Sensitization and activation of intracranial meningeal nociceptors by mast cell mediators

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**Abbreviations:** MC, Mast cells; CV, Conduction velocity; SIF, Synthetic interstitial Fluid; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); kPa, Kilopascal; 5HT, 5-hydroxytryptamine; PGI₂, Prostaglandin I₂; PGD₂, Prostaglandin D₂; LTC₄, Leukotriene C₄.
ABSTRACT

Intracranial headaches such as that of migraine are believed to result from activation of sensory trigeminal pain neurons that supply intracranial blood vessels and the meninges, also known as meningeal nociceptors. Although the mechanism underlying the triggering of such activation is not completely understood, our previous work indicates that the local activation of the inflammatory dural mast cells can provoke a persistent sensitization of meningeal nociceptors. Given the potential importance of mast cells to the pain of migraine it is important to understand which mast cell-derived mediators interact with meningeal nociceptors to promote their activation and sensitization. In the present study, we have used in-vivo electrophysiological single unit recording of meningeal nociceptors in the trigeminal ganglion of anesthetized rats to examine the effect of a number of mast cell mediators on the activity level and mechanosensitivity of meningeal nociceptors. We have found that that serotonin (5HT), PGI₂, and to a lesser extent histamine can promote a robust sensitization and activation of meningeal nociceptors while the inflammatory eicosanoids PGD₂ and LTC₄ were largely ineffective. We propose that dural mast cells could promote headache by releasing 5HT, PGI₂, and histamine.
Intracranial headaches such as that of migraine are believed to result from activation of sensory trigeminal pain neurons that supply intracranial blood vessels and the meninges, particularly the dura mater (i.e. meningeal nociceptors) (Strassman et al., 1996; Burstein, 2001; Pietrobon and Striessnig, 2003; Waeber and Moskowitz, 2005). Although factors that promote such neuronal activation are not completely understood, sterile meningeal inflammation is believed to play a role. Based on this notion, we have shown previously that exogenous application of a mixture of inflammatory mediators (i.e inflammatory soup) to the dura mater can promote in meningeal nociceptors a prolonged increase in their ongoing discharge rate and enhanced responsiveness to mechanical stimulation of the dura (Reviewed in Strassman and Levy, 2006). The cellular origin of such inflammatory stimuli is still under investigation.

While the dura mater is the intracranial structure most heavily innervated by pain fibers, it is also populated by resident mast cells (MCs) in both humans (Artico and Cavallotti, 2001) and rodents (Dimlich et al., 1991; Rozniecki et al., 1999; Strassman et al., 2004). These granulated immunocompetent cells, which reside near blood vessels and in close apposition to the meningeal nociceptive fibers (Rozniecki et al., 1999), have the capacity to initiate or amplify inflammatory responses by releasing a host of mediators such as histamine, serotonin, cytokines, and various lipid mediators, including leukotrienes and prostaglandins (Metcalfe et al., 1997; Mekori and Metcalfe, 2000). Given their pro-inflammatory properties, dural MCs have long been suggested to play a role in the pathophysiology of headaches such as migraine (Sicuteri, 1963; Theoharides, 1983). Experimental work in animals has shown that electrical stimulation of the trigeminal ganglion, leading to activation of meningeal nociceptors, promotes the release
of the granule content (i.e. degranulation) of dural MCs (Dimitriadou et al., 1991; Buzzi et al., 1992) through an axonal reflex mechanism involving sensory neuropeptides, a process leading to a local sterile meningeal inflammation. While MC degranulation has been linked to the neurogenic meningeal inflammation associated with migraine, we have provided evidence for the reverse process, whereby degranulation of dural MCs promotes a prolonged state of excitation of neighboring trigeminal meningeal nociceptors, giving rise to activation of the pain pathway underlying migraine headache (Levy et al., 2007).

