Prolonged reductions in cortical blood flow and tissue oxygen tension do not contribute to the activation and mechanical sensitization of meningeal afferents following CSD

Abbreviated title: Oligemia and hypoxia in CSD-evoked meningeal nociception.

Jun Zhao[1,2] and Dan Levy[1,2]

[1]Department of Anesthesia, Critical Care and Pain Medicine, Beth Israel Deaconess Medical Center and [2]Harvard Medical School, Boston, MA, USA 02215

Corresponding author: Dan Levy, Department of Anesthesia, DA717; 330 Brookline Avenue, Boston MA 02215; Tel: (617) 667-0534, dlevy1@bidmc.harvard.edu

Number of Pages: 41

Number of Figures: 10

Number of tables: 1

Number of Words: Abstract, 231; Introduction, 600; Discussion, 1574.

Conflict of Interest: The authors declare no competing financial interests

Acknowledgments: We thank Dr. A. Strassman for helpful comments on the manuscript. The study was supported by NIH grants NS086830, NS078263, NS101405 to DL.

Author contributions: J.Z., D.L., designed research; J.Z., D.L., performed research; J.Z., D.L., conducted data analyses; D.L. wrote the paper.

.

Abstract

The onset of the headache phase in migraine with aura is believed to involve cortical spreading depression (CSD) and the ensuing activation and sensitization of primary afferent neurons that innervate the intracranial meninges. How CSD enhances meningeal afferent responses remains, nevertheless, unclear. We employed extracellular single-unit recording of meningeal afferents activity and monitored changes in cerebral blood flow and tissue partial pressure of oxygen (tpO_2) in anesthetized male rats to test whether the prolonged cerebral oligemia and reduced tpO_2 that occur in the wake of CSD contribute to meningeal nociception. Inhibition of cortical cyclooxygenase (COX) activity using naproxen suppressed the CSD-induced cortical oligemia and decreased tpO_2 but did not block the associated activation of meningeal afferents. Naproxen, however, distinctly inhibited the CSD-induced afferent mechanical sensitization. Counteracting the CSD-evoked persistent oligemia and reduced (tpO_2), by preemptively increasing cerebral blood flow using the K(ATP) channel opener levcromakalim, inhibited CSD-evoked meningeal afferent activation but had no effect on the mechanical sensitization response. These results suggest that in the wake of CSD, the prolonged reductions in cerebral blood flow and tpO_2 do not contribute directly to the activation and mechanical sensitization of meningeal afferent responses and further point to distinct mechanisms underlying these distinct afferent responses to CSD. Targeting the activation and mechanical sensitization of meningeal afferents by combining COX inhibition and K(ATP)

channel activation could provide a novel therapeutic approach to treat migraine headache.

Significance statement: CSD-evoked activation and mechanical sensitization of meningeal afferents is considered a key mechanism underlying the onset of the headache phase in migraine with aura. We report that blocking the CSD-evoked prolonged cortical oligemia and reduced cortical tpO_2 by COX inhibition is associated with the inhibition of the afferent sensitization but not their activation, while normalization of these CSD-evoked metabolic perturbations by activating K(ATP) channels inhibits afferent activation but not their sensitization. These results question the contribution of cortical metabolic perturbations to the triggering mechanism underlying meningeal nociception and the ensuing headache in migraine with aura and further point to distinct mechanisms underlying the activation and sensitization of meningeal afferents in migraine and highlight the need to target both processes for an effective migraine therapy.

Introduction

Migraine headache affects about 15% of the global adult population. It is considered the seventh most disabling condition, and second leading cause of years lived with disability (Steiner et al., 2013; Disease et al., 2017; Gooch et al., 2017). The development of novel approaches for treating migraine headache is, however, limited by the poor understanding of its underlying mechanisms. It is now well accepted that activation and increased mechanosensitivity of trigeminal afferent neurons that innervate the intracranial meninges and their related large blood vessels play a key role in migraine headache genesis (Strassman et al., 1996; Messlinger, 2009; Olesen et al., 2009; Levy, 2012). However, the endogenous mechanisms the lead to these enhanced meningeal afferent responses in migraine remain incompletely understood.

