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CGRP signaling mediates prolonged meningeal afferent activation evoked by brief local K^+ stimulation but not cortical spreading depression-induced afferent sensitization

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Abstract

Cortical spreading depression (CSD) is believed to promote migraine headache by enhancing the activity and mechanosensitivity of trigeminal intracranial meningeal afferents. One putative mechanism underlying this afferent response involves an acute excitation of meningeal afferents by cortical efflux of K^+ and the ensuing antidromic release of pro-inflammatory sensory neuropeptides, such as calcitonin gene-related peptide (CGRP). We employed extracellular single-unit recording in anesthetized rats to determine whether a brief K^+ stimulus could lead to CGRP-dependent enhancement of meningeal afferent responses. In addition, we tested if CSD-evoked meningeal afferent responses involve CGRP signaling. Acute meningeal K^+ stimulation promoted a 2.3±0.4 fold increase in the ongoing activity level of ~65% of the afferents tested. Neural activation developed following a 23.3±4.1min delay and lasted for 22.2±5.6min. Meningeal afferent mechanosensitivity was not altered by the K^+ stimulation. Pretreatment with the CGRP receptor inhibitor BIBN4096 (333µM, i.v.) suppressed the K^+-related prolonged afferent activation but had no effect on the prolonged increase in ongoing activity and mechanosensitivity of meningeal afferents evoked by CSD. While CGRP-mediated activation of meningeal afferents evoked by cortical efflux of excitatory mediators could promote headache, this mechanism is unlikely to play a key role in mediating the headache of migraine triggered by CSD.

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Introduction

Migraine is the third most prevalent and seventh most disabling disease in the world, affecting about 15% of the adult population worldwide [1,2]. A key migraine theory links the genesis of the headache to a cascade of inflammatory-driven nociceptive events, namely the prolonged activation and increased mechanosensitivity of pain sensitive afferents that innervate the intracranial meninges and their related large blood vessels [3-6].

One fundamental part of this inflammatory hypothesis of migraine, as originally proposed more than 35 years ago [7], implicates the action of proinflammatory sensory neuropeptides, such as calcitonin gene-related peptide (CGRP), that are released from peripheral nerve endings of acutely activated primary afferents through an “axon reflex” process [8]. According to this common theory, the initial stimulation of meningeal afferents, which leads to the antidromic release and action of sensory neuropeptides, subsequently promotes the prolonged increase in meningeal afferent activity and their enhanced mechanosensitivity [5,9-11]. Key support for the this neurogenic inflammation (NI) theory of migraine include the findings of elevated CGRP plasma levels during a migraine attack [12] (but see also [13]) and that triptans and agents that block the action of CGRP, which also inhibit meningeal NI [14-17], are efficacious in the acute treatment of migraine headache [18,19].

A key migraine-related event that has been suggested to initiate meningeal axon reflex is cortical spreading depression (CSD)[20,21]. We reported previously that in a substantial number of meningeal afferents, CSD gives rise to a biphasic pattern of neural activation:

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a short-lasting increase in ongoing activity, during the passage of the CSD wave under the afferents' receptive field that is followed, after some delay, by a prolonged activation and mechanosensitization of the afferents [22-24]. The mechanism underlying the short-lasting activation of meningeal afferents during the CSD is thought to involve the action of excitatory molecules, in particular potassium ions (K^+), that are briefly released in the vicinity of the meninges during the CSD wave [5,22,23]. While the process that contributes to the prolonged and augmented responses of the afferents following CSD remains incompletely, based on the NI theory of migraine, in the wake of CSD, the initial activation of meningeal afferents could indirectly lead to the prolonged afferent response through a mechanism that involves meningeal release of CGRP or other neuropeptides [20,21].

Here, we used single-unit recording of meningeal afferents in anesthetized rats to initially address the question of whether a brief excitation of meningeal afferents using a CSD-related stimulus, namely short-term meningeal elaboration of K^+ [25-28], could lead to prolonged change in the afferents' response properties. Having identified such K^+-driven prolonged activation of meningeal afferents, and given that acute exposure of meningeal afferents to K^+ leads to meningeal CGRP release [29-31] we further examined the relative contribution of CGRP-receptor signaling in mediating this prolonged afferent response. Finally, given that CSD is associated with a CGRP-related meningeal vascular response [32,33], we further examined whether CGRP receptor signaling mediates the prolonged meningeal afferent response to CSD.