Given the potential excitatory effects of MCs on meningeal nociceptors, in this study we used in vivo electrophysiological single unit recording of meningeal nociceptors in anesthetized rats to examine the potential activating and sensitizing effects of a number of MC-related inflammatory mediators. This study reports the effects of the MC-derived bioamines histamine and serotonin, the stable analogue of prostaglandin I₂ (Iloprost), prostaglandin D₂ (PGD₂) and the major MC leukotriene, leukotriene C₄ (LTC₄) (Roberts et al., 1979; Metcalfe et al., 1997).
Materials and Methods

Animals. Sprague-Dawley male rats (250-300 g) were used in compliance with the experimental protocol approved by the institutional Animal Care and Use Committee of the Harvard Medical School.

Surgery and electrophysiological recording. Rats were deeply anesthetized with an initial intraperitoneal dose of 1.8 g/kg urethane with 0.2 g/kg supplemental doses as needed. Single-unit recordings of meningeal nociceptors in the trigeminal ganglion were obtained as described before (Strassman et al., 1996; Levy et al., 2005). Briefly, the rat’s head was mounted in a stereotaxic apparatus (David Kopf) and a 2x2-mm craniotomy was performed to expose the dura over the midsagittal and left transverse sinuses. The exposed dura was bathed with a modified synthetic interstitial fluid (SIF; 135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 5 mM CaCl₂, 10 mM glucose and 10 mM HEPES, pH 7.2). A platinum-coated tungsten microelectrode (impedance 500 Ω; FHC, Bowdoin, ME) was lowered into the trigeminal ganglion through a small circular opening (2-mm wide) that was drilled in the left parietal bone, about 2 mm caudal to the Bregma suture and 2 mm left of the midline. Meningeal nociceptors were then identified by their constant latency response to single shock stimulation (0.5 ms pulse, 5 mA, 0.5 Hz). Response latencies were used to calculate conduction velocity (CV) based on a 12.5 mm distance to the TG (Strassman et al., 1996) and nociceptors were classified as either C-units (CV ≤ 1.5 m/sec) or A-delta units (CV > 1.5 m/sec). A waveform of the action potential evoked by the electrical stimuli was stored as a template using a real-time waveform discriminator (Spike 2, CED, Cambridge, UK) which was used to acquire experimental data and
perform on- and off-line analyses. In all experiments, only one unit was tested in each animal.

**Mechanical stimulation.** Mechanical receptive fields of meningeal nociceptors were mapped initially by stroking the dura with blunt forceps. The lowest threshold point was determined using a series of calibrated von Frey monofilaments ranging from 0.39 to 58.82 mN (38-443 kPa). Response magnitudes to mechanical stimulation were determined quantitatively, using a servo force-controlled mechanical stimulator (Aurora Scientific, Aurora, ON) fitted with a flat-ended plastic cylinder (0.5 mm diameter) aimed at the lowest threshold point on the dura. Stimulus trials for testing changes in mechanical sensitivity consisted of a graded series of three square-wave stimuli (100-msec rise time, 2-sec width, 60-sec inter-stimulus interval) delivered in ascending order, which included a threshold and two suprathreshold stimuli. Stimuli that evoked 1-2 Hz afferent discharge were considered as threshold. Suprathreshold stimuli were usually 2 and 4 times greater than threshold. Such stimulus trials consisting of these 3 stimuli were then delivered repeatedly at 15 minutes intervals. This inter-trial interval was not changed once baseline testing started. A 30 sec interval preceding the threshold stimulus was used for measurement of baseline spontaneous activity. The response to each mechanical stimulus was calculated by subtracting the spontaneous firing rate from the mean firing rate during the stimulus. In all experimental protocols, baseline measurements of spontaneous and mechanically evoked activity were obtained prior to drug administration. Only units that exhibited consistent responses at all stimulus intensities in at least 3 consecutive baseline trials were tested further. These trials also served as
vehicle controls, because the receptive field was bathed in SIF, which was the vehicle for all drugs.

