

Sensitization of meningeal afferents to locomotion-related meningeal deformations in a migraine model

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Andrew S Blaeser, Jun Zhao, Arthur U Sugden, Simone Carneiro-Nascimento, Mark L Andermann , Dan Levy 

Department of Anesthesia, Critical Care and Pain Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, 02215, USA • Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, 02215, USA • Department of Neurobiology, Harvard Medical School, Boston, MA, 02115, USA

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Abstract

Migraine headache is hypothesized to involve the activation and sensitization of trigeminal sensory afferents that innervate the cranial meninges. To better understand migraine pathophysiology and improve clinical translation, we used two-photon calcium imaging via a closed cranial window in awake mice to investigate changes in the responses of meningeal afferent fibers using a preclinical model of migraine involving cortical spreading depolarization (CSD). A single CSD episode caused a seconds-long wave of calcium activation that propagated across afferents and along the length of individual afferents. Surprisingly, unlike previous studies in anesthetized animals with exposed meninges, only a very small afferent population was persistently activated in our awake mouse preparation, questioning the relevance of this neuronal response to the onset of migraine pain. In contrast, we identified a larger subset of meningeal afferents that developed augmented responses to acute three-dimensional meningeal deformations that occur in response to locomotion bouts. We observed increased responsiveness in a subset of afferents that were already somewhat sensitive to meningeal deformation before CSD. Furthermore, another subset of previously insensitive afferents also became sensitive to meningeal deformation following CSD. Our data provides new insights into the mechanisms underlying migraine, including the emergence of enhanced meningeal afferent responses to movement-related meningeal deformations as a potential neural substrate underlying the worsening of migraine headache during physical activity.

eLife assessment

This **fundamental** study explored the impact of migraine-related cortical spreading depression (CSD) on the firing of nerves innervating the coverings of the brain that are considered the putative source of migraine-related pain. Using **convincing** approaches they show that these responses are altered in response to mechanical deformation of the brain coverings. Given that migraine is characterized by worsening head pain in response to movement, the findings offer a potential mechanism that may explain this clinical phenomenon.

Introduction

A large body of evidence supports the notion that migraine headache involves the trigeminal meningeal sensory system (Ashina et al., 2019 [↗](#); Levy and Moskowitz, 2023 [↗](#)). Persistent discharge of meningeal afferents is thought to mediate the ongoing headache, while their augmented mechanosensitivity has been suggested to underlie migraine headache exacerbation during normally innocuous physical activities that cause transient intracranial hypertension, such as coughing and other types of straining (Blau and Dexter, 1981 [↗](#)). Current understanding of migraine-related responses of meningeal afferents is largely based on animal models. For example, triggering an episode of cortical spreading depolarization (CSD), a self-propagating wave of neuronal and glial depolarizations thought to mediate migraine aura, causes persistent activation and mechanical sensitization of meningeal afferents (Zhao and Levy, 2015 [↗](#), 2016 [↗](#)).

Despite the preclinical evidence implicating enhanced responsiveness of meningeal afferents as a driver of migraine headache (Levy and Moskowitz, 2023 [↗](#)), these studies have almost all used acute invasive experiments involving electrophysiological recordings in anesthetized animals with surgically exposed and mildly inflamed meninges (Levy et al., 2007 [↗](#)). Moreover, studies documenting the mechanical sensitization of meningeal afferents were based on findings of increased responsiveness to artificial compressive forces applied to the dura of a depressurized brain. Hence, there is a significant gap in our understanding of whether and how meningeal afferents respond to migraine-related events under more naturalistic conditions in behaving animals with an intact and pressurized intracranial space.

To better understand migraine pathophysiology and improve clinical translation, we leveraged a newly developed approach for two-photon calcium imaging of meningeal afferent responses within the closed intracranial space of an awake-behaving mouse (Blaeser et al., 2022 [↗](#)) in the CSD model of migraine. We imaged changes in afferent ongoing activity and afferent responses to three-dimensional meningeal deformation associated with locomotion (Blaeser et al., 2022 [↗](#)) following the triggering of a single CSD episode. Our data provides new insights into the mechanisms underlying migraine pathophysiology, including acute calcium signaling in meningeal afferent fibers as a potentially critical nociceptive factor contributing to migraine pain and the emergence of enhanced meningeal afferent responses to movement-related meningeal deformations as the neural substrate underlying the worsening of migraine headache during physical activity.

Results

Propagating calcium activity across afferent fibers during CSD

To investigate meningeal afferent responses to CSD, we performed two-photon calcium imaging of GCaMP6s-expressing trigeminal afferent fibers innervating the meninges above the visual cortex ($n = 325$ fibers from 9 fields of view [FOV] from 7 mice, **Figure 1A** [↗](#)). We triggered a single CSD episode in the frontal cortex with a cortical pinprick. In every experiment (9 CSDs in 7 mice), we detected a slow, CSD-like wave of calcium activity in numerous meningeal afferent fibers within one minute following the pinprick (**Figure 1B** [↗](#) and **Supplementary Movie S1**) as well as in background regions (likely reflecting signal from small, out-of-focus afferent branches). These calcium waves proceeded from the pinprick site in an anterior-to-posterior direction across the FOV (**Figure 1C** [↗](#)). We also observed progressive activation of portions of individual afferent fibers aligned to the wave's movement direction. To characterize this phenomenon, we focused on sets of regions of interest (ROIs) belonging to the same long afferent fiber oriented along the

direction of the calcium wave (**Figure 1D** [↗](#), **Supplementary Movie 1**). Compared to baseline afferent calcium signals observed during periods of locomotion, during which all ROIs belonging to an afferent were activated near-simultaneously, as reported previously (Blaeser et al., 2022 [↗](#)), the sequential recruitment of ROIs along a fiber during the CSD-like wave was much slower (**Figure 1E-G** [↗](#)). The proportion of afferents activated during this period exceeded the proportion activated during locomotion bouts (**Figure 1I** [↗](#)). The magnitude of activation was also larger (**Figure 1J** [↗](#)).

Acute afferent activation is not related to CSD-evoked meningeal deformation

CSD gives rise to acute neuronal and glial swelling and shrinkage of the cortical extracellular space (Mazel et al., 2002 [↗](#); Takano et al., 2007 [↗](#); Rosic et al., 2019 [↗](#)). Such cortical mechanical perturbations could lead to acute meningeal deformation, which we have shown previously can activate mechanosensitive meningeal afferents (Blaeser et al., 2022 [↗](#)). We postulated that if CSD leads to meningeal deformations, these deformations could drive the acute afferent response during the CSD wave. Assessment of meningeal deformation parameters (see Methods) revealed severe meningeal scaling and shearing during the CSD-evoked afferent calcium wave (6 CSDs in 6 mice, **Figure S2A-D** [↗](#)). In some experiments, the temporal pattern of meningeal shearing (**Figure S2C** [↗](#)) somewhat resembled that of the acute afferent response. However, the pattern of meningeal scaling (**Figure S2B** [↗](#)) was incongruent with that of the acute afferent response. Surprisingly, the direction of Z-shifts in the meninges during this epoch was inconsistent, resulting in no significant Z-shift on average relative to the pre-CSD epoch (**Figure S2D** [↗](#)). To estimate the relative contribution of the CSD-driven meningeal deformation to the acute afferent responses we observed, we used a general linear model (GLM; see (Blaeser et al., 2022 [↗](#)) and Methods). We focused on afferents whose pre-CSD (baseline) activity could be predicted by the GLM based on deformation predictors (n=145 afferents from 6 mice). We then plugged the peri-CSD deformation data into these GLMs to generate predictions of peri-CSD afferent activity and compared them to the observed activity. Overall, we observed a poor match between the real peri-CSD calcium signals and those predicted by the GLMs trained using pre-CSD deformation data (see example in **Figure S2E** [↗](#) and summary GLM fits in **Figure S2F** [↗](#)), suggesting that the model poorly predicted the magnitude of the activity and/or its temporal pattern in response to CSD. Because ~95% of the afferents were acutely activated by CSD (**Figure 1I** [↗](#)), we propose that this response is mostly driven by other non-mechanical factors, such as the local depolarizing effects of diffusible excitatory molecules.

A minority of afferents exhibit prolonged activation or suppression after CSD

In anesthetized rats with exposed meninges, CSD drives sustained increases in ongoing activity lasting tens of minutes in ~50% of meningeal afferents (Zhao and Levy, 2015 [↗](#)). To directly assess CSD-related changes in afferent ongoing activity in awake mice with intact meninges, we focused on afferent responses during epochs of immobility between locomotion bouts (8 CSDs in 7 mice). We observed low levels of ongoing activity at baseline before CSD (fluorescent events occurring $6.9 \pm 0.3\%$ of the time), consistent with our previous study in naïve mice (Blaeser et al., 2022 [↗](#)). Unexpectedly, most afferents (~70%, 201/288) did not display any change in ongoing activity during the 2 hours following CSD (termed “post-CSD”). However, we identified sustained increases in ongoing activity in ~10% (30/288) of the afferents during this period. Surprisingly, we also observed a larger afferent population (~20%; 57/288) whose activity was suppressed (**Figure 2A-C** [↗](#)). Afferents with sustained activation showed increases in ongoing activity that emerged at a ~25 min delay on average (**Figure 2D** [↗](#)). In contrast, afferents with sustained suppression showed

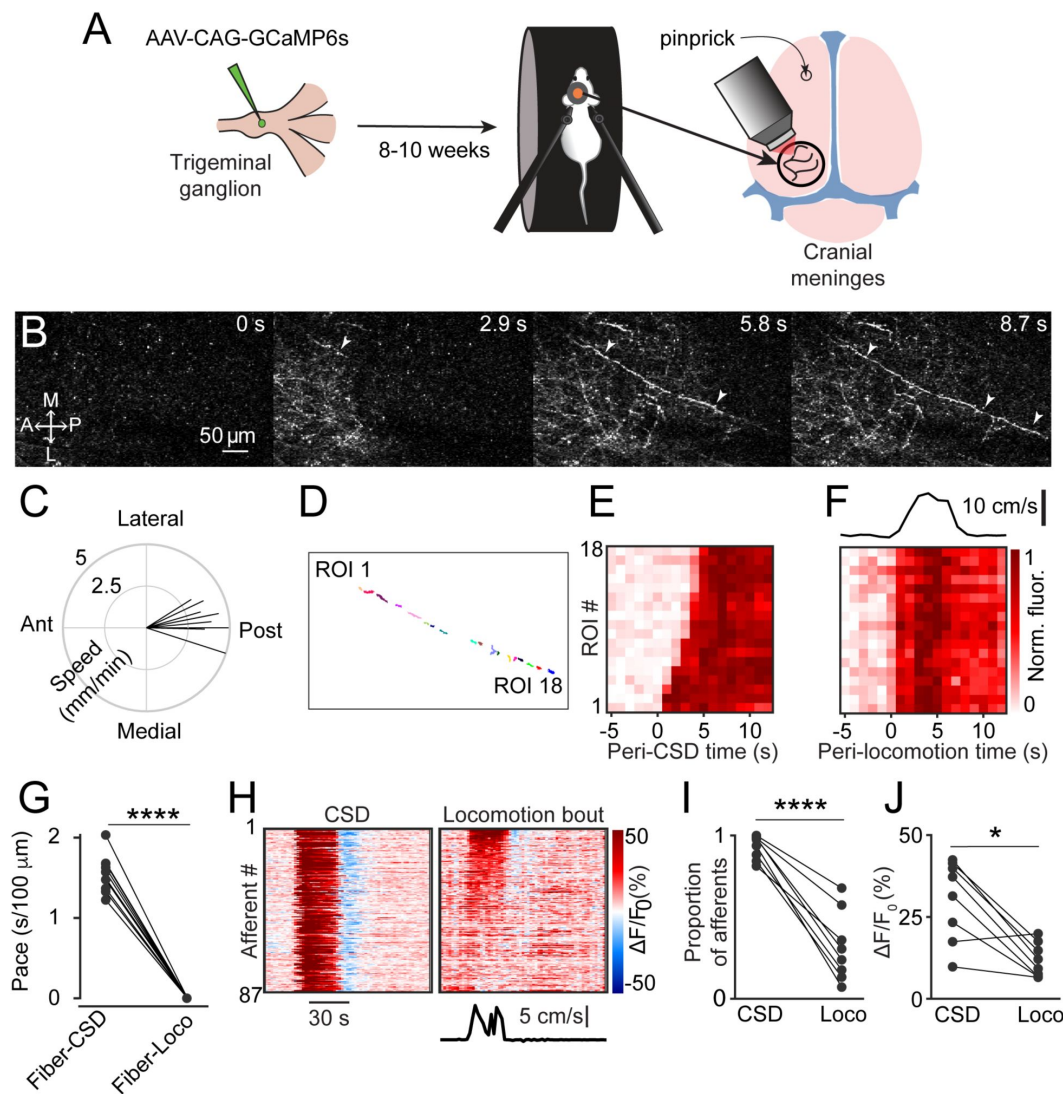


Figure 1.