A key mechanism implicated in the onset of the headache phase in migraine with aura, the second-most prevalent migraine type, is the phenomenon of cortical spreading depression (CSD) (Pietrobon and Moskowitz, 2014). The role of CSD as an endogenous triggering factor underlying the headache phase in migraine with aura is supported by the preclinical finding of CSD evoked persistent activation and increased mechanosensitivity of meningeal afferents (Zhang et al., 2010a; Zhao and Levy, 2015, 2016) and central trigeminal dorsal horn neurons that receive their input (Zhang et al., 2011). The precise mechanisms by which CSD activates and sensitizes meningeal afferents remain, nevertheless, unknown although numerous factors, including dural neurogenic

inflammation (Bolay et al., 2002); see however (Zhao and Levy, 2017) Panx1 and P2X7-related signaling have been hypothesized (Karatas et al., 2013; Chen et al., 2017).

Migraine with aura is associated with cortical hemodynamic changes, including an initial increase (i.e. hyperemia) followed by a sustained reduction in CBF (i.e. oligemia) contemporaneous with the onset of the headache phase (Olesen et al., 1981; Olesen et al., 1982; Lauritzen, 1984; Hadjikhani et al., 2001; Hansen and Schankin, 2017). In animal studies, CSD leads to a similar oligemic response (Fabricius and Lauritzen, 1993; Fabricius et al., 1995; Piilgaard and Lauritzen, 2009). This phase is accompanied by a transient cerebral hypoxia (Takano et al., 2007) that is followed by a prolonged and milder reduction in cerebral tissue oxygen levels (tpO_2) (Piilgaard and Lauritzen, 2009). The prolonged decreases in tpO_2 is likely due to the mismatch between the increase in O_2 consumption that is required for the extremely energetically demanding restoration of the ionic gradients after CSD and the reduced CBF (Shinohara et al., 1979; Piilgaard and Lauritzen, 2009).

The notion that metabolic perturbations associated with reduced blood flow and tissue oxygen tension can promote the activation and sensitization of primary afferent nociceptors in other tissues (MacIver and Tanelian, 1992; Fuchs et al., 2010) prompted us to investigate whether the cortical oligemia and reduced tpO_2 contribute to the enhanced responses of meningeal afferents following CSD. To address this question, we initially examined whether

abrogation of the CSD-evoked cortical oligemia by inhibiting cyclooxygenase (COX) with naproxen (Gariepy et al., 2017) can inhibit the related decrease in tpO_2 and interfere with the associated nociceptive responses of meningeal afferents. Having identified such effects, we also tested whether a preemptive increase in CBF to counteract the CSD-evoked persistent oligemia and reduced tpO_2, using the K(ATP) channel opener levcromakalim (Kleppisch and Nelson, 1995; Shin et al., 2003), could also inhibit the enhanced responses of meningeal afferents. Our data suggests that while COX inhibition and K(ATP) channel opening can abrogate the CSD-related cortical oligemia and related decreases in tpO_2 they exert distinct inhibitory effects on CSD-evoked meningeal afferent responses, thus questioning the contribution of CSD-evoked cortical metabolic perturbations to meningeal nociception and the onset of the headache of migraine with aura.

Materials and Methods

Animals, anesthesia and surgical preparation

The current study employed Sprague-Dawley rats (males, 250–350 g, Taconic). Rats were housed 3 per cage under a 12 h light/dark cycle, in a temperature- and humidity-controlled room in the animal care facility of the Beth Israel Deaconess Medical Center, Boston. Food and water were available *ad libitum.* All experimental procedures followed the “guide for Care and use of Laboratory Animal resources (NIH publication No. 85-23 revised 1996), were approved by

the Animal Care and Use Committee of the Beth Israel Deaconess Medical Center, and were in compliance with the ARRIVE (*Animal* Research: Reporting of In Vivo Experiments) guidelines. 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 were intubated and breathed spontaneously room air enriched with O_2. Physiological parameters were collected throughout the experiments using PhysioSuite (Kent Scientific) and CapStar-100 (CWE). Data used in this report were obtained from animals exhibiting physiological levels of oxygen saturation (>95%), heart rate (350–450 bpm), and end-tidal CO_2 (3.5–4.5%). A saline-cooled dental drill was used to perform 3 separate craniotomies (Figure 1A). One craniotomy was used to expose the left transverse sinus and the posterior part of the superior sagittal sinus, as well as the adjacent cranial dura, extending ~2 mm rostral to the transverse sinus. Another small craniotomy (1 × 1 mm) was made in the calvaria over the right hemisphere, and was centered 2 mm caudal and 2 mm lateral to Bregma to allow insertion of the recording electrode. An additional small burr hole (0.5-mm diameter) was drilled to expose a small area of the frontal cortex to induce CSD (Zhao and Levy, 2015, 2016). The exposed dura was bathed with a modified synthetic interstitial fluid containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.2. The femoral vein was cannulated to allow for intravenous administration.