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Materials and Methods

Animals and anesthesia

The current study employed male Sprague-Dawley rats (250–350 g) so data could be compared with previous in vivo studies of meningeal afferents that were conducted exclusively in males. Animals were handled in compliance with the experimental protocol approved by the Institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Center. Animals were deeply anesthetized with urethane (1.5 g/kg, ip) and mounted on a stereotaxic frame (Kopf Instruments). Core temperature was kept at 37.5-38°C using a homoeothermic control system. Animals breathed spontaneously room air enriched with 100% O_2. Physiological parameters were collected throughout the experiments using PhysioSuite® (Kent Scientific) and CapStar-100 (CWE). Data used in this report were obtained only from animals exhibiting physiological levels of oxygen saturation (>95%), heart rate (350–450 bpm), and end-tidal CO_2 (3.5–4.5%).

Surgery and electrophysiological recordings

A saline-cooled dental drill was used to perform a craniotomy to expose the left transverse sinus as well as the adjacent cranial dura extending ~2 mm rostral to the sinus. In animals tested for the effects of CSD, a small burr hole (0.5-mm diameter) was drilled to expose a small area of dura above the frontal cortex to induce CSD [24]. The exposed dura was bathed with a modified synthetic interstitial fluid (SIF) containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH

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7.2. The femoral vein was cannulated to allow for intravenous administration. Single-unit activity of meningeal afferents (1 unit/rat) was recorded in the ipsilateral (left) trigeminal ganglion using a platinum-coated tungsten microelectrode (50-100 kΩ). The recording electrode was advanced into the left ganglion through a contralateral angled approach, which spares the ipsilateral cortex. Meningeal afferents were identified by their constant latency response to single shock stimulation applied to the dura above the ipsilateral transverse sinus (0.5-ms pulse, 5 mA, 0.5 Hz). The response latency was used to calculate conduction velocity (CV), based on a conduction distance to the trigeminal ganglion of 12.5 mm [34]. Neurons were classified as either A-delta ($1.5 < CV \leq 5$ m/s) or C-afferents ($CV \leq 1.5$ m/sec). All meningeal afferents tested were mechanosensitive when probed with von Frey filaments (0.03–6.9 g, Stoelting), and had at least 1 receptive field (RF) located on the left transverse sinus or its vicinity (<1 mm). Neural activity was digitized and a real-time waveform discriminator (Spike 2 software, CED) was used to create and store a template for the action potential evoked by electrical stimulation, which was used later to acquire and analyze the ongoing activity of the neurons and the activity evoked by mechanical stimulation and CSD.

Mechanical stimulation and detection of sensitization

Mechanical responsiveness was quantitatively determined in each afferent by recording the responses to mechanical stimuli (100-ms rise time, 2-s width, 120-s inter-stimulus interval) delivered using a feedback-controlled mechanical stimulator (Series 300B, Aurora Scientific) and a custom-written script for Spike 2. Stimulus trials for testing changes in mechanosensitivity included one threshold stimulus (TH, which normally

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evoked 1-3-Hz responses) followed by a supra-threshold stimulus (STH, usually X2 of the threshold pressure; 8-10 Hz responses). To minimize response desensitization, stimulus trials were delivered every 15 min throughout the experiment [24,35]. Ongoing afferent discharge rate was recorded continuously between the stimulation trials. Responses to mechanical stimulation were determined during at least four consecutive trials before the elicitation of CSD. Only afferents that exhibited consistent responses (variation of <0.5 Hz for TH responses and <1.5 Hz for STH responses) during baseline recordings were tested further [24].

Induction and monitoring of CSD

A single CSD episode was induced in the frontal cortex by pinpricking the cortex with a fine glass micropipette (diameter 10 µm) at ~2 mm depth for 2 sec. CSD was induced in the frontal cortex to avoid potential damage to the meningeal tissue near the tested RF of the studied afferents, which could have led to their activation and/or sensitization [23]. The occurrence of a CSD episode was determined noninvasively by recording simultaneously changes in cerebral blood flow (CBF) using laser Doppler flowmetry, with the probe positioned just rostral to the RF of the recorded afferent. Induction of CSD was considered successful when the typical hemodynamic signature characterized by a large transient (~1-2 min) cerebral hyperemia, followed by prolonged (>1h) post-CSD oligemia was observed [23,36].