CSD drives wave-like calcium activity in meningeal afferents

(A) Mice received a trigeminal ganglion injection of an AAV to express GCaMP6s in trigeminal meningeal afferents. After 8-10 weeks, following the implantation of a headpost and a cranial window, mice were habituated to head restraint and subjected to two-photon calcium imaging while head-fixed on a running wheel to study the effect of pinprick-triggered CSD on the activity of meningeal afferents. (B) Example of a CSD-associated meningeal calcium wave that spreads across the field of view, with local segments of long afferent fibers becoming sequentially activated as the wave progresses (arrowheads). M: medial, L: lateral, A: anterior, P: posterior. (C) Summary of speed and direction of CSD-associated meningeal calcium waves, typically from anterior ('Ant.') (closer to where CSD was triggered anterior to the cranial window) to more posterior locations ('Post.'). Speed estimates were obtained using the analysis method described in [Figure S1A](#). On average, the wave progressed at 3.8 ± 0.2 mm/min. (D) Map of 18 ROIs belonging to a single meningeal afferent fiber visible in B. (E) Activity heatmap of the afferent ROIs indicated in D illustrates progressive activation in response to CSD. (F) In contrast, the same afferent ROIs became activated simultaneously during a locomotion bout. Top trace depicts locomotion speed. (G) The pace of the CSD-associated afferent calcium wave was much slower than the spread of activity along the same afferent fibers during locomotion-evoked activity pre-CSD (**** $P < 0.0001$, paired, two-tailed t-test). (H) Example heatmaps of afferent activity observed during CSD showing different timecourse and magnitudes when compared to the activity observed during a locomotion bout. Bottom trace depicts locomotion speed. (I) Comparisons across all FOVs indicate a higher proportion of afferents exhibiting acute activation during the CSD vs. during locomotion (**** $P < 0.0001$, iterated bootstrap). (J) A higher proportion of afferents also displayed increased magnitudes of activation (* $P < 0.05$, paired, two-tailed t-test). See also Movie S1 and [supplementary Fig. 1B](#).

decreases in ongoing activity beginning shortly after the passage of the acute calcium wave (**Figure 2D** [↗](#)). The durations of the afferent activation or suppression were similar, lasting ~25 min on average (**Figure 2E** [↗](#)).

CSD augments afferent responsiveness associated with meningeal deformations

Meningeal deformation associated with locomotion bouts can lead to the activation of mechanosensitive meningeal afferents (Blaeser et al., 2022 [↗](#)). We wondered whether, following CSD, afferent responses to a given level of mechanical deformation would be enhanced (i.e., sensitization). If so, this could explain the exacerbation of migraine headaches during physical activity. CSD suppresses cortical activity leading to decreased motor function (Houben et al., 2017 [↗](#)), including reduced locomotion in head-fixed mice (Enger et al., 2017 [↗](#)). CSD-related vascular changes and reduced extracellular space (Mazel et al., 2002 [↗](#); Takano et al., 2007 [↗](#)) could also affect meningeal deformations and the associated afferent response. Hence, we first analyzed the effect of CSD on wheel running activity and the associated meningeal deformation. In most sessions, mice stopped locomoting following the CSD (8/9 CSDs in 7 mice) but resumed their wheel-running activity ~6 min later on average (range 0.5-16.5 min). However, the duration of time that the mice spent running during the 2 hours post-CSD was overall reduced compared to the baseline period (**Figure 3B** [↗](#)). Locomotion bout analysis also revealed an overall reduction in bout rate during the post-CSD period (**Figure 3C** [↗](#)). Remarkably, despite the reduction in locomotion following CSD, we observed similar bout characteristics at baseline and post-CSD, including bout duration (**Figure 3D** [↗](#)) and peak velocity (**Figure 3E** [↗](#)). Given that CSD had minimal effect on locomotion bout characteristics, we next examined its effect on meningeal deformations. Surprisingly, CSD did not affect bout-related meningeal deformations: we observed similar scaling, shearing, and Z-shift values during the 2 hours post-CSD (**Figure 3F-H** [↗](#)).

Having shown that locomotion and the related meningeal deformations are not altered during the two hours following CSD, we next compared afferent responses during locomotion bouts before and after CSD. Initial observations of afferent activation during locomotion indicated enhanced responsiveness following CSD (**Figure 4A** [↗](#)). To systematically investigate this augmented afferent response, we used GLMs (see Methods and (Blaeser et al., 2022 [↗](#))) to assess whether meningeal afferent activity becomes sensitized to the state of locomotion and/or to various aspects of meningeal deformation following CSD. We modeled each afferent's activity based on variables that describe (i) the binary state of locomotion, (ii) mouse velocity, or (iii) aspects of meningeal deformation, including scaling, shearing, and Z-shift.

We first focused on afferents whose activity could be predicted by the same variables both at baseline and following CSD (i.e., afferents that exhibited sensitivities to locomotion and/or deformation signals both before and after CSD, $n = 67/325$ afferents, 9 CSDs in 7 mice). Higher GLM coefficients for a given variable post-CSD indicate greater afferent response during an equal expression level of that variable. Thus, we defined an afferent as sensitized by CSD if its GLM coefficients post-CSD were larger than at baseline (i.e., stronger activation of afferents per unit deformation or locomotion; for example, see **Figure 4C** [↗](#)). Using these criteria, we identified elevated locomotion and/or deformation-related activity (i.e., sensitization) post-CSD in ~51% of afferents (34/67; **Figure 4D** [↗](#)). In contrast, only 12% of afferents (8/67) showed reduced locomotion- and deformation-related activity (i.e., desensitization) post-CSD. Sensitivity was unchanged in the remaining 37% of afferents (25/67).

Meningeal afferent sensitization following CSD may reflect increased sensitivity to mechanical deformation and/or to other physiological processes that occur in response to locomotion (Blaeser et al., 2022 [↗](#)). Because locomotion and meningeal deformations are partially correlated (Blaeser et al., 2022 [↗](#)), we next estimated their relative contributions to the augmented afferent

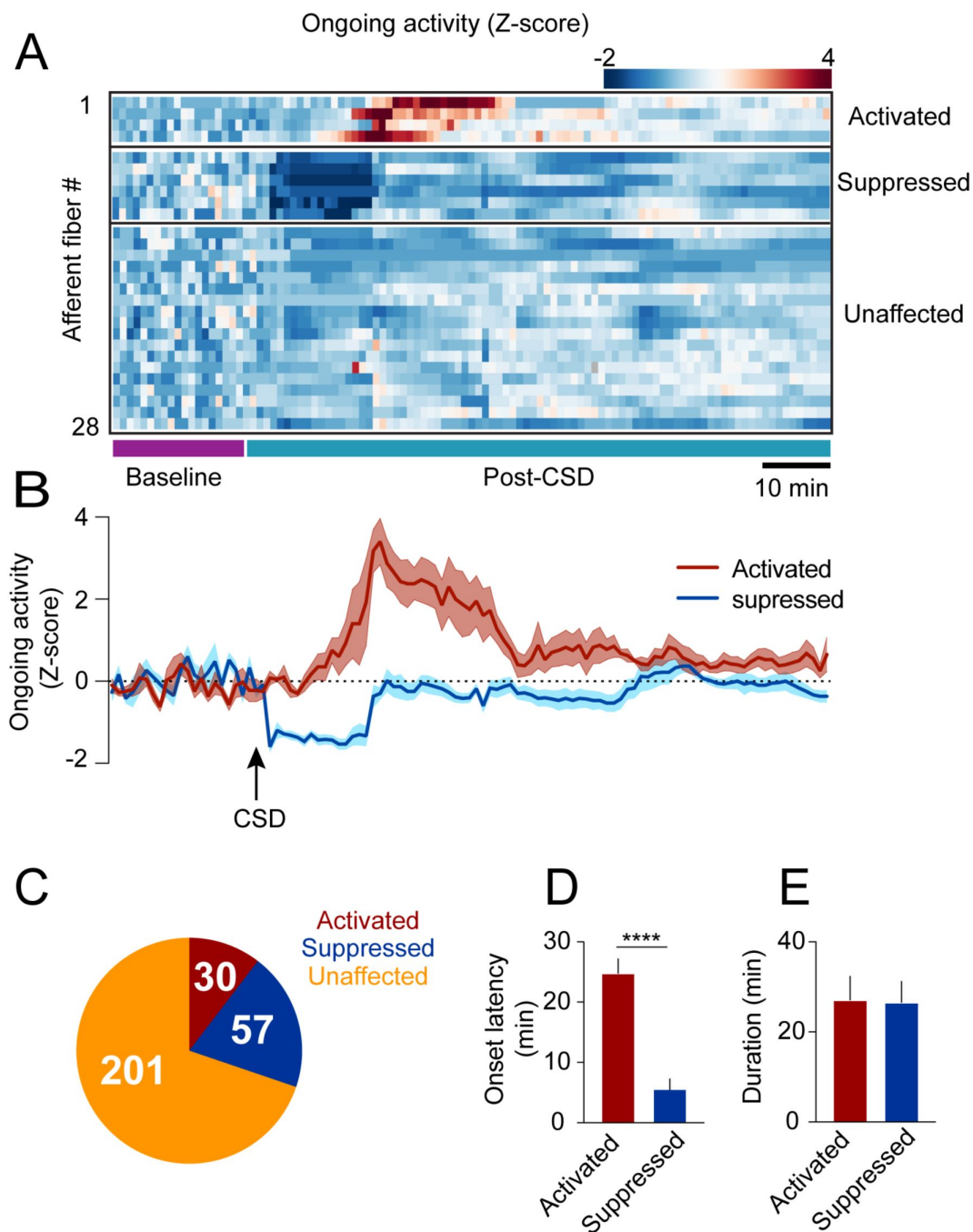


Figure 2.

CSD-related persistent changes in the ongoing activity of meningeal afferents

(A) Example heatmap of standardized ongoing activity (fraction of time afferents exhibited calcium events when the mouse is not locomoting) for all afferent fibers from a single FOV during baseline and up to 120 min following CSD (termed “post-CSD”). Data shows concatenated 1 min bins of activity. Afferents were either activated, suppressed, or unaffected by CSD. Note the delayed activation and immediate suppression in two small subsets of fibers. **(B)** Mean activity time course of the activated and suppressed afferents from the same population depicted in A. **(C)** Pie chart depicting the breakdown of the afferent subpopulations based on their change in ongoing activity following CSD. Most afferents were not affected (orange), while two smaller populations either exhibited prolonged activation (maroon) or suppression (blue) of ongoing activity following CSD (8 events in 7 mice). **(D)** Afferents exhibiting prolonged activation had a longer onset latency than those exhibiting suppression (**** $P < 0.0001$, Mann-Whitney t-test. Error bars: SEM). **(E)** The duration of increases in ongoing activity and suppressions in activity were similar ($P = 0.97$, two-tailed t-test. Error bars: SEM).