**Drugs and solutions.** The following drugs were used: histamine (1-1000 µM.), serotonin (5HT, 0.1-100 µM), prostaglandin D$_2$ (PGD$_2$, 1-1000µM), the PGI$_2$ stable analogue Iloprost (0.1-100 µM) and leukotriene C$_4$ (LTC$_4$, 2-200 µM). Histamine and 5HT were obtained from Sigma Aldrich (Saint Louis MO), PGD$_2$ from EMD Biosciences (San Diego, CA), and Iloprost and LTC$_4$ from Cayman Chemicals (Ann Arbor, MI). Stock solutions for all drugs were prepared by dissolving the drugs in either distilled water (histamine, 5HT) or 100% ethanol (for the eicosanoids) and kept frozen (-20°C). Drugs were further diluted on the day of the experiments with fresh SIF. Final concentration of ethanol never exceeded 0.1%

**Experimental paradigm.** The effects of the MC-related mediators on the activity and mechanosensitivity of meningeal nociceptors was tested by applying the agents topically to the dural receptive field using a small piece of cotton soaked with approximately 40 µl of the tested agent. Individual neurons were tested with ascending doses of a specific MC mediator, with each dose applied for 1 trial (~15 min). In cases in which a certain dose was found to affect at least one parameter (threshold, suprathreshold or ongoing activity), the dura was washed for 15 min before the next dose was applied. To examine the duration of the response, in sensitized neurons recording was maintained for up 60 min during the last wash period. In cases when the highest dose was found ineffective in one trial, it was left for up to 1 hour in order to examine potential indirect effects of the agent
due to an inflammatory action in the dura. In most neurons only one agent was tested while in a few neurons two agents were tested. A second agent was tested only if no response was elicited by the first agent, and it was applied to the dura following at least 2 hours of a wash period. At the end of experiment, rats were euthanized with an intravenous bolus of 1 M KCl.

**Data analysis.** Data are displayed as the median and interquartile range (IQR). For each neuron, an increase in threshold or suprathreshold responses, or ongoing discharge level was defined as an increase in firing rate that exceeded the mean plus two times the standard deviation (SD) of the baseline. Group comparisons were made between baseline and the various doses using the Friedman test. Post-hoc paired comparisons between the different doses and baseline were performed using a one-tailed Wilcoxon matched-pairs signed-ranks test. Effects were analyzed separately for A-delta and C-units. The level of significance was set at 0.05. The Mann Whitney U-test was used to analyze differences between the baseline mechanical threshold and spontaneous activity of the A-delta and C-units tested.

**Results**

**Population of units tested.** Extracellular unit recordings were obtained from 69 mechanosensitive meningeal nociceptors in the trigeminal ganglion that were identified by their response to single shock stimulation of the dura overlying the ipsilateral transverse sinus. All neurons in this study exhibited mechanical receptive fields on the dura overlying or immediately adjacent to the ipsilateral transverse sinus or the caudal
most part of the superior sagittal sinus. The baseline response properties of the neurons tested are depicted in Table 1.

**Effects of Histamine.** The effect of topical application of histamine was examined in 9 A-delta and 10 C-units. Among the A-delta units tested only 2/9 units showed an increase in their threshold and suprathreshold responses while among the C-units tested 7/10 units were mechanically sensitized and both threshold and suprathreshold responses increased (Fig 1). Overall, histamine significantly increased threshold and suprathreshold responses only at the two highest doses tested. Histamine-induced mechanical sensitization was brief, with mechanical sensitivity returning to baseline values 15 minutes after wash with SIF (Fig 1). Histamine induced an increase in the ongoing activity rate only in 1/9 A-delta units tested but affected 6/10 C-units. Overall, the increase in ongoing discharge rate within the C-unit population was achieved only at the two highest histamine doses tested, was brief and always returned to baseline following 15 min wash with SIF.

**Effect of 5-HT.** The effect of topical application of 5-HT was examined in 6 A-delta and 8 C-units. Topical application of 5-HT for 15 min affected the mechanosensitivity of both the A-delta and C-unit populations. Among the A-delta units tested, 5HT increased threshold responses in 4/6 units and suprathreshold responses in 5/6 units. Among the C-units tested, 5-HT increased threshold responses in 7/8 units and suprathreshold responses in 5/8. Overall, 5-HT induced a significant mechanical sensitization already at 0.1µM within the C-units and at 1µM within the A-delta units (Fig 2). The increase in threshold responses was dose-dependent only within the C-unit population. In most sensitized neurons responses remained elevated during the last wash period for an
additional 30-45 min. Topical application of 5-HT also increased the ongoing discharge rate in 4/6 of the A-delta units and in 5/8 of the C-units. 5-HT increased the level of spontaneous activity only at the two highest doses in both the A-delta and C-units tested. In all activated neurons the increased ongoing discharge rates remained elevated for at least 30 min during the last wash period.