Drugs

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BIBN4096BS (Tocris) was dissolved in 1N HCl, further diluted with saline (0.9%) and titrated to pH 6.5–7.0 by 1N NaOH. Potassium chloride (Sigma Aldrich) solution, diluted in SIF, was used for K^+ stimulation.

Data analysis

Offline analyses for afferent responses were conducted using template matching in Spike 2. Statistical analyses were conducted using Prism 7 software. Average data are presented in the text as the mean ± SEM. Average data in figures is presented as the mean ± 95% confidence interval (CI). Criteria used to consider meningeal afferent activation and sensitization responses were based on our previous studies [23,24]. In brief, an increase in afferents ongoing activity was considered if the firing rate increased above the upper end point of the 95% CI calculated for the baseline mean. Acute afferent activation was considered when ongoing activity level increased during the K^+ stimulus or the arrival of the CSD wave (i.e. the hyperemia stage) under the RF of the studied afferent and subsided within 2 minutes. Prolonged activation was considered when ongoing activity rate was increased for >10 min. Mechanical sensitization was considered only if the afferent responses changed according to the following criteria: TH and/or STH responses increased to a level greater than the upper endpoint of the 95% CI calculated for the baseline mean; increased responsiveness began during the first 60 min post-CSD; and sensitization lasted for at least 30 min. For analyses of CSD-related changes in the afferent responses, data from A-delta and C afferents were combined given their comparable values (see [24]). Group differences were analyzed using two-tailed, Fisher's exact test. Statistical differences were analyzed using two-tailed

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unpaired *t*-test or Mann Whitney U test for datasets that failed normality test. Results were considered to be significant at $p < 0.05$.

Results

Brief meningeal stimulation with a CSD-related K^+ stimulus promotes prolonged activation of meningeal afferents

We first asked whether a brief, local meningeal stimulation with K^+ (50 to 5 mM in 60 sec, See Fig. 1), to mimic the K^+ ionic change the occurs during the pass of the CSD wave under the meninges [25-28], could promote long-term changes in the activity and/or mechanosensitivity of meningeal afferents. Stimulation with K^+ gave rise, as expected [34], to a robust yet short-term (< 70 sec, see an example in Fig. 1) increase in the ongoing activity rate (8.7±2.4 fold) of 12/14 afferents tested (4/5 A-delta, 8/9 C). Following a wash with SIF, 9/14 of the afferents (3/5 A-delta 6/9 C) developed a prolonged increase in their ongoing activity rate (see an example in Fig. 1). As Figs. 2A-C depict, this K^+-evoked neuronal response developed with an average delay of 23.3±4.1 min (range 5-40 min), lasted 22.2±5.6 min (range 10-55 min) and was associated with a 2.3±0.4 (range 1.8-5.0) fold increase in the afferent firing rate. When compared to data collected in time-control experiments (n=12, 1 sensitized C afferent, for 30 min), K^+ stimulation (n=14) did not affect the afferents' mechanosensitivity (Fig. 2D,E) with only 1 C afferent exhibiting a brief (30 min) increase in TH and STH responses and another C afferent exhibiting 30 min increase only in its STH response.

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K^+ - induced prolonged activation of meningeal afferents is CGRP-dependent

Previous studies reported that a similar brief meningeal K^+ stimulation evokes meningeal CGRP release [29,30,37]. We therefore asked next whether the K^+ - driven prolonged activation of meningeal afferents is CGRP-dependent, and thus potentially related to meningeal afferent axonal reflex and NI. To examine the role of CGRP receptor signaling we administered the CGRP-receptor antagonist BIBN4096 (333 μg/kg, i.v) one hour before testing the afferent response to local K^+ stimulation. The dose of BIBN4096 was adapted from previous studies showing efficacy in inhibiting neurogenically-evoked meningeal vasodilation [15,38]. As indicated in Table 1, neither BIBN4096 (19 A-delta, 18 C afferents) nor its vehicle (25 A-delta, 25 C afferents) affected the afferents' baseline ongoing activity rate, which is in agreement with the pervious study of Fischer et al. [39]. BIBN4096 also did not influence the baseline mechanosensitivity of the afferents (Table 2). The effect of BIBN4096 on K^+ -evoked responses was tested on 13 afferents (6 A-delta, 7C). BIBN4096 did not inhibit the initial short excitatory response to K^+ (10/13 afferents developed a 7.6±3.7 fold increase in activity, $p=0.64$). BIBN4096, however, significantly inhibited the K^+-driven prolonged increase in ongoing activity with only 2/13 afferents (2 C) displaying prolonged activation (compared with 9/14 units affected in vehicle control experiments, $p<0.05$ two-tailed, Fisher's exact test). The onset latencies for the prolonged activation in these two afferents were 35 and 10 min with matching durations of 20 and 10 min. The magnitude of change in afferent activity in the units tested from animals administered

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with BIBN4096 prior to K^+ stimulation was also lower than that observed in units treated with the vehicle prior to K^+ stimulation (Fig 2C; $p<0.05$).