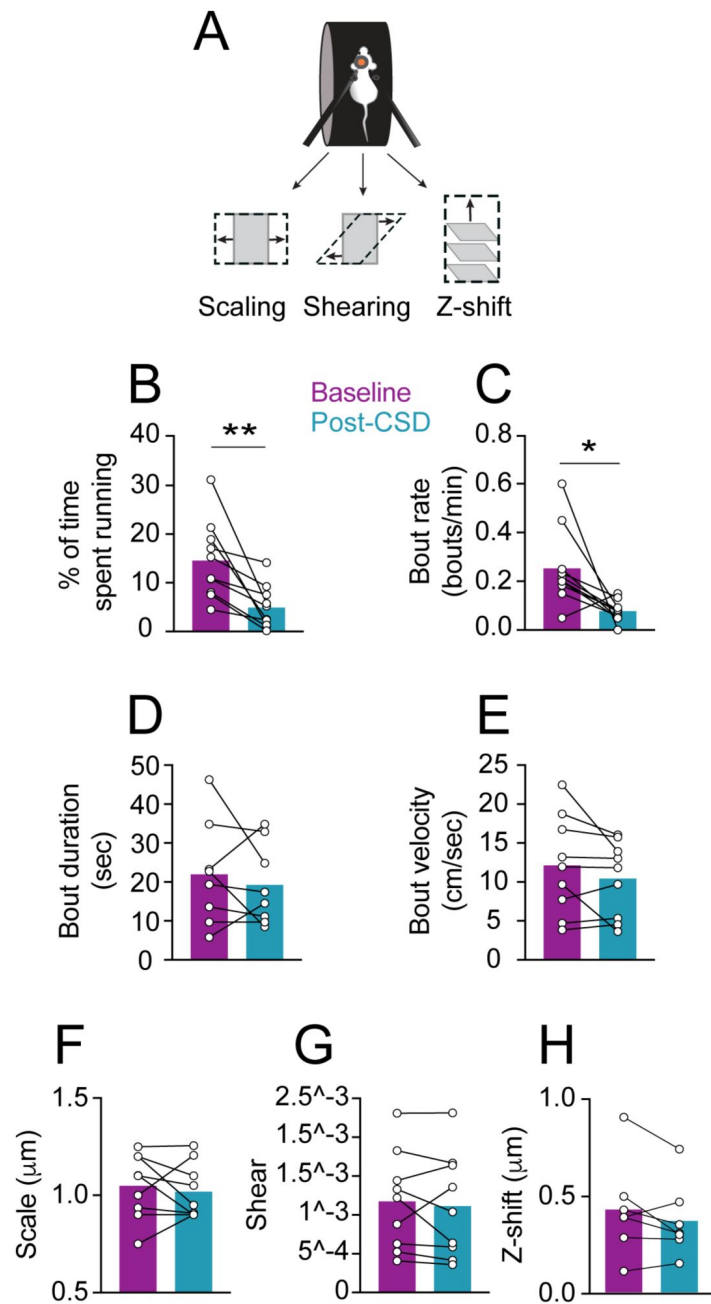


Figure 3.

Locomotion and related meningeal deformations changes post CSD

(A) In head-fixed mice, wheel running is associated with meningeal scaling, shearing, and positive Z-shift (i.e., meningeal movement toward the skull). **(B)** When compared to the baseline period, there was an overall reduction in the time mice spent running during the 2-hour post-CSD observation period (** $P < 0.01$, paired t-test, 9 CSDs in 7 mice). **(C)** CSD also led to decreased locomotion bout rate (* $P < 0.05$, Wilcoxon, signed-rank test). **(D, E)** CSD, however, did not affect bout duration ($P = 0.50$, paired t-test) or bout peak velocity ($P = 0.18$, paired t-test). **(F, G, H)** CSD also did not affect subsequent locomotion bout-evoked meningeal scaling, shearing, or Z-shift ($P = 0.56$; $P = 0.55$, $P = 0.18$, paired t-tests, respectively, 9 CSDs, in 7 mice for scale and shear, 7 CSD in 7 mice for Z-shift). Bars depict the mean.

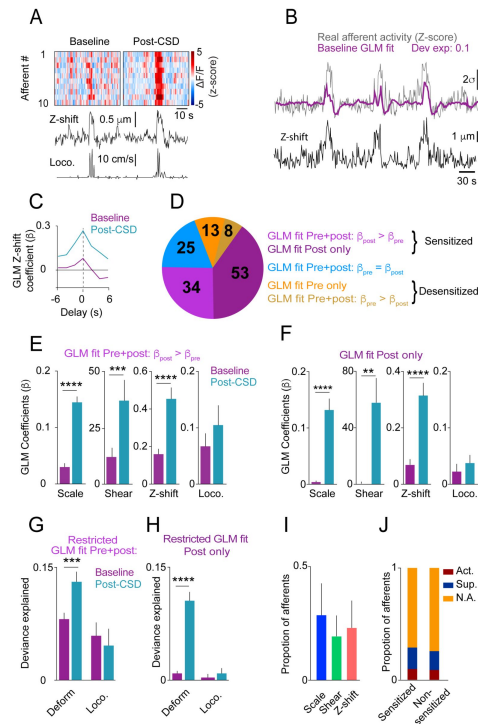


Figure 4.

CSD leads to sensitization of meningeal afferents to local deformation signals

(A) Example of meningeal afferent sensitization following CSD. Locomotion and its related Z-shift (bottom traces) are comparable before (left) and after (right) CSD, but afferent fibers exhibit greater responses associated with the Z-shift after CSD (heatmaps, top panels). (B) Example GLM fit of afferent activity in response to Z-shift before CSD. A raw calcium activity trace recorded pre-CSD (gray traces, z-scored; σ : 1 standard deviation) is plotted along with the model fit (purple). The deviance explained ('dev exp') is a metric of GLM fit quality and is above the threshold (0.05) for classifying an afferent's activity as reasonably well-fit by the GLM. The activity of this example afferent could not be predicted by other deformation or locomotion variables (not shown), suggesting unique sensitivity to Z-shift (C) GLM β coefficients used as a metric of the coupling between the Z-shift and the activity of the example afferent shown in B across different delays. A maximal coefficient at zero delay indicates the alignment of activity with Z-shifts. Note the greater afferent activation per unit Z-shift after CSD relative to baseline, indicative of an augmented or sensitized response. (D) Pie chart indicating the numbers and distribution of all afferents that were well-fit by deformation and/or locomotion signals either before and/or after CSD. Afferents categorized as sensitized if they (i) had significant GLM fits both pre- and post-CSD and higher coefficients for a given deformation and/or locomotion predictor post-CSD (purple) or (ii) were well-fit only post-CSD (magenta). Two small subsets of afferents categorized as desensitized had worse GLM fits post-CSD (mustard) or were no longer well-fit post-CSD (orange). The incidence of afferent sensitization exceeded that of desensitization ($P < 0.001$, χ^2 test). (E, F) Comparisons of pre- and post-CSD GLM coefficients for each of the deformation and locomotion predictors. Data are shown for sensitized afferents with well-fit models pre- and post-CSD (corresponding to the purple population in D) and for afferents with well-fit models only post-CSD (i.e., silent pre-CSD, corresponding to the magenta population in D). Mouse velocity coefficients were close to 0 in all cases and are not shown. In the two sensitized afferent populations, only coefficients related to deformation predictors increased post-CSD (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, Wilcoxon sign rank test with correction for multiple analyses). (G, H) The response bias of sensitized afferents to meningeal deformation was further observed when comparing these GLMs to restricted GLMs that included only the group of deformation predictors or the group of locomotion predictors. The deviance explained by the deformation response component (estimated as the difference between the full GLM and the GLM lacking deformation variables) was significantly greater than for the locomotion response component in sensitized afferents that were well-fit pre- and post-CSD and for those that were well-fit only post-CSD (*** $P < 0.001$ and **** $P < 0.0001$, Wilcoxon test for G and H respectively). Bars depict mean; error bars indicate SEM. (I) Among the sensitized afferents with enhanced sensitivity to deformation variables, we observed a similar sensitization to scale, shear, and Z-shift variables. Bars depict mean; error bars indicate SEM. (J) There was no difference in the incidence of sensitized afferents among afferents that showed prolonged activation, prolonged suppression, or no change in ongoing activity post-CSD ($P = 0.9$, χ^2 test; cf. Figure 2).

responsiveness post-CSD by comparing, for each sensitized afferent, the GLM coefficients generated for baseline epochs and for post-CSD epochs. Surprisingly, we found that only the deformation coefficients were increased post-CSD (**Figure 4E**), suggesting that meningeal afferent sensitization following CSD reflects primarily an increased sensitivity to local mechanical deformations.

We next considered the possibility that sensitization is also manifested in the unmasking of responsiveness to locomotion and/or meningeal deformation in previously silent (i.e., insensitive) meningeal afferents (Levy and Strassman, 2002; Levy and Moskowitz, 2023). Indeed, among the 245 afferents that were not well-fit before CSD (i.e., insensitive), we detected a substantial population that developed a sensitivity to locomotion and deformation variables following CSD (53/245; i.e., afferents whose activity could be well-predicted by locomotion and deformation variables following CSD). By contrast, far fewer neurons (13/245) lost their sensitivity to these variables after CSD (i.e., afferents that were well fit before but not after CSD). Overall, the above finding show that four times more afferents displayed increased sensitivity than decreased sensitivity following CSD (87 vs. 21 afferents). This sensitized afferent population also displayed post-CSD increases in GLM coefficients related to deformation but not to locomotion (**Figure 4F**).

To further quantify the importance of the locomotion and deformation variables to the afferent sensitization, we estimated their relative contributions to the overall ability of the models to predict the afferent activity pre- vs. post-CSD. To this end, we calculated the difference in model fit using the full model with all variables or models lacking either the set of deformation variables or locomotion variables. We found that the impact of deformation variables on the model fit was greater post-CSD than pre-CSD, while the impact of locomotion variables was similar pre- and post-CSD. This was true for afferents with models that were well fit both at baseline and following CSD (**Figure 4G**; purple subset in **Figure 4D**) and for those with models that were well fit only post-CSD (**Figure 4H**; magenta subset in **Figure 4D**). Further analysis revealed that scale, shear, and Z-shift deformations were, on average, equally important in predicting the activity patterns of sensitized afferents (**Figure 4I**). Taken together, these data suggest that the afferent sensitization following CSD primarily involves increased afferent responsiveness to a mix of meningeal deformation variables rather than to other locomotion-associated processes.

Previous studies in anesthetized rats suggested that the mechanisms underlying meningeal afferent mechanical sensitization are independent of those responsible for increased ongoing discharge in several migraine models, including CSD (Levy and Strassman, 2002; Zhang et al., 2011; Zhang et al., 2013; Zhao and Levy, 2018; Zhao et al., 2021). Here, using the CSD model in awake mice, we also found no association between sensitization and sustained changes in ongoing activity, as similar proportions of sensitized and non-sensitized afferents were activated, suppressed, or did not display any change in their ongoing activity following CSD (**Figure 4H**).

Discussion

Prior studies suggested that CSD drives meningeal nociception that can lead to the headache phase in migraine with aura. These studies, which mostly involved invasive experiments in anesthetized rats with surgically exposed meninges, showed prolonged activation and mechanical sensitization of meningeal afferents (Carneiro-Nascimento and Levy, 2022). To better understand migraine pathophysiology and improve clinical translation, we used two-photon calcium imaging to characterize, for the first time, CSD-related changes in the responsiveness of individual meningeal sensory afferents at the level of their peripheral nerve fibers in the closed cranium of a behaving mouse. We show that a single CSD episode drives a wave of calcium activity across most afferents while producing a more prolonged change in ongoing activity in only a small subset. We then

combined afferent calcium imaging with behavioral tracking of locomotion and estimates of local meningeal deformations. This approach revealed that CSD causes prolonged augmentation of afferent responsiveness to meningeal deformations associated with locomotion in previously sensitive afferents and emergent mechanical responses in previously silent afferents. These data support the notion that enhanced responsiveness of meningeal afferents to local meningeal deformation is the neural substrate for headache pain associated with physical activity following migraine onset.

