for the absence of a detectable IM-induced increase in the excitability of roughly half of the male dural afferents: 1) male afferents lack functional receptors for the mediators employed, 2) the wrong combination of mediators was employed, and 3) there are differences in downstream mechanisms of excitability. Our data argue against the absence of functional receptors for the mediators employed based on the significant increase in TTX-R \( I_{\text{Na}} \) in 13 of 14 male dural afferents. Similarly, while a different combination of mediators might have sensitized a larger proportion of male dural afferents, the failure to detect an increase in excitability despite the increase in TTX-R \( I_{\text{Na}} \) in the majority of these afferents argues that the combination of mediators used was not the primary reason for the observation. Thus we suggest that the third possibility is the most likely to account for the absence of a detectable increase in excitability in half of the male dural afferents. Consistent with this suggestion, there is evidence for sex differences in the second messenger pathways underlying inflammatory hypersensitivity, which could influence the pattern of ion channel changes and subsequently the changes in excitability of dural afferents (Dina et al. 2003; Levine et al. 2001). For example, an IM-induced increase in \( K^+ \) conductance may have masked the impact of an increase in TTX-R \( I_{\text{Na}} \) in unresponsive male dural afferents. We and others have described an IM-induced increase in intracellular Ca\(^{2+}\) in dural afferents as well as evidence of several different Ca\(^{2+}\)-modulated/dependent \( K^+ \) currents in sensory neurons (Gold et al. 1996b; Vaughn and Gold 2010; Zhang et al. 2010), suggesting that a substrate for such an increase in \( K^+ \) current is present in dural afferents. Given the absence of a detectable decrease in \( R_f \) and/or depolarization of membrane potential in unresponsive neurons, a more likely explanation for the absence of sensitization of a subpopulation of male dural afferents is a lack of \( I_{\text{IM-Cl}} \) activation in these neurons, underscoring the importance of this conductance to the IM-induced sensitization of dural afferents.

Features of the IM-induced changes in passive and active electrophysiological properties reported here point to specific ion channels as underlying the increase in dural afferent excitability. Sensitization of female dural afferents was associated with an increase in AP overshoot, a decrease in \( R_f \), and depolarization of \( V_{\text{rest}} \). These changes are consistent with both an increase in TTX-R \( I_{\text{Na}} \) and the activation of \( I_{\text{IM-Cl}} \) as we have described previously in female rats (Vaughn and Gold 2010). Both currents appear to contribute to the sensitization of male dural afferents. Interestingly, the magnitude of \( I_{\text{IM-Cl}} \) was also comparable in dural afferents from males and females sensitized by IM, supporting the suggestion that \( I_{\text{IM-Cl}} \) is a
common target that could have efficacy for the treatment of migraine in males and females. However, in male afferents, there was also an increase in AHP and AP duration. An increase in AHP magnitude is generally reflective of a relative increase in K$^+$ conductance during the falling phase of the AP. However, such an increase in K$^+$ conductance should also result in a decrease in AP duration, while the opposite was observed in the present study. Because Ca$^{2+}$ influx via voltage-gated Ca$^{2+}$ channels also contributes to the AP duration (Blair and Bean 2002), an explanation for these apparently discrepant changes in AP waveform would be an IM-induced increase in Ca$^{2+}$ influx that subsequently results in the increased activation of a Ca$^{2+}$-dependent K$^+$ current. Such an explanation would be inconsistent with previous data indicating that PGE$_2$ results in the suppression of Ca$^{2+}$ current (I$_{Ca}$) in trigeminal sensory neurons (Borgland et al. 2002) and in marked contrast to our previous description of IM-induced changes in I$_{Ca}$ and Ca$^{2+}$-dependent K$^+$ currents in female dural afferents (Vaughn and Gold 2010). Nevertheless, there is evidence, albeit from avian sensory neurons, suggesting that PGE$_2$ can increase I$_{Ca}$ (Nicol et al. 1992), raising the possibility that the underlying mechanisms of dural afferent sensitation in males and females are truly distinct.

The epidemiology of migraine, with onset at menarche and resolution with menopause, suggests that gonadal hormones contribute significantly to the prevalence of migraine in women. Interestingly, a growing body of evidence suggests that it is not just the levels of gonadal hormones that are the problem, but fluctuations in hormone levels, in particular the drop in 17β-estradiol that occurs with ovulation that may be the most effective trigger for migraine (Shuster et al. 2011). These clinical data raise the intriguing possibility that acute deprivation of estrogens associated with the harvest and dissociation of dural afferents from female rats may contribute to the higher proportion of afferents sensitized by IM from females compared with males. It is also tempting to suggest that such a mechanism contributes to the sex difference in the manifestation of migraine given evidence that gonadal hormones, in particular estrogens, can directly influence the properties [i.e., L-type Ca$^{2+}$ channels (Meremelstein et al. 1996)] and/or expression pattern [i.e., splice variants of the BK Ca$^{2+}$-modulated K$^+$ channels (Poulsen et al. 2009)] of ion channels that influence neuronal excitability. However, increasing awareness of childhood migraine (Bigal and Arruda 2010) and the persistence of migraine in a significant proportion of postmenopausal women (MacGregor 2009) suggests that other mechanisms are also likely to contribute to the sex difference in migraine. Whether the sex differences observed in the present study are due to genetics (SRY, X-inactivation) or organizational and/or activational influences of gonadal hormones has yet to be determined (Arnold and Chen 2009; Huang et al. 2008). Nevertheless, the fact that we have identified a sex difference in intact animals provides compelling support for subsequent mechanistic analysis.

In summary, we have identified a sex difference in the proportion of afferents sensitized by IM as well as potential differences underlying mechanisms of excitability. Further exploration into the underlying mechanisms of excitability in responsive neurons will be necessary to more clearly define the role of I$_{IM-C[I]}$ in vivo and to identify the basis for observed sex differences. If differences in the relative contribution of different ion channels in dural afferents from males and females are substantiated, the difference suggests that it may ultimately be possible, if not necessary, to develop different approaches for the treatment of migraine tailored specifically to men and women.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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