Enhanced meningeal afferent responses following CSD do not require CGRP signaling

Given the ability of BIB4096 pretreatment to inhibit the K^+ - driven prolonged afferent activation, we next tested the hypothesis that CGRP receptor activation also mediates the prolonged meningeal afferents' responses in the wake of CSD. We initially verified that in the setting of CSD, BIBN4096 treatment inhibits a previously recognized CGRP-mediated meningeal response, namely the brief pial hyperperfusion [40,41]. Induction of CSD in BIBN4096 pretreated animals gave rise to a hyperemic response that was significantly lower than that observed in vehicle-treated animals (158.1±8.2% (n=8) vs 198±9.4% (n=9); $p<0.05$) confirming the efficacy of BIBN4096 in this model. In BIBN4096-treated animals, CSD resulted in the development of prolonged activation in 9/24 (37.5%) of the afferents tested (4/13 A-delta; 5/11 C). This response rate, however, was not statistically different than that observed in animals treated with vehicle (9/19 A-delta and 9/17 C; p=0.80 overall; p=0.47 for A-delta afferent population; p=1.00 for the C-afferent population). BIBN4096 treatment also did not influence the characteristics of the afferents' responses to CSD. As Fig. 3A depicts, the average onset latency for CSD-evoked afferent activation in the BIBN treated group was 3.9±1.6 min (range 0-15 min) and was not statistically different than that observed in the control treated group (average onset 8.8±2.6 min; range 0-35 min; p=0.29). The duration of the post-CSD

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evoked afferent discharge in the BIBN4096 treatment group (average 30.0±5.3 min; range 10-55) was also not different than that observed in the vehicle control group (average 32.8±3.8 min; range 10-55; p=0.15; Fig 3B). Finally, the magnitudes of the increase in afferent discharge rate following CSD were also not different between the two treatment groups (BIBN4096; average 2.9±0.7; range 1.5-8.3 fold vs. control; average 2.9±0.5 fold; range 1.2-10.4 fold; p=0.41; Fig 3C).

To examine the effect of BIB4096 pretreatment on the CSD-evoked meningeal afferent mechano-sensitization we analyzed data collected from 21 afferents (11 A-delta and 10 C), all of which displayed consistent responses at baseline. In BIBN4096-treated animals, CSD evoked mechanical sensitization in 11/21 afferents (5/11 A-delta and 6/10 C). This rate of response was not different than that observed in animals treated with vehicle (7/10 A-delta and 9/13 C; p=0.35 overall; p=0.39 for the A-delta afferent population; p=0.69 for the C afferent population). Overall, BIBN4096 also did not affect the CSD-evoked sensitization characteristics (Fig. 4). The onset latency for the CSD-evoked TH sensitization in the BIB4096-treated group averaged 35.6±6.3 min (range 15-60 min), which was not statistically different than that observed in the control group (average 21.0±3.6 min, range 15-45 min; p=0.09; Fig. 4A). The onset latency for the increase in STH responses in the BIBN4096 group averaged 31.5±5.7 min (range 15-60 min), which was also not statistically different than that observed in the control group (average 24.2±3.2 min; range 15-60; p=0.25; Fig. 4B). The duration of the post-CSD TH sensitization response in the BIBN4096-treated group averaged 73.8±9.9 min (range 30-120 min) was similar to that observed in the control group (average 71.3±10.5

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min; range 30-105 min, p=0.89; Fig 4C). The duration of the STH sensitization response in the BIBN4096 group averaged 64.5±9.8 min (range 30-120 min) and also was not statistically different than that observed in the control group (average 73.9±8.7 min; range 30-120; p=0.54; Fig 4D). Finally, the magnitudes of the TH and STH sensitization responses in the BIBN treated group (TH; average 1.6±0.5 fold; range 0.3-4.8 fold; STH, average 1.6±0.1 fold; range 1.2-2.3) were not different than those observed in the control group (TH; average 1.8±0.4; range TH, range 0.3-4.8; STH, average 1.6±0.1 fold range 1.2-2.0; p=0.92 for TH; p=0.74 for STH; Fig. 4E,F).