Recording of meningeal afferent activity

Single-unit activity of meningeal afferents (1 afferent/rat) was recorded from their cell body in the ipsilateral (left) trigeminal ganglion using a contralateral approach, as described recently (Zhao and Levy, 2014, 2015, 2016) (and see Figure 1A). A platinum-coated tungsten microelectrode (impedance 50-100 kΩ, FHC) was then advanced through the right hemisphere into the left trigeminal ganglion using an angled (22.5°) trajectory. The insertion of the recording electrode using this approach does not produce CSD in the left cortical hemisphere (Zhao and Levy, 2015, 2016). Meningeal afferents were identified by their constant latency response to electrical stimuli 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 (Strassman et al., 1996). Neurons were classified as A-delta ($1.5 \leq CV \leq 5$ m/sec) or C afferents ($CV < 1.5$ m/sec). Neural activity was digitized and sampled at 10KHz using power 1401/spike 2 interface (CED). 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 to acquire and analyze afferent activity.

Assessment of changes in meningeal afferent mechanosensitivity

Mechanical receptive fields (RF) of meningeal afferents were first identified by probing the exposed dura with a 2.0 g von Frey filament (Stoelting). The site of

lowest mechanical threshold was further determined using monofilaments that exert lower forces (≥0.02 g). For quantitative determination of mechanical responsiveness, we applied mechanical stimuli to the site with the lowest threshold, using a servo force-controlled mechanical stimulator (Series 300B Dual Mode Servo System, Aurora Scientific), controlled via the power 1401 interface. The stimulus was delivered using a flat-ended cylindrical plastic probe that was attached to the tip of the stimulator arm. One of three probe diameters (0.5, 0.8 or 1.1 mm) was selected for each neuron, depending on the sensitivity of the neuron. Stimulus trials for testing mechanically sensitive were made using a custom-written script for Spike 2 and consisted of 'ramp and hold' stimuli (rise time 100 msec, stimulus width 2 sec, inter-stimulus interval 120 sec). Each trial included a threshold stimulus (which normally evoked at baseline 1-3 Hz responses) followed by a supra-threshold stimulus (usually X2 of the threshold pressure). To minimize response desensitization, stimulus trials were delivered every 15 min throughout the experiment (Zhang et al., 2013; Zhao and Levy, 2016). Ongoing afferent discharge rate was recorded continuously between the stimulation trials. Responses to mechanical stimuli were determined during at least four consecutive trials before the elicitation of CSD. Data was analyzed only from afferents that exhibited consistent responses (variation of <0.5 Hz for threshold responses and <1.5 Hz for suprathreshold responses) during baseline recordings (Zhao and Levy, 2016).

Induction of CSD

A single CSD episode was induced by quickly inserting a fine glass micropipette (diameter 10 μm) ~2 mm deep in the cortex for 2 sec (Zhang et al., 2010a). CSD was triggered 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 (Zhao and Levy, 2015).

Measurement of CSD-evoked changes in cerebral blood flow (CBF) using laser Doppler flowmetry

Changes in CBF (Gariepy et al., 2017) were documented using a needle probe (0.8 mm diameter) connected to a laser Doppler flowmeter (Vasamedic, St Paul MN). The probe was positioned just above the intact dura, ~1 mm from the mechanical RF of the recorded afferent in an area devoid of large pial blood vessels (>100 μm). Such system (using a near IR 785 nm probe and fiber separation of 0.25 mm) is expected to record CBF changes at a cortical depth of ~1 mm (Fabricius et al., 1997). The laser Doppler signal was digitized (100Hz) and recorded using power 1401/Spike 2. Laser Doppler recordings were obtained with the ambient lights turned off. Baseline data were based on recording conducted for at least 30 min prior to CSD.

Measurement of cortical partial pressure of oxygen (tpO_2)

Cortical tissue partial pressure of O_2 (tpO_2) was recorded using a modified Clark-type polarographic oxygen microelectrode (OX-10; Unisense) with a guard cathode. The oxygen electrode was connected to a high-impedance Pico-amperometer (PA2000; Unisense) that sensed the currents of the oxygen electrodes. The tpO_2 signal was then digitized (100 Hz) and recorded using the Power 1401/Spike 2 interface. The microelectrode responded linearly to changes in oxygen concentration and was calibrated before and after each experiment in both air-saturated saline and in oxygen-free solution consisting of 0.1 M of sodium-ascorbate and 0.1 M NaOH dissolved in saline. This approach has been used previously to record changes in cerebral tpO_2 in response to CSD (Takano et al., 2007; Piilgaard and Lauritzen, 2009; Thrane et al., 2013). Changes in tpO_2 values were recorded at a cortical depth of 100-200 μm, with the electrode placed <0.5 mm away from the LDF probe. Baseline tpO_2 values were recorded for at least 30 min prior to CSD.