**Effect of PGI$_2$** The effect of the stable PGI$_2$ analogue iloprost was tested in 6 A-delta and 7 C-units. Overall, topical application of iloprost produced a significant increase in the threshold, but not suprathreshold responses in both the A-delta and C-unit populations (Fig. 3). Among the A-delta units tested, 4/6 neurons had increased threshold responses while only 1/6 had increased suprathreshold responses. Among the C-units tested, 5/7 units had increased threshold responses while only 1/7 had increased suprathreshold responses. The lowest effective dose that promoted increases in threshold responses was 0.1 µM for the A-delta and 1 µM for the C-units. The sensitizing effect of iloprost was dose-dependent within both the A-delta and C-unit populations. In most sensitized neurons, threshold responses remained elevated for at least 30 minutes during the wash period. Iloprost also significantly increased the level of ongoing discharge in 3/6 A-delta and 5/7 C-units. The minimal dose that elicited this response was 100 µM for the A-delta and 0.1µM for the C-units. In all neurons in which iloprost promoted activation, the increased ongoing discharge rate, similar to the mechanical sensitization, persisted for at least 30 minutes during the last wash period.
Effect of PGD₂. The effect of PGD₂ was tested in 6 A-delta and 6 C units. Topical application of PGD₂ evoked minimal mechanical sensitization in 1/6 A-delta and 1/6 C-units which lasted for only one trial (~15 min). PGD₂ also had a minimal effect on the level of spontaneous activity, in 1/6 C-units with spontaneous activity returning to baseline levels 15 min after wash. Overall there was no significant affect of PGD₂ on either the A-delta nor C-units tested (Fig. 4, left).

Effect of LTC₄. The effect of LTC₄ was tested in 6 A-delta and 5 C-units. Topical application of LTC₄ at all doses tested, and up to 1 hour of treatment did not affect the mechanosensitivity or the level of spontaneous discharges in any of the meningeal nociceptors tested (Fig. 4, right).

Discussion

We have previously reported that dural MC degranulation can promote activation of meningeal nociceptors (Levy et al., 2007). The results of the present study suggest that 5HT, PGI₂, and to a lesser extent histamine may play a role in MC-dependent activation of meningeal nociceptors. In addition to nociceptor activation, we also show that these MC mediators can also promote mechanical sensitization of meningeal nociceptors. Among all of the MC mediators tested in this study 5HT was the most potent one, promoting both activation and mechanosensitization of both threshold and suprathreshold responses in both the A-delta and C-unit meningeal nociceptor populations at a micromolar range. Our findings also suggest that the MC-derived eicosanoids PGD₂ and LTC₄ do not affect the response properties of meningeal nociceptors, at least at the doses and time course tested in the current study. Given that activation of meningeal
nociceptors may contribute to the ongoing intracranial pain of migraine, and mechanical sensitization to its throbbing nature we propose that in conditions where MCs are activated during a migraine attack, 5HT, PGI₂ and histamine may play a role in promoting the intracranial headache of migraine.

Historically, histamine is considered the main effector molecule underlying MC-related inflammatory actions (Metcalfe et al., 1997). Clinical evidence suggests the involvement of histamine in migraine based on findings showing elevated histamine levels during migraine (Heatley et al., 1982; Haimart et al., 1987), the ability of histamine infusion to trigger migraine-like headache by activating the H1 receptor (Lassen et al., 1995), and the prophylactic effect of antihistamines in a subset of migraine patients (Rossi et al., 2003; Lewis et al., 2004; Togha et al., 2006).