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Discussion

The major findings of the following study are 1) Brief (~ 1min) strong stimulation of meningeal afferents using a CSD-related stimulus, namely local elaboration of K^+ concentration, gave rise to a delayed and prolonged increase in their ongoing activity but did not change their mechanosensitivity, 2) pretreatment with the CGRP receptor antagonist BIBN4096, blocked the development of the K^+ - related prolonged activation of meningeal afferents, and 3) a similar BIBN4096 pretreatment did not inhibit the prolonged activation or mechanical sensitization of meningeal afferents in response to CSD.

Despite the widely-held belief [11], the role of trigeminal axon reflex and the ensuing release and action of sensory pro-inflammatory neuropeptides as peripheral mediators of migraine pain remains unclear. Previous studies which examined the question of whether acute stimulation of primary afferent neurons, leading to axon reflex and release of proinflammatory sensory neuropeptides, influence the sensitivity of primary afferent nociceptive neurons have yielded opposing data. For example, in studies conducted in monkeys [42] and rats [43], antidromic stimulation of cutaneous nociceptive afferents did not subsequently alter their ongoing activity, mechanosensitivity or heat sensitivity. A study in rabbits, however, reported the development of heat sensitization of nociceptive afferents following antidromic stimulation [44]. Finally, a rat study which employed a local capsaicin stimulation to evoke acute excitation of cutaneous afferents also documented a delayed and prolonged increase in the afferents' ongoing activity and mechanosensitivity that were suggested to involve CGRP signaling [45]. Our present

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finding support a mechanism by which a strong, yet relatively brief (~60 sec), excitation of meningeal afferents at the level of their receptive field can give rise to a prolonged increase in their ongoing activity level, but does not affect their mechanosensitivity. The discrepancy between the development of prolonged ongoing activity and lack of mechanical sensitization in the current study is in agreement with previous finding from our lab which suggest that different mechanisms underlie these two nociceptive responses in meningeal afferents [24,35,46]. It may also reflect differences between the magnitude, or characteristics of the cutaneous response evoked by capsaicin and the meningeal response induced in by local K^+ stimulation. Testing and interpreting the effects of capsaicin on meningeal afferents in the context of axon reflex nevertheless will be difficult given the presence of vascular TRPV1 receptors [47]. Finally, the lack of intact tissue mast cells, which contain many afferent sensitizing molecules, in our meningeal afferent preparation [48-52] could have also contributed to the lack of mechanical sensitization in response to K^+ stimulation.

We employed meningeal of K^+ stimulation [5,22,23] as a surrogate stimulus for CSD to investigate whether acute excitation of meningeal afferents could lead to prolonged afferent responses. Such K^+ stimulus is also likely to promote meningeal CGRP release [29,30,37] thus potentially also linking the local release and action of CGRP to CSD [40,41]. Our finding that BIBN4096 treatment abrogated the delayed and prolonged activation of meningeal afferents evoked by K^+ stimulation suggests that meningeal release of CGRP and its action on non-neuronal meningeal receptors [53] is a key process underlying this afferent response. However, given that BIBN4096 was administered systemically (to

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allow action also on meningeal tissue that remained unexposed in our preparation) we cannot exclude the possibility that inhibition of CGRP receptors localized to the cell body of meningeal afferents in the trigeminal ganglion [53,54] also played a role. Nonetheless, while meningeal CGRP release is an established phenomenon [29,30,37], evidence supporting CGRP release in the TG as well as its action receptors localized to the TG cell bodies *in vivo* are currently lacking.

In addition to the evoked meningeal CGRP release, local stimulation with K^+ could have also stimulated adrenergic postganglionic autonomic fibers that innervate the meningeal vasculature [55] leading to local release and action of norepinephrine [31]. Such local sympathetic response could have influenced the excitability of meningeal afferents indirectly by promoting the release of prostaglandins [56] or other sensitizing factors from non-neuronal meningeal constituents, such as fibroblasts [57]. However, the earlier finding that norepinephrine signaling inhibit CGRP release from trigeminal afferents [58,59] suggest that activation of meningeal postganglionic sympathetic neurons did not play a role in mediating the prolonged afferent response following K^+ stimulation.