The current study represents the first characterization of a CSD-associated calcium wave across meningeal afferent fibers and along the length of individual fibers. A rise in intracellular calcium detected by the GCaMP sensor normally indicates an action potential-evoked calcium influx (Chen et al., 2013 [DOI](#)). In contrast, the seconds-long wave of calcium elevation along individual afferent fibers is incongruent with the generation of action potentials. It may instead be related to subthreshold depolarizations (Li et al., 2022 [DOI](#)) and the opening of voltage-gated calcium channels (Awatramani et al., 2005 [DOI](#)). Our data thus support the notion that meningeal afferents can generate spatially localized and subthreshold yet robust calcium transients during CSD. In turn, increased intracellular calcium could drive the release of sensory neuropeptides, such as CGRP, that can promote a local neurogenic inflammatory response linked to migraine pain (Akerman et al., 2003 [DOI](#); Amrutkar et al., 2011 [DOI](#); Levy and Moskowitz, 2023 [DOI](#)).

The mechanisms underlying the acute CSD-related afferent calcium wave remain unclear. We observed short-lasting meningeal deformation in response to CSD, likely due to the swelling of cortical cells and decreased extracellular space. However, this deformation was not associated with the acute afferent response, and thus unlikely to be its primary driver. A mechanism involving local depolarizing effects of diffusible excitatory molecules, such as potassium ions, whose cortical levels show a wave of elevation coincident with the propagation of the CSD wave (Suryavanshi et al., 2022 [DOI](#)) is more likely to play a role. Since we also observed instantaneous elevation in calcium activity across subregions of individual afferent fibers oriented perpendicular to the calcium wave, we cannot exclude the possibility that some afferents also signal via action potentials during the passage of the CSD wave.

Electrophysiological recordings in anesthetized rats previously demonstrated prolonged elevations in spiking in ~50% of the trigeminal somata of meningeal nociceptive afferents following CSD. In most recordings, increased activity emerged after a ~10 min delay and persisted for nearly an hour following CSD (Zhao and Levy, 2015 [DOI](#)). In the awake mouse, the propensity for these prolonged afferent responses was considerably smaller and their duration was notably shorter, raising questions about the relevance of this response to migraine pain. While species differences and the effects of anesthesia in the rat studies might play a role, meningeal irritation due to the acute large craniotomies used in previous electrophysiological recording studies could be a major contributing factor. Craniotomy increases meningeal permeability (Roth et al., 2014 [DOI](#); Zhao et al., 2017 [DOI](#)), which could facilitate the transfer of algescic signals from the cortex. An acute craniotomy also leads to a meningeal inflammatory response via the activation of local immune cells (Levy et al., 2007 [DOI](#)) and could prime meningeal afferents to develop prolonged activation following CSD. If such a priming mechanism occurs in susceptible individuals who suffer from migraines, it could facilitate the activation of meningeal afferents and drive the headache during a migraine attack. These processes are likely less prevalent when using our chronic cranial window approach, as we have previously shown that this approach is not associated with meningeal vascular and cortical inflammation (Goldey et al., 2014 [DOI](#); Blaeser et al., 2022 [DOI](#)). The very low level of ongoing activity (fluorescent events) we observed at baseline also suggests a lack of an inflammatory response and associated afferent priming before CSD.

Unexpectedly, a separate afferent population exhibited suppressed calcium activity that began immediately after the acute response and lasted for approximately 25 min. This pattern of afferent responses resembles the rapid loss of cortical activity in the wake of CSD. However, its faster

recovery points to a mechanism distinct from the pre- and post-synaptic changes responsible for the silencing of cortical activity (Sawant-Pokam et al., 2017 [DOI](#); Kucharz and Lauritzen, 2018 [DOI](#)). Whether CSD drives the local release of mediators capable of reducing the excitability and spiking dynamics in a subset of afferents will require further studies.

A major finding of this study is the post-CSD development of augmented meningeal afferent responsiveness to meningeal deformations associated with locomotion bouts in awake mice with closed and pressurized meninges. We previously found that the activity of most meningeal afferents around the time of locomotion is driven by mixed sensitivities to meningeal deformation signals and to the binary state of locomotion. However, the activity of about a third of the afferents is more closely associated with the moment-to-moment deformation than with the state of locomotion per se (Blaeser et al., 2022 [DOI](#)). Here, we demonstrate that the amplification of afferent responsiveness following CSD relates primarily to enhanced neural gain in response to meningeal deformation rather than to other physiological processes associated with locomotion, such as cerebral vasodilation and a minor increase in intracranial pressure (Gao and Drew, 2016 [DOI](#)). This increased sensitivity suggests that the afferent population sensitive to meningeal deformation has unique properties that render it most susceptible to becoming sensitized following CSD. Migraine headaches are often worsened by abrupt maneuvers, such as coughing and sneezing, which result in a larger increase in intracranial pressure compared to that during locomotion. Thus, our observation of heightened responsiveness to locomotion-related meningeal deformation may underestimate the increased afferent responsivity post-CSD during other behaviors, such as coughing.

Meningeal afferents responding to physiological meningeal deformations may constitute a population of low-threshold mechanoreceptor (LTMR) afferents (von Buchholtz et al., 2020 [DOI](#)) whose activation under normal conditions is unlikely to produce headache. Our finding that CSD can augment the mechanical sensitivity of these afferents suggests that they may also possess nociceptive properties. The discovery of a subset of cutaneous A-LTMR trigeminal afferents that also respond to noxious mechanical stimuli (von Buchholtz et al., 2021 [DOI](#)) supports this view. Our data further suggest that about half of all afferents deemed to be sensitized following CSD are likely higher-threshold mechanosensitive afferents, as they were not driven by meningeal deformations or locomotion at baseline. Accordingly, local inflammation, which occurs following CSD, could recruit “silent” meningeal nociceptive afferents to become functional mechanonociceptors (Strassman and Levy, 2006 [DOI](#)). Overall, we propose that the sensitization of silent nociceptors, as well as of afferents with basal responsiveness to acute meningeal deformations, could produce a state of intracranial mechanical allodynia that underlies the exacerbation of migraine headaches during physical exertion and associated meningeal deformations.

Materials and methods

Animals

All experimental procedures complied with the ARRIVE and were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. All experiments were conducted on adult (8-16 weeks old) C57BL/6J mice (5 males, 2 females, Jackson Laboratory). Mice were group housed with standard mouse chow and water provided *ad libitum* before viral injection (see below). Mice used for *in vivo* two-photon imaging were singly housed and provided a running wheel, a hut, and a chew bar.

Surgical procedures and CSD induction

All surgical procedures were performed in anesthetized mice (isoflurane in O₂; 3.5% for induction, 1.5% for maintenance). Animals were given Meloxicam SR (4 mg/kg s.c.) for post-surgical analgesia. For monitoring calcium activity in meningeal afferents, 2.0 µl of AAV2/5.CAG.GCaMP6s.WPRE.SV40 (titer: 1X10¹³; Addgene) was injected into the left trigeminal ganglion (TG) using the following stereotaxic coordinates: 1.5 mm lateral and 0.3–0.8 mm anterior to Bregma and 7.0–7.2 mm ventral to the dura at a lateral-to-medial tilt with an angle of 22.5° relative to the dorsal-ventral axis. We previously verified this approach by examining GCaMP6s expression in TG somata and meningeal afferent fibers 8 weeks after injection using immunohistochemistry (Blaeser et al., 2022 [DOI](#)). Mice used for *in vivo* two-photon imaging were instrumented with a titanium headpost and a 3 mm cranial window (Goldey et al., 2014 [DOI](#); Blaeser et al., 2022 [DOI](#)) covering the posterior cortex (window centered roughly 1.5 mm lateral and 2 mm posterior to Bregma over the left hemisphere) 6–8 weeks after AAV injection. For CSD induction, a burr hole (~0.5 mm diameter) was drilled 1.5 mm anterior to the edge of the window until the brain's surface was barely visible. The burr hole was then plugged using a silicone elastomer (Kwik-Cast, WPI), and the mouse was allowed to recover. To trigger a single CSD episode, a glass micropipette (50 µm diameter) was briefly inserted through the silicon plug ~1 mm deep into the cortex for 2 seconds (Zhao and Levy, 2016 [DOI](#)).

Running wheel habituation

After at least one week of recovery following cranial window implantation, mice were head-fixed on a 3D-printed running wheel for gradual habituation (10 minutes to 1 hour over 3–4 days). To minimize downward forces on the bone and meninges produced while the mouse pushed upwards against the headpost, the running wheel was mounted on a cantilever (Ramesh et al., 2018 [DOI](#); Blaeser et al., 2022 [DOI](#)). The position of the headpost, anterior to where the mouse's paws touch the wheel, also made it hard for the mouse to push straight up and apply forces to the skull. The strong cement used to bind all skull plates and headpost together (Goldey et al., 2014 [DOI](#)) further mitigated any movement-induced strain on the skull that might affect the underlying meninges. Mice displaying signs of stress were immediately removed from head fixation, and additional habituation days were added until mice tolerated head fixation without visible signs of stress. Mice received a high-calorie liquid meal replacement (Ensure) via a syringe as part of the habituation process.

Two-photon imaging

Calcium imaging was performed as recently described (Blaeser et al., 2022 [DOI](#)) while mice were head-fixed on the running wheel. We used a Nikon 16X, 0.8 NA water immersion objective on a resonant-scanning two-photon microscope (NeuroLabware) and a MaiTai DeepSee laser, set to 920 nm with 25–40 mW power for GCaMP6s visualization. Digital zoom was set at 2.4X (626×423 µm² FOVs). In 7 experiments, we imaged a 60 mm volume (3D) using an electrically tunable lens (Optotune) at 1.03/s. We employed volumetric imaging for three main reasons: 1) To capture the activity of afferents throughout the meningeal volume. In our volumetric imaging approach, including in this work, we observed afferent calcium signals throughout the meningeal thickness (see Figure 5 in Blaeser et al. 2022 [DOI](#)). However, the majority of afferents were localized to the most superficial 20 microns (Figure S1E [DOI](#) in Blaeser et al. 2022 [DOI](#)), suggesting that we mostly recorded the activity of dural afferents; 2) to enable simultaneous quantification of three-dimensional deformations and the activity of afferents throughout the thickness of the meninges. This allowed us to determine whether changes in mechanosensitivity could involve augmented activity to intracranial mechanical forces that produced meningeal deformation along the Z-axis of the meninges (e.g., increased intracranial pressure); 3) to provide a direct means to confirm that the afferent GCaMP fluorescent changes we observed were not due to artifacts related to meningeal motion along the Z-axis. In two experiments, due to technical issues, only single-plane (2D) data were collected at the cranial dura level at 15.5 Hz. In every experiment, we conducted two imaging runs (30 min each) to collect baseline data, followed by four more 30-minute runs

post-CSD induction. In a subset of experiments in which the FOV included a visible large pial artery ($n = 4$), we verified the induction of a CSD by visualizing its vascular signature, including a brief vasoconstriction followed by dilation (Rosic et al., 2019 [DOI](#)) (**Supplementary Fig. 1A** [DOI](#)).

Locomotion signals

Wheel position during each imaging run was recorded using an Arduino Uno board at 15.5 Hz. The instantaneous velocity was calculated as the time derivative of this signal and was downsampled to match the sampling rate of volume scans. Locomotion state was determined using a two-state Hidden Markov Model. Locomotion bouts were defined as periods when the locomotion state was sustained for at least two seconds (Blaeser et al., 2022 [DOI](#)).