Previous electrophysiological studies conducted in visceral tissues including testis (Koda et al., 1996), heart (Nishi et al., 1977), and airways (Matsumoto et al., 1992; Matsumoto et al., 1993; Riccio et al., 1996) have shown that histamine can excite polymodal nociceptors having both A-delta and C-unit CVs, likely through activation of the H1 receptor. The results of our studies, however suggest that histamine excites mainly C-unit meningeal nociceptors. Such preferential activation is reminiscent of the effect of MC degranulation on C-unit meningeal nociceptors (Levy et al., 2007). We found that histamine also preferentially promotes mechanical sensitization of C-units, findings which are in agreement with its effect on testicular nociceptors (Koda and Mizumura, 2002). Our study further showed that both the activation and sensitization mediated by histamine are short-lived suggesting that histamine alone may not be able to sustain the
prolonged activation of meningeal nociceptors evoked by MCs degranulation. Such a sustained effect therefore may require the action of histamine in combination with other MC mediators.

Although platelets are a major source of peripheral 5HT, significant quantities are also present in the granules of MCs, in both rodent and human (Metcalfe et al., 1997; Kushnir-Sukhov et al., 2007). Previous studies conducted in humans and rats have shown that direct administration of 5HT promotes mechanical hyperalgesia (Jensen et al., 1990b; Jensen et al., 1990a; Taiwo et al., 1992; Taiwo and Levine, 1992), an effect that is believed to be mediated by the activation and sensitization of nociceptors. However, previous electrophysiological studies have shown that although 5HT is capable of promoting relatively short duration excitation of high threshold A-delta and C nociceptive afferents innervating skin (Beck and Handwerker, 1974), knee joint (Herbert and Schmidt, 1992), jejunum (Brunsden and Grundy, 1999) and colon (Blackshaw and Grundy, 1993a; Coldwell et al., 2006) it does not promote mechanical sensitization (Blackshaw and Grundy, 1993a; Coldwell et al., 2006), unless the application of 5HT is preceded by local inflammation (Herbert and Schmidt, 1992; Coldwell et al., 2006). In our study 5HT, being the most potent of all the MC mediators tested, promoted both activation and mechanosensitization in both the C and A-delta meningeal nociceptor populations. Overall, 5HT was slightly more potent in the C-unit population, eliciting effects at a lower dose. The ability of 5HT to promote mechanical sensitization of meningeal nociceptors may reflect a different repertoire of 5-HT receptors on trigeminal nociceptors, in particular meningeal nociceptors, which could mediate mechanical sensitization. Alternatively, the induction of mechanical sensitization may be related to a
dural irritation induced by the craniotomy needed to expose the receptive field. Such irritation may also explain the presence of baseline spontaneous activity seen frequently in this preparation (Levy and Strassman, 2002a; Levy and Strassman, 2002b).

Activation of at least 4 types of 5-HT receptors, including 5-HT$_{1A}$ (Taiwo and Levine, 1992; Cardenas et al., 1997b), 5-HT$_{2A}$ (Wei et al., 2005; Sasaki et al., 2006), 5-HT$_3$ (Blackshaw and Grundy, 1993b; Zeitz et al., 2002) and 5-HT$_4$ (Cardenas et al., 1997a) were implicated in the peripheral nociceptive actions of 5-HT. It is however, unclear which receptor system mediate neuronal activation and which mechanosensitization. Activation of the 5HT$_{1A}$ and 5-HT$_4$ receptors that are coupled to activation of the cAMP-PKA signaling cascade has been suggested to promote sensitization by enhancing TTX-resistant Na$^+$ currents (Gold et al., 1996; Cardenas et al., 2001). We however, have shown that activation of this cascade promotes only mechanical sensitization in meningeal nociceptors without activation (Levy and Strassman, 2002a), suggesting that 5HT promotes excitation of meningeal nociceptors through a different receptor and/or signaling cascade. A recent study has shown that activation of the 5-HT3 receptor promotes an increase in firing rate in both A-delta and C-units (Zeitz et al., 2002). However, 5-HT3 agonist does not promote mechanical sensitization (Taiwo and Levine, 1992). Further studies are needed to examine which 5-HT receptors promote activation and sensitization in meningeal nociceptors.