Our previous findings that local or systemic application of vasodilating doses of CGRP did not influence the ongoing activity of meningeal afferents [60] seem to contradict the current data. The possibility that the levels of CGRP elaborated in response to the K^+ stimulation in the current study exceeded the concentrations of CGRP employed in our previous work may be entertained. However, previous data suggest that stimulation of meningeal afferents either electrically or chemically with a mixture of inflammatory

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mediators or with 50mM K^+ leads to increased CGRP levels at the picomolar range [30,37,61], which is much lower than the micromolar range that failed to influence meningeal afferent responsiveness in our previous study. Differences in the durations of CGRP action between the studies is also an unlikely explanation. In our previous study, local CGRP action (measured using changes in local blood flow) lasted for at least 10 minutes when CGRP was applied locally and for ~15 min following systemic application. Thus, the duration of CGRP action in our previous work was likely to be longer than that induced by the release of CGRP in response to the 1 min excitation of the afferents by the K^+ stimulus in the present study. As a potential explanation for the seemingly discrepancy between the two studies we propose that the process underlying the K^+ evoked prolonged afferent activation, which requires CGRP, is multifactorial. The release of CGRP from activated meningeal afferents is likely to be accompanied by the release of other neuropeptides, and potentially other factors such as glutamate. These mediators, in turn could act upon meningeal immune, vascular and Schwann cells, which produce the final algesic mediators that enhance the activity of meningeal afferents. We proposed that CGRP action plays a key part in this cascade of events, through a synergistic effect [62,63], by reinforcing or enhancing the release of the algesic mediators from meningeal non-neuronal cells which express CGRP receptors [53].

Our data supports the view that activation of meningeal CGRP receptor signaling is unlikely to constitute the major contributing factor underlying the prolonged activation and sensitization of meningeal afferents following CSD. Our finding that BIBN4096 inhibited the CSD-evoked increase in CBF, a previously recognized CGRP mediated

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event [40,41] together with its ability to block neurogenic meningeal vasodilatation per se [15,38] also support the claim that acute CGRP-related responses (including its vasodilatory effect) are also not major contributing factors that mediated the meningeal nociceptive effect of CSD. That meningeal vasodilation (whether CGRP-dependent or not) may not be responsible for the CSD-related prolonged meningeal afferents' responses is also congruent with our previous finding that ablation of the meningeal parasympathetic innervation, which contributes to the prolonged post-CSD meningeal vascular response [20] did not inhibit the prolonged afferent response to CSD [22]. The discrepancy between the involvement of CGRP receptor signaling in mediating the K^+-driven prolonged afferents activation data but not in response to CSD also calls into question the notion that the initial short-lasting cortical efflux of excitatory molecules, in particular K^+, during the CSD wave directly contributes to the meningeal nociceptive response via a CGRP-dependent mechanism. Further studies are required to determine whether the CSD-evoked parenchymal release of K^+ or other excitatory molecules influence the activity and mechanosensitivity of meningeal afferents via different mechanisms.

While CSD can be efficiently induced by a cortical pin-prick stimulus, another common induction method, which has been used in numerous in vivo studies of headache mechanisms, involves epidural application of K^+ at high concentration (usually 1M). Our finding of acute and prolonged activation of meningeal afferents following epidural K^+ stimulation at a much lower dose, that does not promote CSD, thus have important implications for the interpretation of previous studies that used K^+ stimulation to study

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the effect of CSD on trigeminal/meningeal pain (e.g. [22,64-66]). Future studies that employ the CSD paradigm to study related trigeminal responses and their underlying mechanisms thus should be conscientious about the CSD induction method employed. The use of less invasive methods that induce CSD remotely, such as optogenetics [67], which may not influence meningeal afferents responsiveness *per se* may provide a better choice to study mechanisms of CSD-related meningeal nociception and its involvement in migraine headache.

Conflict of Interest: Authors report no conflict of interest

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Table 1:

	Before treatment		**60 min post treatment**	
	A-delta	**C**	**A-delta**	**C**
Control	0.7±0.1	1.1±0.2	0.6±0.1	1.0±0.2
BIBN4096	0.5+0.1	1.2±0.3	0.5±0.3	1.1±0.3

Table 1: Effect of pretreatment with BIBN4096 or its vehicle on the rate of ongoing activity (spikes/sec) of meningeal afferents prior to meningeal K^+ stimulation or CSD.