Image processing and calcium signal extraction

All image processing and analyses were performed in MATLAB 2020a and ImageJ (FIJI, NIH) as described previously (Shipley et al., 2020 [DOI](#); Blaeser et al., 2022 [DOI](#)). In brief, imaging movies were subjected to several preprocessing steps. For single-plane experiments, all imaging runs were concatenated into a single movie. A reference image was defined as the mean projection over the middle 50% of frames from the second run pre-CSD. Each frame of the concatenated movie was then affine registered to the reference using the TurboReg plugin for ImageJ. For volumetric imaging, we first corrected the lensing effect due to the electrically tunable lens. We next subjected the movies to rigid registration, using the discrete Fourier transform to correct for within-volume (2D) translations and translations along the z-axis. We next used z-interpolation to correct for z-translations of individual imaged planes within each volume. The last step involved affine registration using a reference volume formed from the mean projection of the middle 50% of frames from the second run.

Registered 2D movies were analyzed using a PCA/ICA package (Mukamel et al., 2009 [DOI](#)) to extract masks of pixels with correlated activity. Users screened each prospective region of interest (ROI) for quality of morphology and fluorescence signal. Afferent ROIs with < 50 pixels were rejected. For each ROI included in subsequent analyses, we generated a dilated mask extending 8-21 pixels from the outer edge of the ROI, excluding any pixels that belonged to another ROI. This “neuropil” mask was used for the subtraction of background signals. In volumetric imaging, we generated a mean projection over all planes containing afferents for each volume. Then we ran the resulting 2D movie through the PCA/ICA procedure, yielding an initial set of 2D masks representing putative 3D ROIs. An initial fluorescence trace extracted from each 2D mask was calculated by averaging fluorescence across all pixels in the mask. To identify which voxels in the original volumetric dataset contributed most strongly to each fluorescence trace, we calculated the Pearson correlation of this trace with the fluorescence timecourse of each voxel, resulting in a 3D volume of correlation values. These 3D correlation volumes were then screened manually for quality of morphology and signal. The surviving volumes were thresholded at the 75th percentile of correlation across all voxels to form putative 3D ROI masks.

Fluorescence signals

We calculated raw fluorescence signals at each time point for the i^{th} ROI (F_{ROI}^i) and its corresponding neuropil mask (F_{np}^i), as the simple arithmetic means of all pixels/voxels within each ROI mask. Next, we calculated $F^i = F_{\text{ROI}}^i - F_{\text{np}}^i + \langle F_{\text{np}}^i \rangle$, where brackets denote the mean across the entire recording. We then calculated the corresponding baseline signal F_0^i as the 10th percentile of a moving window for the last 32 seconds (Sugden et al., 2020 [DOI](#)). We then calculated the normalized, baseline-subtracted time series $\Delta F/F_0 = (F^i - F_0^i)/F_0^i$. This signal was standardized akin to a Z-score operation by subtracting the median value and dividing by the standard deviation (calculated during quiet wakefulness, an epoch with low levels of evoked activity). Fluorescence events were defined as periods where the signal consistently exceeded a value of 1 for at least one second and where peak fractional change in fluorescence ($\Delta F/F_0$) was at least 5%.

Identifying calcium activity in afferent fibers

To analyze calcium activity related to an afferent fiber, sets of ROIs putatively belonging to the same axon were initially identified using a previously described method (Liang et al., 2018; Blaeser et al., 2022). Briefly, we calculated the pairwise fluorescence event correlation between ROIs during quiet wakefulness, thresholding at 0.7 correlation. We then calculated the cosine dissimilarity between the full set of correlation coefficients for each pair of ROIs, which in turn was used to calculate the linkage between each pair. Finally, hierarchical clustering was performed using a cutoff value of 2. This procedure generated sets of ROIs that were mutually highly correlated. We then visually inspected each cluster and manually identified the subsets of ROIs that unambiguously covered the same specific afferent fiber without any branching. We used the mean activity of these ROI subsets to analyze each afferent's calcium activity.

CSD-associated calcium wave characterization

To detect the CSD-associated meningeal calcium wave (see **Supplementary Fig. 1B**), we first calculated the mean fluorescence signal over all voxels in the FOV, $F_{\text{FOV}}(t)$, and its time derivative dF_{FOV}/dt , in the first 5 minutes after cortical pinprick. To identify the timing of the CSD, we first defined an initial period, t_{initial} , between the derivative's maximum and minimum. We then calculated baseline fluorescence, F_{pre} , as the 10th percentile value of F_{FOV} in the 30 sec before the initial period and a normalized fluorescence signal, $\Delta F/F_{\text{pre}} = (F_{\text{FOV}} - F_{\text{pre}})/F_{\text{pre}}$. Next, we defined a threshold value as $0.1 \cdot \max(\Delta F(t_{\text{initial}})/F_{\text{pre}})$. The wave's final onset and offset times were defined as the points around the peak where $\Delta F_{\text{FOV}}/F_{\text{pre}}$ crossed the threshold value.

To measure the propagation of the calcium wave throughout the meninges, we estimated the wave's onset time at different X and Y positions within the FOV. Briefly, we gathered all the data from the peri-CSD wave from -6 to 14 s relative to the calcium wave onset time determined above. All voxels belonging to or neighboring (within 8 pixels) ROIs were excluded to focus on the overall wavelike advancement in the background 'neuropil' fluorescence signal (which integrates background fluorescence across depths and thus reflects a smooth estimate of mean activity in a given region). The resulting series of images was then divided into 40×40 pixel spatial bins, and mean fluorescence signals $F_{\text{np}}^{\text{bin}}(t)$ were calculated. The time of estimated onset of the CSD-associated wave at a given bin was estimated by fitting $F_{\text{np}}^{\text{bin}}(t)$ to a logistic function, $A/(1 + \exp(-(t - t_{\text{onset}})/\tau)) + K$. Fits with $R^2 < 0.5$, or $\tau > 2$ s, or $\tau < 0$ s, were excluded. The wave's speed was estimated by linear regression of onset times as a function of distance. The direction of the wave was determined by estimating the contour of the wavefront at the median bin onset time, fitting this contour to a line, and then calculating the vector orthogonal to that line.

To specifically examine the propagation of the fluorescence signals along long (>200 mm) individual afferent fibers, we determined the timing of CSD-associated wave onset by fitting each ROI's peri-wave fluorescence signals to a sigmoidal function and then estimating the speed of propagation by linear regression of onset times as a function of distance. For comparison, this procedure was repeated using the peri-locomotion bout signals. Since locomotion-associated activation occurred essentially simultaneously (i.e., $\Delta t = 0$ s), even for distant ROIs, we report these results in terms of the pace (the inverse of speed) to avoid dividing by zero.

Assessment of CSD-evoked changes in afferent ongoing activity

To determine changes in ongoing afferent activity, we focused on the periods of immobility between locomotion bouts (stillness). We minimized any residual effects of locomotion-related activity by excluding 30-second epochs before and following the locomotion bouts. We then analyzed fluorescence events as above. We estimated levels of ongoing activity rate from the normalized Z-score timecourses as the fraction of time the afferents exhibited fluorescent events (defined as above) during each one-minute interval. We defined afferents with post-CSD increases or suppression of activity if changes in ongoing activity lasted >10 consecutive min and began within 30 min post the CSD wave (Zhao et al., 2021).

Meningeal deformation signals

To estimate the degree of meningeal deformation in locomoting mice, we extracted the values produced from the affine registration procedure used to correct intra-volume (XY) and inter-volume (Z) image displacements (see above). Corrections made to account for XY displacements were used to quantify the amount of scaling (expansion and compression) and shearing. Corrections made along the z-axis measured how much each plane moved up or down relative to a reference volume (“Z-shift”). Scaling, and Z-shifts were converted from pixels to microns. Positive Z-shifts indicate meningeal movement toward the skull.

General linear models

To classify afferent responses, we fit Gaussian general linear models (GLM) for locomotion, deformation, and fluorescence using the `glmnet` package in Matlab. To allow for the possibility of a delay between the predictor and response, we expanded the set of predictors to include variables at varying delays relative to the calcium activity (Driscoll et al., 2017 [↗](#); Ramesh et al., 2018 [↗](#); Blaeser et al., 2022 [↗](#)). Specifically, for each variable, we generated a set of temporally shifted versions spanning a time window from -6 s to +6 s. These sets of arrays of temporal shifts for each variable were joined to form an array of temporally shifted predictor signals. The GLM was fit on 75% of the data for each cell with elastic net regularization ($\alpha = 0.01$). We then used the GLM coefficients to measure the deviance explained on the remaining 25% of the data. The relative explanatory value of locomotion or deformation variables was calculated by refitting the GLM after excluding the family of predictors (locomotion state and velocity for evaluating the effect of locomotion and scale, shear, and Z-shift for examining the effect of deformation) and calculating the difference in deviance explained by the full model versus the model lacking a given family of predictor variables. All GLM models underwent 10-fold cross-validation.

Data analysis and statistics

Data analyses were performed in MATLAB 2020a and Prism 9. Sample sizes were not predetermined by power analysis but are similar to previous studies (Sugden et al., 2020 [↗](#); Blaeser et al., 2022 [↗](#)). Two-tailed paired *t*-tests and one-way analysis of variance (ANOVA) followed by a post-hoc Tukey test were used for all parametric data. Data with non-Gaussian distributions were analyzed using a Wilcoxon matched-pairs signed rank sum test or a Mann-Whitney test. Corrections for multiple comparisons were adjusted using the False Discovery Rate approach. Bootstrapped confidence intervals and hypothesis tests were generated using the ‘iBoot’ iterated bootstrapping package (Penn, 2020 [↗](#)). Data are presented as averages $\pm$ standard error of the mean (SEM) unless otherwise noted. *P*-values are indicated as follows: *P* < 0.05 (*), *P* < 0.01 (**), *P* < 0.001 (***), *P* < 0.0001 (****).

Data availability

The code used to analyze locomotion data is available at: <https://github.com/ablaeser/LocomotionAnalysis> [↗](#). Code for organizing and processing two-photon imaging data is available at: <https://github.com/ablaeser/MovieProcessing> [↗](#). The code for analysis of calcium imaging is available at: <https://github.com/ablaeser/CalciumAnalysis> [↗](#). Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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Supplemental material

Movie S1. CSD-associated afferent calcium wave. Related to **Figure 1** [↗](#) and **Supplemental Fig 1** [↗](#). Movies of the CSD-associated calcium waves spread from anterior to posterior, as shown for two example waves from different mice. Note the spread of calcium activation along individual afferents in each movie. Scale bars: 50 μ m.

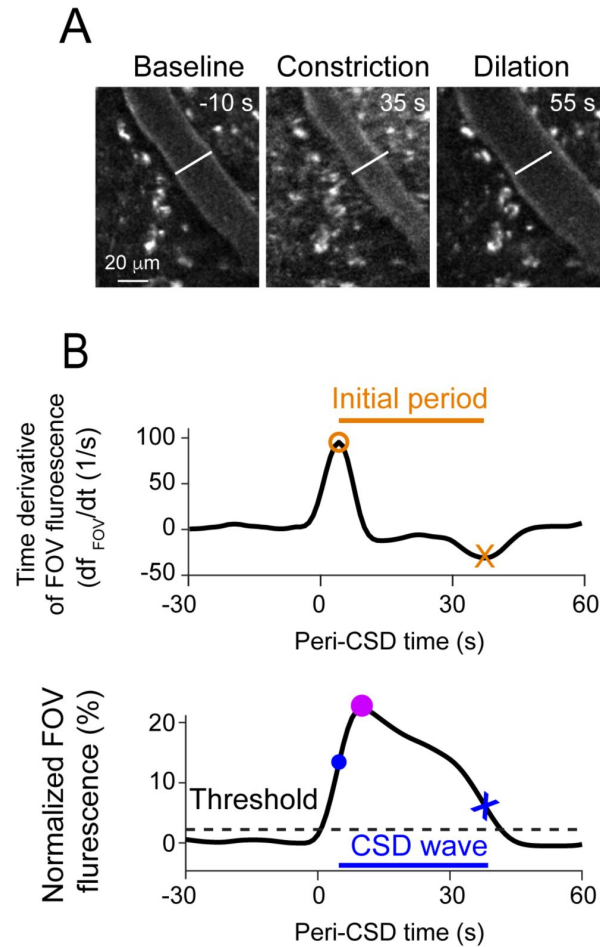


Figure S1.