We have shown recently that the migraine drug sumatriptan, an agonist at the 5HT$_{1B/D/F}$ receptors, can also activate and sensitize meningeal nociceptors at micromolar concentrations (Strassman and Levy, 2004; Burstein et al., 2005). Since activation of the 5HT$_{1B}$ receptor does not produce hyperalgesia (Taiwo and Levine, 1992) sumatriptan
may promote enhanced excitability of meningeal nociceptors via another excitatory 5-HT receptor. Activation of both the 5HT$_{1A}$ and 5HT$_7$ receptors, both of which are positively coupled to cAMP, and which sumatripan is capable of activating at a similar dose range (Schoeffter and Hoyer, 1989; Bard et al., 1993) may play a role in promoting mechanical sensitization.

Previous studies indicated that PGI$_2$, which can be secreted from MCs (Metcalf et al., 1997) is capable of promoting activation and sensitization of articular mechanonociceptors (Birrell et al., 1991; Birrell et al., 1993) but capable of only weakly activating visceral testicular nociceptors (Mizumura et al., 1991). Our results suggest that the PGI$_2$ sensitivity of meningeal nociceptors is similar to that of the knee joint with PGI$_2$-mediated activation and sensitization of both A-delta and C-units. Given that the prostacyclin receptor (IP) is primarily coupled to activation of adenylyl cyclase, increased cAMP may a play in the mechanical sensitization elicited by the PGI$_2$ analogue Iloprost (Pitchford and Levine, 1991; Nakae et al., 2005). However, the IP receptor may also be coupled to activation of phospholipase C (PLC) and increased intracellular calcium (Hayes et al., 1999; Lawler et al., 2001) which may play a role in eliciting meningeal nociceptor activation.

PGD$_2$, synthesized by the prostaglandin D2 synthase, is the major prostanoid secreted by MCs (Metcalf et al., 1997). Activation of visceral MCs has been shown to promote enhanced excitability of sensory vagal afferents, in part by promoting inhibition of a Ca$^{2+}$-dependent K$^+$ current underlying post-spike hyperpolarization (AHP$_{slow}$) which controls repetitive spike firing (Weinreich and Wonderlin, 1987; Greene et al., 1988;
Cordoba-Rodriguez et al., 1999). The ability of PGD₂ to promote inhibition of \( AHP_{\text{slow}} \) in vagal afferents (Greene et al., 1988; Cordoba-Rodriguez et al., 1999) suggests a potential role for this MC mediator in promoting sensitization of visceral sensory neurons. In our \textit{in vivo} preparation of meningeal nociceptors we found however that PGD₂ had no effect on the response properties of most meningeal nociceptors. While our results are in discrepancy to those found in vagal afferents they are in agreement with other electrophysiological studies showing no, or minimal effect of PGD₂ on sensory dorsal root ganglion neurons (Rueff and Dray, 1993; Bley et al., 1998; Hwang et al., 2000).

Although currently it is unknown whether nociceptive neurons expresses functional PGD₂ receptors (i.e DP₁, DP₂), the lack of increase in cAMP levels in dorsal root ganglion neurons following exposure to PGD₂ (Smith et al., 1998) suggests that dorsal root, and trigeminal ganglion nociceptors may not express such receptors.

Although PGD₂ may be devoid of direct action on most meningeal nociceptors it has been shown to promote a rapid increase in vascular permeability (Woodward et al., 1993; Nishimura et al., 2001) and vasodilatation of meningeal arterioles (Ellis et al., 1979), both of which have been suggested as putative mechanisms underlying vascular headaches such as that of migraine. Given that topical application of PGD₂ for up to 1 hour did not affect the responsiveness or ongoing activity of meningeal nociceptors it is questionable whether such PGD₂-related inflammatory responses may promote headache.

Similar to PGD₂, the cysteinyi leukotriene LTC₄, another major MC-constituent has been shown to play a role in the excitatory action of MCs on vagal afferent neurons (Undem et al., 1993; Cordoba-Rodriguez et al., 1999) together with promoting plasma
extravasation in rat skin. (Morimoto et al., 1989). LTC₄ however, does not affect the mechanosensitivity of airway afferents (Riccio et al., 1996). In our current study we found that LTC₄ did not affect meningeal nociceptors even when applied at the highest dose and for up to 1 hour. Although there is evidence to suggest that leukotriene receptor modifiers, such as montelukast which blocks signal transduction through the leukotriene receptor CysLT1 (Funk, 2005) can serve as prophylactic migraine drugs (Sheftell et al., 2000) our results suggest that if LTC₄, or its metabolites play a role in migraine precipitation, their action may not be mediated by promoting the activation or sensitization of meningeal nociceptors.
References