Table 2:

	Pre-treatment		**60 min post-treatment**	
	TH	STH	TH	STH
Control	1.3±0.3	8.2±1.0	2.0±0.7	8.3±0.9
BIBN4096	2.1±0.3	8.7±0.9	2.8±0.6	9.9±1.2

Table 2: Effect of pretreatment with BIBN4096 (n=33) or its vehicle (n=28) on baseline TH and STH responses (spikes/sec) prior to meningeal K^+ stimulation or CSD.

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Figure legends:

Figure 1. Raw data example of the delayed and prolonged activation of one C meningeal afferent following acute meningeal stimulation with K^+. **A.** Traces represent peristimulus time histograms (PSTH, 10-min epochs, 10-sec bin size). Average ongoing activity rate is denoted in parentheses. **B.** The protocol employed for meningeal application of K^+ (serial dilutions of KCl from 50 to 5mM/60 sec). Note the acute increase in afferent activity during the stimulation. **C.** Time course data depicting the ongoing activity level of the same afferent during baseline and every 5 min after K^+ stimulation. Red color areas represent the acute and delayed phases of neuronal activation.

Figure 2. Characteristics of the K^+ - driven prolonged activation of meningeal afferents and lack of effect on afferents' mechanosensitivity and the effect of BIBN4096. **A**, onset latency of the prolonged activation. **B**, Duration of the prolonged afferent activation. **C**, Magnitude of the K^+ - driven prolonged increase in ongoing activity of meningeal afferents in control animals (open symbols denote units not activated by K^+) and BIBN4096 pretreated animals. *** $p<0.01$. Meningeal K^+ - stimulus did not affect the responses of the afferents to threshold stimuli (TH, which normally evoked 1-3-Hz responses, **D.**) or suprathreshold stimuli (STH, which were usually ×2 of the threshold; 8-10 Hz responses, **E.**).

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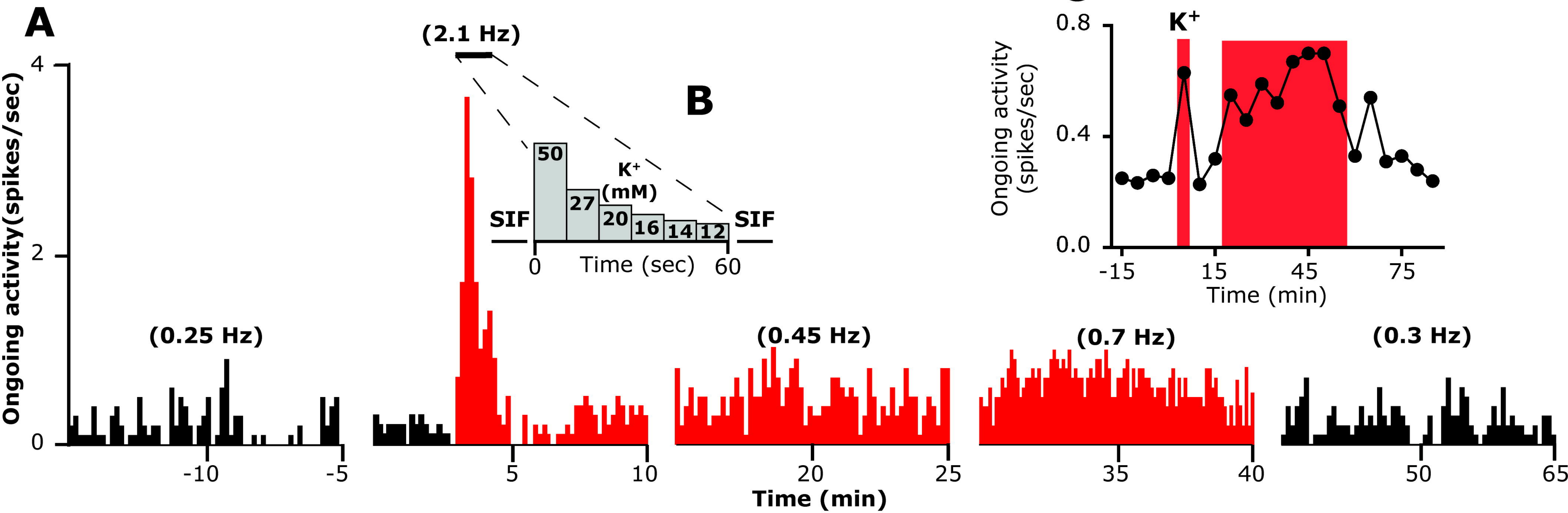
Figure 3. BIBN4096 does not affect the characteristics of the CSD-evoked prolonged activation of meningeal afferents. **A**, onset latency of the prolonged activation. **B**, Duration of the prolonged afferent activation. **C**, Magnitudes of the prolonged increase in ongoing activity rate.