Electrophysiological recording of CSD

A tungsten microelectrode (impedance 1-2Ω, FHC), was used to record multi-unit activity at a depth of 200-400 μM in the right visual cortex (S1). A silver ground electrode was placed in the neck muscle. Signal was amplified and filtered (X20K, 300Hz low frequency; 10KHz high frequency, DAM-80/BMA400, WPI/CWE), and then digitized and sampled at 10KHz. Cortical activity was

recorded simultaneously with CBF changes, with the recording electrode placed <0.5 mm away from the LDF probe.

Drugs

Naproxen sodium and Levcromakalim were purchased from Tocris Biosciences. Naproxen sodium was made in 0.9% immediately before administration. Levcromakalim stock solution (10 mM) was made in DMSO and was further diluted with synthetic interstitial fluid (final concentration 500μM) immediately before its application. The vasoactive concentration of levcromakalim expected to be attained at the superficial cortex was estimated to be 100-1000 times lower, based on a recent meningeal permeability study (Zhao et al., 2016).

Data analyses and statistics

All data were analyzed and plotted using Prism 7 software. Data is presented as [median (IQR)]. Group data in graphs shows all data points and is plotted as box and whiskers with min and max values. For CSD-related changes in CBF and tpO_2, raw data were processed with a custom-written script made for Spike 2. Baseline values were based on the average of the values collected during the 600 secs prior to the induction of CSD. Offline analyses for afferent responses were conducted using template matching in Spike 2. Criteria used to consider meningeal afferent activation and sensitization responses were based on our previous studies (Zhao and Levy, 2015, 2016). In brief, prolonged 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 for >10 min. Acute afferent activation during the CSD wave was considered as an increased ongoing discharge rate that occurred ≥30 sec following the pinprick and lasted ≤120 sec. Mechanical sensitization was considered only if the afferent responses changed according to the following criteria: threshold and/or suprathreshold 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. Changes in CSD-evoked responses of meningeal afferents in the presence of naproxen or levcromakalim were compared to data obtained in control animals using the same surgical approach and CSD induction method. Control data was obtained from experiments in animals treated intravenously with the naproxen vehicle (0.9% saline, 60 min prior to CSD induction, n=24), or locally (i.e. continuous dural bathing) with synthetic interstitial fluid (n=36). The two data sets were comparable and were combined to increase power. Differences in CSD-evoked afferent activation and sensitization propensities were analyzed using two-tailed, chi-square tests. Within- and between group comparisons were made using Wilcoxon test and Mann Whitney U-test respectively. Results were considered to be significant at $p<0.05$.

Results

CSD-evoked prolonged activation of meningeal afferents coincides with cerebral oligemia and reduced tpO_2

Protracted cerebral oligemia and reduced cerebral tpO_2 were recorded previously (Piilgaard and Lauritzen, 2009; Fordsmann et al., 2013), at time points corresponding to the prolonged activation of meningeal afferents (Zhang et al., 2010a; Zhao and Levy, 2015). We therefore initially verified, using simultaneous recording of meningeal afferent activity with CBF and tpO_2 (n=6), that CSD-evoked afferent activation indeed coincides with these cerebral metabolic perturbations. As the example in Figure 1B demonstrates, persistent meningeal afferent activation could be observed during the prolonged post-CSD cerebral oligemia and reduced tpO_2 stage.

Naproxen blocks CSD-evoked decrease in cortical tpO_2.

We have shown previously that naproxen, which does not block CSD induction, abrogates the prolonged cerebral oligemia that develops following CSD (Gariepy et al., 2017). Because cortical tpO_2 depends in large part on cortical perfusion, we examined whether the blockade of the cerebral oligemia by naproxen is associated with the inhibition of the CSD-induced changes in cortical tpO_2. We initially verified the effects of naproxen treatment on the CSD-evoked CBF changes. When compared to control animals (n=22), systemic treatment with naproxen (n=22) enhanced the CSD-related cerebral hyperemic responses and