Further analyses of hemodynamics and calcium activity related to the CSD wave

(A) Example cropped subregion of a field-of-view (FOV) demonstrating a typical pial vascular response to CSD, including an initial constriction (middle) followed by dilation (right). **(B)** Analysis used for detecting the CSD-associated meningeal calcium wave. Top: Time derivative of the FOV-averaged fluorescence signal over the first 90 s of imaging after the cortical pinprick. An initial period was defined between the derivative's maximum (orange circle) and minimum (orange X) and was used to define the baseline fluorescence value (F_{pre}). Bottom: Normalized FOV fluorescence signal. The maximum within the initial period (purple circle) is taken as the peak of the CSD-associated wave, and a threshold value (dashed line) is defined as 10% of that peak value. The threshold crossings before (blue circle) and after (blue X) the peak define the wave's onset and offset, respectively.

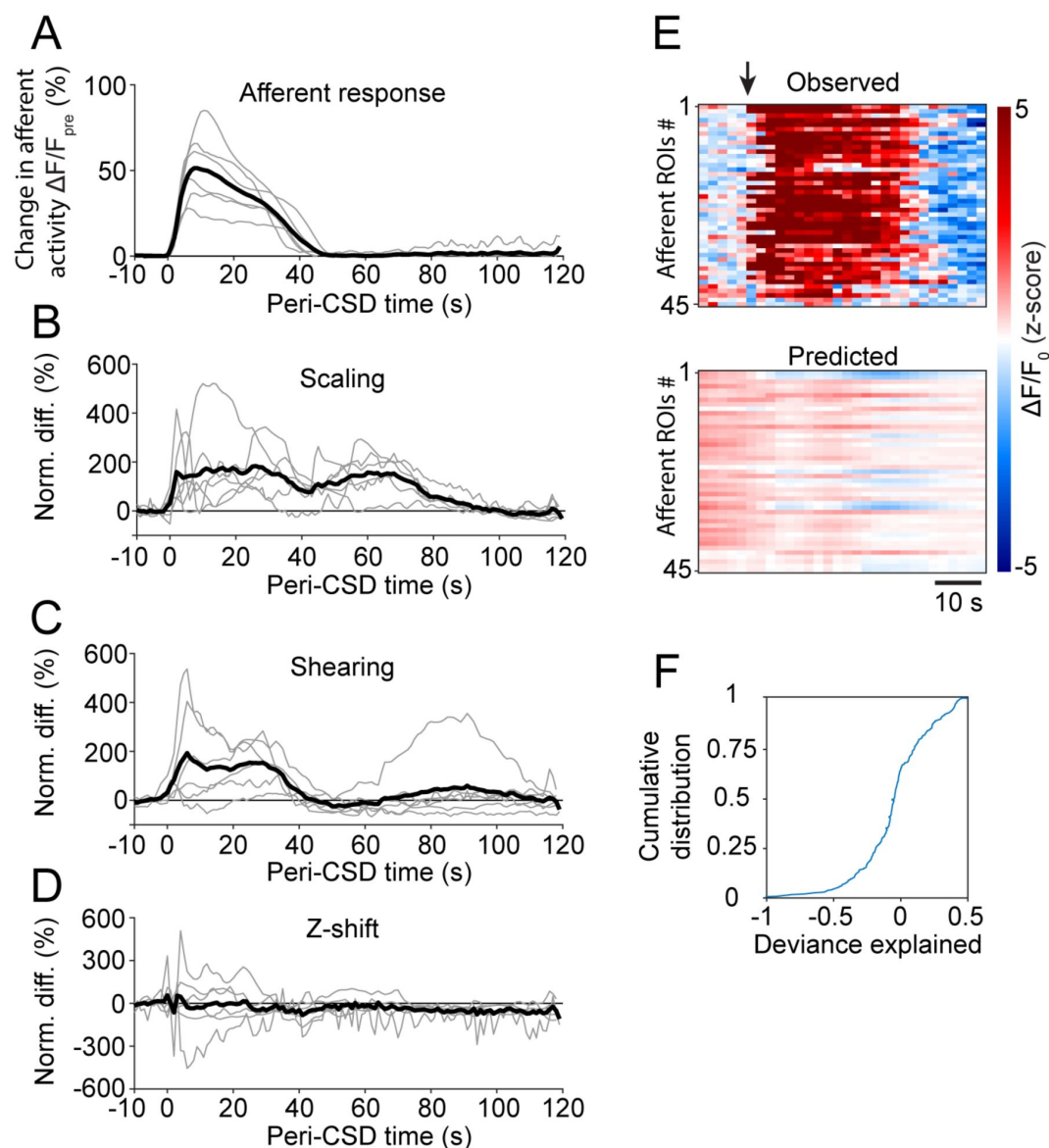


Figure S2

CSD-associated wave deformation and acute afferent responses.

(A) Time course of peri-CSD afferent GCaMP fluorescence signals normalized to the 10-second pre-wave period. Gray traces represent the means of all afferent responses for a given CSD experiment. Black trace represents the average across all volumetric imaging experiments (6 CSDs in 6 mice). **(B-D)** Time courses of peri-CSD meningeal deformation signals normalized to the 10-second pre-wave period. Gray traces represent averages over all well-registered planes for a given FOV. Black traces represent the average across FOVs (6 CSDs in 6 mice). **(E)** Example comparison of the peri-CSD fluorescence signals observed (top) to those predicted by the GLM model trained using pre-CSD meningeal deformation data. The arrow indicates the onset of the CSD-associated wave. **(F)** Cumulative distribution of the GLM deviance explained by the peri-CSD deformation predictors. Overall, the activity of only a small fraction of afferents was somewhat well fit by the model (based on threshold deviance explained > 0.05), suggesting that the acute afferent response is mostly driven by other non-mechanical factors, such as the excitatory action of locally released mediators.

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Article and author information

Andrew S Blaeser

Department of Anesthesia, Critical Care and Pain Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, 02215, USA

Jun Zhao

Department of Anesthesia, Critical Care and Pain Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, 02215, USA

Arthur U Sugden

Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, 02215, USA

Simone Carneiro-Nascimento

Department of Anesthesia, Critical Care and Pain Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, 02215, USA

Mark L Andermann

Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, 02215, USA, Department of Neurobiology, Harvard Medical School, Boston, MA, 02115, USA

For correspondence: manderma@bidmc.harvard.edu

Dan Levy

Department of Anesthesia, Critical Care and Pain Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, 02215, USA

For correspondence: dlevy1@bidmc.harvard.edu

ORCID iD: [0000-0003-0630-6660](https://orcid.org/0000-0003-0630-6660)

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Editors

Reviewing Editor

Samuel Pleasure

University of California, San Francisco, San Francisco, United States of America

Senior Editor

Sacha Nelson

Brandeis University, Waltham, United States of America

Reviewer #1 (Public Review):

Summary:

Herein, Blaeser et al. explored the impact of migraine-related cortical spreading depression (CSD) on the calcium dynamics of meningeal afferents that are considered the putative source of migraine-related pain. Critically previous studies have identified widespread activation of these meningeal afferents following CSD; however, most studies of this kind have been performed in anesthetized rodents. By conducting a series of technically challenging and compelling calcium imaging experiments in conscious head fixed mice they find in contrast that a much smaller proportion of meningeal afferents are persistently activated following CSD. Instead, they identify that post-CSD responses are differentially altered across a wide array of afferents, including increased and decreased responses to mechanical meningeal deformations and activation of previously non-responsive afferents following CSD. Given that migraine is characterized by worsening head pain in response to

movement, the findings offer a potential mechanism that may explain this clinical phenomenon.

Strengths:

Using head fixed conscious mice overcomes the limitations of anesthetized preps and the potential impact of anaesthesia on meningeal afferent function which facilitated novel results when compared to previous anesthetized studies. Further, the authors used a closed cranial window preparation to maximize normal physiological states during recording, although the introduction of a needle prick to induce CSD will have generated a small opening in the cranial preparation, rendering it not fully closed as suggested. However, technical issues with available AAV's and alternate less invasive triggering methodologies necessitate the current approach.

Weaknesses:

Although this is a well conducted technically challenging study that has added valuable knowledge on the response of meningeal afferents the study would have benefited from the inclusion of more female mice. Migraine is a female dominant condition and an attempt to compare potential sex-differences in afferent responses would undoubtedly have improved the outcome. The authors report potential sex-specific effects on AAV transfection rates between males and females which have contributed to this imbalance.

The authors imply that the current method shows clear differences when compared to older anaesthetized studies; however, many of these were conducted in rats and relied on recording from the trigeminal ganglion. Attempts to address this point have proven difficult due to limited GCaMP signalling in anaesthetised mice, meaning that technical differences cannot be ruled out.

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Reviewer #2 (Public Review):

This is an interesting study examining the question of whether CSD sensitizes meningeal afferent sensory neurons leading to spontaneous activity or whether CSD sensitizes these neurons to mechanical stimulation related to locomotion. Using two-photon in vivo calcium imaging based on viral expression of GCaMP6 in the TG, awake mice on a running wheel were imaged following CSD induction by cortical pinprick. The CSD wave evoked a rise in intracellular calcium in many sensory neurons during the propagation of the wave but several patterns of afferent activity developed after the CSD. The minority of recorded neurons (10%) showed spontaneous activity while slightly larger numbers (20%) showed depression of activity, the latter pattern developed earlier than the former. The vast majority of neurons (70%) were unaffected by the CSD. CSD decreased the time spent running and the numbers of bouts per minute but each bout was unaffected by CSD. There also was no influence of CSD on the parameters referred to as meningeal deformation including scale, shear, and Z-shift. Using GLM, the authors then determine that there is an increase in locomotion/deformation-related afferent activity in 51% of neurons, a decrease in 12% of neurons, and no change in 37%. GLM coefficients were increased for deformation related activity but not locomotion related activity after CSD. There also were an increase in afferents responsive to locomotion/deformation following CSD that were previously silent. This study shows that unlike prior reports, CSD does not lead to spontaneous activity in the majority of sensory neurons but that it increases sensitivity to mechanical deformation of the meninges. This has important implications for headache disorders like migraine where CSD is thought to contribute to the pathology in unclear ways with this new study suggesting that it may lead to increased mechanical sensitivity characteristic of migraine attacks.

Reviewer #3 (Public Review):

Summary: In this manuscript, Blaeser et al. explore the link between CSD and headache pain. How does an electrochemical wave in the brain parenchyma, which lacks nociceptors, result in pain and allodynia in the V1-3 distribution? Prior work had established that CSD increased the firing rate of trigeminal neurons, measured electrophysiologically at the level of the peripheral ganglion. Here, Blaeser et al. focus on the fine afferent processes of the trigeminal neurons, resolving Ca^{2+} activity of individual fibers within the meninges. To accomplish these experiments, the authors injected AAV encoding the Ca^{2+} sensitive fluorophore GCamp6s into the trigeminal ganglion, and 8 weeks later imaged fluorescence signals from the afferent terminals within the meninges through a closed cranial window. They captured activity patterns at rest, with locomotion, and in response to CSD. They found that mechanical forces due to meningeal deformations during locomotion (shearing, scaling, and Z-shifts) drove non-spreading Ca^{2+} signals throughout the imaging field, whereas CSD caused propagating Ca^{2+} signals in the trigeminal afferent fibers, moving at the expected speed of CSD (3.8 mm/min). Following CSD, there were variable changes in basal GCamp6s signals: these signals were unchanged in the majority of fibers, signals increased (after a ~20 min delay) in 10% of fibers, and signals decreased in 20% of fibers. Bouts of locomotion were less frequent following CSD, but when they did occur, they elicited more robust GCamp6s signals than pre-CSD. These findings advance the field, suggesting that headache pain following CSD can be explained on the basis of peripheral cranial nerve activity, without invoking central sensitization at the brain stem/thalamic level. This insight could open new pathways for targeting the parenchymal-meningeal interface to develop novel abortive or preventive migraine treatments.