Figure 4. BIBN4096 does not affect the prolonged increased in the mechanical sensitivity of meningeal afferents following CSD. Onset latency of the prolonged TH **(A)** and STH **(B)** sensitization responses. Durations of the prolonged TH (**C)** and STH **(D)** sensitization responses**.** Magnitudes of the prolonged TH **(E)** and STH **(F)** sensitizations.


A
(2.1 Hz)
Ongoing activity(spikes/sec)
4
2
0
B
50
K+
(mM)
27
20
16
14
12
SIF
SIF
0
Time (sec)
60
(0.25 Hz)
(0.45 Hz)
(0.7 Hz)
(0.3 Hz)
-10
-5
5
10
20
25
35
40
50
65
Time (min)
C
K+
0.8
0.4
0.0
Ongoing activity
(spikes/sec)
-15
15
45
75
Time (min)

A

Onset latency (min)

0 10 20 30 40

B

Duration (min)

0 20 40 60

C

Fold increase in responses

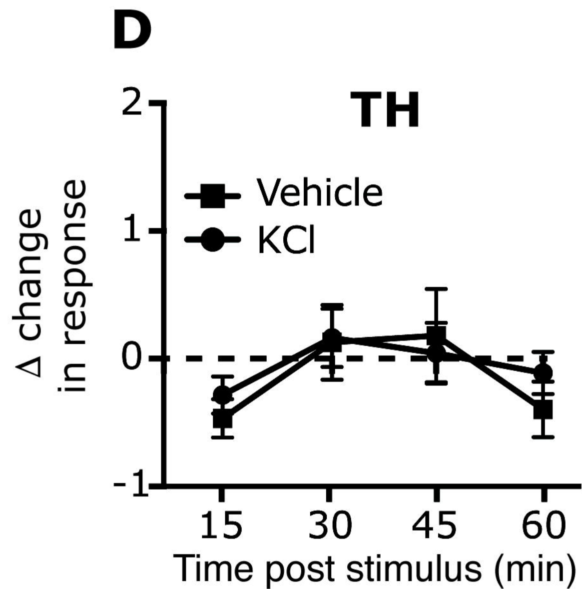
0 2 4 6

**

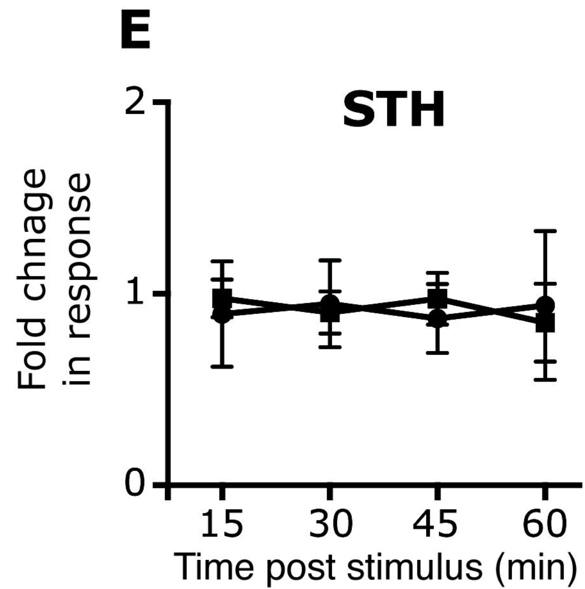
K+

BIBN/K+

D



E



A

Onset latency (min)

40
30
20
10
0

B

Duration (min)

60
40
20
0

C

Fold increase in Magnitude

8
6
4
2
0

Vehicle BIBN4096

TH

STH

A

Onset latency (min)

0
20
40
60

B

Onset latency (min)

0
20
40
60

C

Duration (min)

0
50
100
150

D

Duration (min)

0
50
100
150

E

Δ chnage in response

0
2
4
6

F

Fold increasei in responses

0
1
2
3

Vehicle BIBN4096

Vehicle BIBN4096