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Footnotes:

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Legends for Figures:

**Figure 1.** Effects of histamine of the activity and mechanosensitivity of meningeal nociceptors. On the left is an example of the response of one C-unit to 1mM histamine. The top panels illustrate the neuron’s response to an electrical stimulus and the mechanical stimulation paradigm with the pressures applied to the neuron’s mechanical receptive field. The bottom panels indicate the responses of the neurons to mechanical stimulation at baseline, 15 min after histamine and 15 min after wash with SIF. The numbers in parentheses indicate mean spikes/sec for each of the stimuli. Note the short duration of the activation and sensitization induced by histamine. On the right, the median and interquartile range of the responses to threshold (TH) and suprathreshold (STH) mechanical stimuli and spontaneous activity (SA) of A-delta and C-unit meningeal nociceptors to increasing doses of histamine. * p<0.05, one-tailed Wilcoxon matched-pairs signed-ranks test compared to application of SIF.

**Figure 2:** Effects of 5HT on the activity and mechanosensitivity of meningeal nociceptors. On the left is shown the response of a C-unit that was sensitized following administration of 100μM 5HT to its dural receptive field. Note the increased responsiveness to mechanical stimulation at both the threshold and suprathreshold levels and the increase in spontaneous activity. Also note the persistence of the sensitization 45 minutes after wash with SIF. On the right, the median and interquartile range of the responses to mechanical stimuli and spontaneous activity level following local application of increasing doses of 5HT. Note the effect on both the A-delta and C-units.
* p<0.05, one-tailed Wilcoxon matched-pairs signed-ranks test compared to application of SIF.

**Figure 3:** Effects of PGI$_2$ on the activity and mechanosensitivity of meningeal nociceptors. On the left is shown the response of a C-unit that was sensitized by application of 100µM PGI$_2$ to its dural receptive field. Note that while threshold responses and spontaneous activity were increased, the response to higher pressure suprathreshold stimuli did not change. Also note that sensitization was still maintained 30 min during the last washing with SIF. On the right, the median and interquartile range of the responses to mechanical stimuli and spontaneous activity level following local application of increasing doses of PGI$_2$. Note the dose-dependent effect on the TH responses of the C-units and the lack of effect on the suprathreshold responses. * p<0.05, one-tailed Wilcoxon matched-pairs signed-ranks test compared to application of SIF.

**Figure 4:** Lack of effect of topical application of PGD$_2$ (left) and LTC$_4$ (right) to the receptive fields of A-delta and C-unit meningeal nociceptors. Note that even at the highest dose tested both agents did not change the mechanosensitivity of meningeal nociceptors or promote their activation.
Table 1. Properties of the A-delta and C-units meningeal nociceptors used in the current study. * p < 0.05 Mann-Whitney U-test between A-delta and C-units.

<table>
<thead>
<tr>
<th></th>
<th>CV (m/sec) Median (IQR, range)</th>
<th>Von Frey threshold (kPa) Median (IQR, range)</th>
<th>Baseline ongoing discharge (spikes/sec) Median (IQR, range)</th>
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</thead>
<tbody>
<tr>
<td>A-delta</td>
<td>33 3.13 (1.27, 1.56-4.17)</td>
<td>133 (81, 38–263)</td>
<td>0.1 (0.50, 0 - 1.15)</td>
</tr>
<tr>
<td>C-units</td>
<td>36 0.70 (0.39, 0.27 - 1.49)</td>
<td>133 (81, 63 - 372)</td>
<td>0.3 (0.62, 0 - 3.24) *</td>
</tr>
</tbody>
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Figure 3

This figure shows the neuronal responses of C-units (0.66 m/sec) over time with different pressure levels. The C-units were tested under three conditions: Baseline, 15 min PGI₂, and 30 min wash. The responses are measured in spikes/sec and are compared to TH and STH conditions. The diagram also includes bar charts showing the neuronal responses at different PGI₂ dose concentrations from 0 to 100 μM.