Strengths: The manuscript is well-written. The studies are broadly relevant to neuroscientists and physiologists, as well as neurologists, pain clinicians, and patients with migraine with aura and acephalgic migraine. The studies are well-conceived and appear to be technically well-executed.

Weaknesses: In the present study, conclusions are based entirely on fluorescence signals from GCamp6s. Fluorescence experiments should be interpreted cautiously in the context of CSD. GCamp6 fluorophores are strongly pH dependent, with decreased signal at acidic pH values (at matched Ca^{2+} concentration). CSD induces an impressive acidosis transient in the brain parenchyma, so one wonders whether the suppression of activity reported in the wake of CSD (Figure 2) in fact reflects decreased sensitivity of the GCamp6 reporter, rather than decreased activity in the fibers. If intracellular pH in trigeminal afferent fibers acidifies in the wake of CSD, GCamp6s fluorescence may underestimate the actual neuronal activity.

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Author Response

The following is the authors' response to the original reviews.

We thank the reviewers for their thorough assessment of our study, their overall enthusiasm, and the helpful suggestions for clarifying the methods and results, additional analyses, and discussion points. We have made earnest efforts to address the weaknesses raised in the public review and other recommendations made by the reviewers.

Public Reviews:

Reviewer #1 (Public Review):

Herein, Blaeser et al. explored the impact of migraine-related cortical spreading depression (CSD) on the calcium dynamics of meningeal afferents that are considered the putative source of migraine-related pain. Critically previous studies have identified widespread activation of these meningeal afferents following CSD; however, most studies of this kind have been performed in anesthetized rodents. By conducting a series of technically challenging calcium imaging experiments in conscious head fixed mice they find in contrast that a much smaller proportion of meningeal afferents are persistently activated following CSD. Instead, they identify that post-CSD responses are differentially altered across a wide array of afferents, including increased and decreased responses to mechanical meningeal deformations and activation of previously non-responsive afferents following CSD. Given that migraine is characterized by worsening head pain in response to movement, the findings offer a potential mechanism that may explain this clinical phenomenon.

Strengths:

Using head fixed conscious mice overcomes the limitations of anesthetized preps and the potential impact of anaesthesia on meningeal afferent function which facilitated novel results when compared to previous anesthetized studies. Further, the authors used a closed cranial window preparation to maximize normal physiological states during recording, although the introduction of a needle prick to induce CSD will have generated a small opening in the cranial preparation, rendering it not fully closed as suggested.

Weaknesses:

Although this is a well conducted technically challenging study that has added valuable knowledge on the response of meningeal afferents the study would have benefited from the inclusion of more female mice. Migraine is a female dominant condition and an attempt to compare potential sex-differences in afferent responses would undoubtedly have improved the outcome.

Our study included only two females, largely reflecting the much higher success rate of AAV-mediated meningeal afferent GCaMP expression in males than in females. The reason for the lower yield in female mice is unclear to us at present but may involve, at least partly, sex-specific differences in the mechanisms responsible for efficient transduction with this AAV vector observed in peripheral tissues (Davidoff et al. 2003). While our study did not address sex differences, a recent study (Melo-Carrillo et al. 2017) reported CSD equally activating and sensitizing second-order dorsal horn neurons that receive input from meningeal afferents in male and female rats.

The authors imply that the current method shows clear differences when compared to older anaesthetized studies; however, many of these were conducted in rats and relied on recording from the trigeminal ganglion. Inclusion of a subgroup of anesthetized mice in the current preparation may have helped to answer these outstanding questions, being is this species dependent or as a result of the different technical approaches.

We have tried to address the anesthesia issue by conducting imaging sessions in several isoflurane-anesthetized mice. However, during these experiments, we observed a substantial decrease in the GCaMP fluorescence signal with a much lower signal-to-noise ratio that made the analyses of the afferents' calcium signal unreliable. Reduced GCaMP signal in meningeal axons during anesthesia may be related to the development of respiratory acidosis, since

lower pH leads to decreased GCaMP signal, as also mentioned by Reviewer #3. Of note, urethane anesthesia, which was used in all previous rat experiments, also produces respiratory acidosis.

The authors discuss meningeal deformations as a result of locomotion; however, despite referring to their previous work (Blaeser et al., 2022), the exact method of how these deformations were measured could be clearer. It is challenging to imaging that simple locomotion would induce such deformations and the one reference in the introduction refers to straining, such as cough that may induce intracranial hypertension, which is likely a more powerful stimulus than locomotion.

As part of the revision, we now provide a better description of the methodology (“Image processing and calcium signal extraction” section) used to determine meningeal deformations, including scaling, shearing, and Z-shift. In our previous paper (Blaeser et al. 2023), we provided an extensive description of the types of meningeal deformations occurring in locomoting mice. It should also be noted that locomotion drives cerebral vasodilation and intracranial pressure increases (Gao and Drew, 2016), which likely mediate, at least in part, the movement of the meninges towards the skull (positive Z-shift) and potentially other meningeal deformation parameters. We also agree with the reviewer that sudden maneuvers such as coughing and sneezing that lead to a larger increase in intracranial pressure are likely to be even more powerful drivers of endogenous intracranial mechanical stimulation than locomotion. Thus, our finding of increased responsiveness to locomotion-related meningeal deformation post-CSD may underestimate the increased afferent responsivity post-CSD during other behaviors such as coughing. We added this point to the discussion.

More recently, several groups have used optogenetic triggering of CSD to avoid opening of the cranium for needle prick. Given the authors robustly highlight the benefit of the closed cranium approach, would such an approach not have been more appropriate.

We agree with the reviewer that optogenetic methods used for CSD induction in non-craniotomized animals will further ensure accurate pressurization and, thus, will be an even better approach that avoids the burr hole used for pinprick. It should be noted, however, that the burr hole used for the pinprick likely had a minimal effect on intracranial pressure, as we minimized depressurization by plugging the burr hole throughout the experiments with a silicone elastomer. We have added this information to the revised Methods section.

It is also worth noting that the optogenetic methodology used by others to provoke CSD was optimized only recently and relies on transgenic mice with a strong expression of YFP (Thy1.ChR2-YFP mice) within the superficial cortex that is not compatible with the afferent GCaMP imaging of meningeal afferents. Modifications using red-shifted opsins may allow the use of this strategy in the future.

It was not clear how deformations predictors increased independent of locomotion (Figure 4D) as locomotion is essentially causing the deformations as noted in the study. This point was not so clear to this reviewer.

As noted in our previous paper (Blaeser et al., 2023), deformation variables often exhibit different time courses than locomotion, even when a deformation is initially induced by the onset of locomotion. Most notably, the scaling-related deformation ramps up slowly and often persists for tens of seconds after the onset and termination of locomotion, which may be related to the recovery dynamics of the meningeal vascular response to locomotion. Overall, while locomotion serves as a predictor of meningeal deformation, we observed previously (Blaeser et al. 2023) many afferents whose responses were more closely associated with the

moment-to-moment deformations than with the state of locomotion per se, suggesting that a unique set of stimuli is responsible for the activation of this deformation-sensitive afferent population. The increased sensitivity to deformation signals we observed following CSD suggests that the afferent population sensitive to deformation has unique properties that render it most susceptible to becoming sensitized following CSD. We now discuss this possibility.

Reviewer #2 (Public Review):

This is an interesting study examining the question of whether CSD sensitizes meningeal afferent sensory neurons leading to spontaneous activity or whether CSD sensitizes these neurons to mechanical stimulation related to locomotion. Using two-photon in vivo calcium imaging based on viral expression of GCaMP6 in the TG, awake mice on a running wheel were imaged following CSD induction by cortical pinprick. The CSD wave evoked a rise in intracellular calcium in many sensory neurons during the propagation of the wave but several patterns of afferent activity developed after the CSD. The minority of recorded neurons (10%) showed spontaneous activity while slightly larger numbers (20%) showed depression of activity, the latter pattern developed earlier than the former. The vast majority of neurons (70%) were unaffected by the CSD. CSD decreased the time spent running and the numbers of bouts per minute but each bout was unaffected by CSD. There also was no influence of CSD on the parameters referred to as meningeal deformation including scale, shear, and Z-shift. Using GLM, the authors then determine that there is an increase in locomotion/deformation-related afferent activity in 51% of neurons, a decrease in 12% of neurons, and no change in 37%. GLM coefficients were increased for deformation related activity but not locomotion related activity after CSD. There also was an increase in afferents responsive to locomotion/deformation following CSD that were previously silent. This study shows that unlike prior reports, CSD does not lead to spontaneous activity in the majority of sensory neurons but that it increases sensitivity to mechanical deformation of the meninges. This has important implications for headache disorders like migraine where CSD is thought to contribute to the pathology in unclear ways with this new study suggesting that it may lead to increased mechanical sensitivity characteristic of migraine attacks.

- 1. It would be helpful to know what is meant by "post-CSD" in many of the figures where a time course is not shown. The methods indicate that 4, 30 min runs were collected after CSD but this would span 2 hours and the data do not indicate whether there are differences across time following CSD nor whether data from all 4 runs are averaged.*

While we monitored time course changes in ongoing activity (see Figure 2), it was challenging to evaluate post-CSD changes in locomotion-related deformation responses at a fine temporal scale, as running bouts resumed at different time points post-CSD and occurred intermittently throughout the post-CSD analysis period. Our experiments were also not sufficiently powered to break out analyses at multiple different epochs post-CSD, partly because there wasn't much locomotion. To allow comparisons using a sufficient number of bouts, we conducted our GLM analyses using all data collected during running bouts in the 2-hour post-CSD period (termed "post-CSD") versus in the 1-hour pre-CSD period. We have now clarified this further in the main text and figure legends.

- 1. Why is only the Z-shift data shown in Figures 4A-C? Each of the deformation values seems to contribute to the activity of neurons after CSD but only the Z-shift values are shown.*

In many afferents, only one deformation variable best predicted the activity at both the pre- and post-CSD epochs. However, at the population level, all deformation variables were equally predictive. In the examples provided, the afferent developed augmented sensitivity

that could only be predicted by the Z-shift variable, and the other deformation variables were not included to keep the figure legible. This is now clarified in the figure legend.

1. How much does the animal moving its skull against the head mount contribute to deformations of the meninges if the skull is potentially flexing during these movements? Even if mice are not locomoting, they can still attempt to move their heads thus creating pressure changes on the skull and underlying meninges. The authors mention in the methods that the strong cement used to bind the skull plates and headpost together minimize this, but how do they know it is minimized?

We did not measure skull flexing during locomotion and its potential effect on meningeal deformation. However, we would like to point out several considerations. It is evident from numerous imaging studies across various brain regions in freely moving animals, utilizing brain motion registration, that brain motion of the same scale (a few microns), as that observed in our studies, also occurs in the absence of head fixation (e.g., Glas et al, 2019; Zong et al 2021). In our system, the head-fixed mouse is locomoting on a cantilevered (spring-like) running wheel (see also Ramesh et al., 2018), which dissipates most, albeit not all, upward and forward forces applied to the skull during locomotion. Furthermore, the position of the headpost, anterior to where the mouse's paws touch the wheel, makes it hard for the mouse to push straight up and apply forces to the skull. We have updated the text in the methods section (Running wheel habituation) to address this. In our previous work (See Figure 2B in Blaeser et al. 2023), we found a substantial subset of afferents showing an increase in calcium activity that began after each bout of locomotion had terminated, and that lasted for many seconds, suggesting that skull flexing during locomotion may not play a leading role. Finally, we proposed in that study that meningeal deformations play a major role in the afferent response, given our findings of (i) sigmoidal stimulus-response curves between afferent activity and meningeal deformation and (ii) of different afferents that track scaling deformations along different axes. It is unlikely that all of these are related to any residual forces generated from skull deformations.

1. What is the mechanism by which afferents initiate the calcium wave during the CSD itself? Is this mechanical pressure due to swelling of the cortex during the wave? If so, why does the CSD have no impact on the deformation parameters? It seems that this cortical swelling would have some influence on these values unless the measurements of these values are taken well after cortical swelling subsides. Related to point 1 above, it is not clear when these measurements are taken post-CSD.

We provide, for the first time, evidence that CSD evokes local calcium elevation in meningeal afferent fibers in a manner that is incongruent with action potential propagation, as the activity gradually advances along individual afferents across many seconds during the wave. As indicated in Figure 1H, we measured these changes during the first 2 minutes post-CSD. Based on the reviewer's question, we have now addressed whether mechanical changes occurring in the cortex in the wake of CSD might be responsible for the acute afferent activation we observed. We now include new data (Results, "Acute afferent activation is not related to CSD-evoked meningeal deformation" and Figure S2) showing an acute phase of meningeal deformation (as expected given the changes in extracellular fluid volume) lasting 40-80 seconds following the induction of CSD. Our data suggests, however, that these meningeal deformations are unlikely to be the main driver of the acute afferent calcium response. We propose that, based on the speed of the afferent calcium wave propagation and the distinct dynamics of calcium activity as compared to the dynamics of the deformations, the acute afferent response is more likely to be mediated by the spread of algescic mediators (e.g., glutamate, K⁺ ATP) and their diffusion into the overlying meninges.

Because the peri-CSD meningeal deformations return to baseline soon after the cessation of the CSD wave, they are unlikely to affect our analyses of post-CSD changes in afferent sensitivity in the following 2 hours. This is also supported by our data (see Figure 3F-H) showing similar locomotion-related deformations pre- and post-CSD, which were measured after the deformations related to the CSD itself had subsided.

1. How does CSD cause suppression of afferent activity? This is not discussed. It is probably a good idea in this discussion to reinforce that suppression in this case is suppression of the calcium response and not necessarily suppression of all neuronal activity.

The mechanism underlying the suppression of afferent activity remains unclear. We now discuss the following points:

First, the pattern of afferent responses resembles the rapid loss of cortical activity in the wake of a CSD, but its faster recovery points to a mechanism distinct from the pre- and post-synaptic changes responsible for the silencing of cortical activity (Sawant-Pokam et al., 2017; Kucharz and Lauritzen, 2018). Whether CSD drives the local release of mediators capable of reducing afferent excitability and spiking dynamics will require further studies.

Second, the reviewer proposes that the suppressed calcium activity we observed in ~20% of the afferents immediately following CSD may reflect a decreased calcium response independent of afferent spiking activity. Such a process could theoretically involve factors influencing the GCaMP fluorescence (see also our response to Reviewer #3) and/or factors modifying the afferents' spiking-to-calcium coupling. We note that if a CSD-related factor could modify the calcium response independent of afferent spiking, one would expect a more consistent effect across axons, reflected as a reduced signal in a larger proportion of the afferents, which we did not observe.

1. How do the authors interpret the influence of CSD on locomotor activity? There was a decrease in bouts but the bouts themselves showed similar patterns after CSD. Is CSD merely inhibiting the initiation of bouts? Is this consistent with what CSD is known to do to motor activity? And again related to point 1, how long after CSD were these measurements taken? Were there changes in locomotor activity during the actual CSD compared to post-CSD?

To the best of our knowledge, there is very little data on the effect of CSD on motor activity, making it challenging to engage in further speculation regarding the mechanisms underlying the preservation of running bouts patterns post-CSD. Houben et al. (2017) described a similar reduction in locomotion in mice, corresponding to decreased motor cortex (M1) activity, and preservation of intermittent locomotion bouts. In the revised Results section, we now provide information about the cessation of locomotor activity during the CSD wave and have added information regarding the measurement of locomotion following CSD.

1. The authors mention the caveats of prior work where the skull is open and is thus depressurized. Is this not also the case here given there is a hole in the skull needed to induce CSD?

Unlike previous electrophysiological studies, which involved several large openings (~2x2 mm), including at the site of the afferents' receptive field, our study involved only a small burr hole located remotely (1.5 mm) from the frontal edge of our imaging window. As noted in our response to Reviewer #1, this burr hole (~0.5 mm diameter) was unlikely to produce inflammation at the imaging site or cause depressurization as it was sealed with a silicone plug throughout the experiment.

1. The authors should check the %'s and the numbers in the pie chart for Figure 4. Line 224 says 53 is 22% but it does not look this way from the chart.

The 22% reported is the percentage of afferents that developed sensitivity post-CSD among all the non-sensitive ones pre-CSD. The pie chart illustrates only afferents that were deemed sensitive before and/or after the CSD. We removed the % to clarify.

1. Line 319 mentions that CSD causes "powerful calcium transients" in sensory neurons but it is not clear what is meant by powerful if there are no downstream effects of these transients being measured. The speculation is that these calcium transients could cause transmitter release, which would be an important observation in the absence of AP firing, but there are no data evaluating whether this is the case.

We changed the term to "robust"

Reviewer #3 (Public Review):

Summary:

Blaeser et al. set out to explore the link between CSD and headache pain. How does an electrochemical wave in the brain parenchyma, which lacks nociceptors, result in pain and allodynia in the V1-3 distribution? Prior work had established that CSD increased the firing rate of trigeminal neurons, measured electrophysiologically at the level of the peripheral ganglion. Here, Blaeser et al. focus on the fine afferent processes of the trigeminal neurons, resolving Ca²⁺ activity of individual fibers within the meninges. To accomplish these experiments, the authors injected AAV encoding the Ca²⁺ sensitive fluorophore GCaMP6s into the trigeminal ganglion, and 8 weeks later imaged fluorescence signals from the afferent terminals within the meninges through a closed cranial window. They captured activity patterns at rest, with locomotion, and in response to CSD. They found that mechanical forces due to meningeal deformations during locomotion (shearing, scaling, and Z-shifts) drove non-spreading Ca²⁺ signals throughout the imaging field, whereas CSD caused propagating Ca²⁺ signals in the trigeminal afferent fibers, moving at the expected speed of CSD (3.8 mm/min). Following CSD, there were variable changes in basal GCaMP6s signals: these signals decreased in the majority of fibers, signals increased (after a 25 min delay) in other fibers, and signals remained unchanged in the remainder of fibers. Bouts of locomotion were less frequent following CSD, but when they did occur, they elicited more robust GCaMP6s signals than pre-CSD. These findings advance the field, suggesting that headache pain following CSD can be explained on the basis of peripheral cranial nerve activity, without invoking central sensitization at the brain stem/thalamic level. This insight could open new pathways for targeting the parenchymal-meningeal interface to develop novel abortive or preventive migraine treatments.

Strengths:

The manuscript is well-written. The studies are broadly relevant to neuroscientists and physiologists, as well as neurologists, pain clinicians, and patients with migraine with aura and acephalgic migraine. The studies are well-conceived and appear to be technically well-executed.

Weaknesses:

1. Lack of anatomic confirmation that the dura were intact in these studies: it is notoriously challenging to create a cranial window in mouse skull without disrupting or even removing the dura. It was unclear which meningeal layers were captured in the imaging plane. Did the visualized trigeminal afferents terminate in the dura, subarachnoid space, or pia (as suggested by Supplemental Fig 1, capturing a pial artery in the imaging plane)? Were z-stacks obtained, to maintain the imaging plane, or to follow visualized afferents when they migrated out of the imaging plane during meningeal deformations?

We agree that avoiding disruption of the dura is challenging. Indeed, it took many months of practice before conducting the experiments in this manuscript to master methods for a craniotomy that spared the dura.

We addressed the issue of meningeal irritation due to cranial window surgery in our previous work (Blaeser et al., 2023). In brief, we conducted vascular imaging using the same cranial window approach and showed no leakage of macromolecules from dural or pial vessels anywhere within the imaging window at 2-6 weeks after the surgery (Figure S1D in Blaeser et al. 2022). This data suggested no ongoing meningeal inflammation below the window. The very low level of ongoing activity we observed at baseline also suggests a lack of an inflammatory response that could lead to afferent sensitization before CSD. This is now mentioned in the Discussion.

We conducted volumetric imaging for three main reasons: 1) To capture the activity of afferents throughout the meningeal volume. In our volumetric imaging approach, including in this work, we observed afferent calcium signals throughout the meningeal thickness (see Figure 5 in Blaeser et al. 2022). However, the majority of afferents were localized to the most superficial 20 microns (Figure S1E in Blaeser et al. 2022), suggesting that we mostly recorded the activity of dural afferents; 2) to enable simultaneous quantification of three-dimensional deformation and the activity of afferents throughout the thickness of the meninges. This allowed us to determine whether changes in mechanosensitivity could involve augmented activity to intracranial mechanical forces that produced meningeal deformation along the Z-axis of the meninges (e.g., increased intracranial pressure); 3) to provide a direct means to confirm that the afferent GCaMP fluorescent changes we observed were not due to artifacts related to meningeal motion along the Z-axis. We have now added this information to the “Two-photon imaging” section of the Methods.

1. Findings here, from mice with chronic closed cranial windows, failed to fully replicate prior findings from rats with acute open cranial windows. While the species, differing levels of inflammation and intracranial pressure in these two preparations may contribute, as the authors suggested, the modality of measuring neuronal activity could also contribute to the discrepancy. In the present study, conclusions are based entirely on fluorescence signals from GCaMP6s, whereas prior rat studies relied upon multiunit recordings/local field potentials from tungsten electrodes inserted in the trigeminal ganglion.

As a family, GCaMP6 fluorophores are strongly pH dependent, with decreased signal at acidic pH values (at matched Ca²⁺ concentration). CSD induces an impressive acidosis transient, at least in the brain parenchyma, so one wonders whether the suppression of activity reported in the wake of CSD (Figure 2) in fact reflects decreased sensitivity of the GCaMP6 reporter, rather than decreased activity in the fibers. If intracellular pH in trigeminal afferent fibers acidifies in the wake of CSD, GCaMP6s fluorescence may underestimate the actual neuronal activity.

Previous in vivo rodent studies observed a tissue acidosis transient that peaks during the DC shift corresponding to the wavefront of the spreading depolarization, and lasting for ~ 10 min. (Mutch and Hansen, 1984). Since we observed a massive increase in afferent calcium activity with a propagation pattern resembling the cortical wave, it is unlikely that the cortical acidosis during the CSD wave strongly affected the GCaMP signal in the overlying meninges. Furthermore, if cortical acidosis non-discriminately affects the GCaMP signal, one would expect a more consistent effect across axons, reflected as a reduced calcium signal in a larger proportion of the afferents, which we did not observe. Finally, the finding that in affected afferents, decreased calcium activity lasted for > 20 min – a time point when cortical acidosis has fully recovered - points to a distinct underlying mechanism. We also note that any residual acidosis would not confound our main finding of increased calcium responses to meningeal deformation at later periods post-CSD, as acidosis should, if anything, decrease calcium-related fluorescence.

The authors might consider injecting an AAV encoding a pHi sensor to the trigeminal ganglion, and evaluating pHi during and after CSD, to assess how much this might be an issue for the interpretation of GCaMP6s signals. Alternatively, experiments assessing trigeminal fiber (or nerve/ganglion) activity by electrophysiology or some other orthologous method would strengthen the conclusions.

Please see our comment above regarding the short duration of the pH changes post-CSD.

N's are generally reported as # of afferents, obscuring the number of technical/biological replicates (# of imaging sessions, # of locomotion bouts, # of CSDs induced, # of animals).

We now report the number of replicates (# of afferent, # of CSD events, and # of mice).

Fig 1F trace over the heatmap is not explained in the figure legend. Is this the speed of the running wheel? Is it the apparent propagation rate of the GCaMP6s transient through the imaging field?

We have added to the legend of Figure 1 that the trace in panel F depicts locomotion speed.