blocked, as expected the prolonged cerebral oligemic response (Figure 2). The inhibition of the cortical oligemia by naproxen was associated with a blockade of the CSD-evoked changes in cortical tpO_2. In the presence of naproxen (n=6) there was an initial increase in tpO_2 [55.74 mmHg (33.66) [median (IQR)] to 68.2 mmHg (30.52) [median (IQR)]; W=6.0; p=0.031; phase 1 in Figure 3B, and arrowhead in the insert], which was smaller when compared to that observed in control animals [n=11; 48.76 mmHg (15.52) [median (IQR)] to 68.73 mmHg (32.24) [median (IQR)]; W=66.0; p=0.006] (U=7.0; p=0.007, Figure 3C). While in control animals, there was an acute decrease in tpO_2 to hypoxic levels [17.66 mmHg (18.64) [median (IQR)]; W= -66.0; p=0.001)] lasting 110.0 sec (70.30) [median (IQR)]; (phase 2, Figure 3A)], naproxen also completely blunted the acute hypoxic phase and tpO_2 increased significantly in all animals [113.4 mmHg (90.24) [median (IQR)]; W=21.0; p=0.015; phase 2, Figure 3B) before returning to baseline levels at 15-25 min [59.02 mmHg (39.63) [median (IQR)]; W=7.0; p=0.288; phase 3, Figure 3B]. Overall, CSD-evoked changes in tpO_2 were significantly different than those in control animals at both phase 2 (U=0; p<0.001; Figure 3D) and phase 3 (U=3; p=0.005; Figure 3E).

Suppression of CSD-evoked cortical oligemia and hypoxia by naproxen is not associated with the inhibition of CSD-evoked activation of meningeal afferents.

To determine whether suppression of the CSD-evoked cerebral oligemia and decrease in tpO_2 by naproxen were associated with the inhibition of the CSD-evoked acute and/or prolonged activation of meningeal afferents, we next investigated the effect of naproxen on CSD-evoked changes in 31 meningeal afferents (15 A-delta and 16 C). In the presence of naproxen, the baseline ongoing activity rate in the A-delta afferents was not different than that recorded in control animals [0.04 spikes/sec (0.20) [median (IQR)] vs 0.05 (0.25) [median (IQR)]; U=189.0; p=0.87]. The baseline ongoing activity of the C afferents was also not different between the naproxen and control groups [1.08 spikes/sec (1.58) [median (IQR)] vs 0.57 spikes/sec (1.35) [median (IQR)]; U=215.0; p=0.236]. In naproxen-treated animals, CSD evoked acute activation in 5/15 A-delta and 6/16 C, ratios which were not significantly different than those observed in control experiments (9/26 A-delta, 14/34 C; χ^2 =0.007; p=0.99 for the A-delta population; χ^2 =0.061; p=0.804 for the C afferent populations; χ^2=0.071; p=0.79 for the combined population). When compared to the control group, naproxen also did not reduce the magnitude of the immediate activation, neither in the A-delta [4.2 fold (3.50) [median (IQR)] vs 3.6 fold (2.10) [median (IQR)]; U=22.0;

p=0.99] nor in the C afferent [1.6 fold (0.50) [median (IQR)] vs 2.1 fold (1.40) [median (IQR)]; U=30.0; p=0.353, Figure 4C] populations.

The likelihood of developing a prolonged increase in ongoing afferent activity following CSD in naproxen-treated animals (see examples in Figure 5A, B) was also not different when compared to that observed in control animals (7/15 vs 13/26, χ^2 =0.04;2 p=0.837 for the A-delta population; 7/16 vs 22/34, χ^2 =1.961; p=0.164 for the C-afferent population; χ^2 =1.427; p=0.232 for the combined population). Naproxen treatment also did not affect any of the CSD-evoked afferent activation parameters: persistent afferent activation developed in the A-delta population with a delay of 25 min (40) [median (IQR)], no different than the onset latency observed in control animals [10 min (5.0) [median (IQR)]; U=33.0; p=0.34, Figure 5C]; C afferents also displayed a similar delay in activation [15 min (31.25) [median (IQR)] vs 5 min (17.5) [median (IQR)]; U=67.5; p=0.58, Figure 5C]. The duration of CSD-evoked prolonged activation was also unaffected by naproxen: A-delta afferents were activated for 15 min (15.0) [median (IQR)], vs 30 min (22.5) [median (IQR)] in the control group (U=22.0; p=0.07, Figure 5D); C-afferents were activated for 30 min (31.25) [median (IQR)] vs 35 min (30.0) [median (IQR)] in the control group (U=58.0; p=0.34, Figure 5D]. Finally, when compared to controls, naproxen treatment did not reduce the magnitude of the CSD-evoked activation observed in either afferent population [A-delta; 2.3 fold (3.0) [median (IQR)] vs 3 fold (3.8) [median (IQR)]; U=39.0; p=0.64; C afferents; 1.8 fold (3.0) [median (IQR)] vs. 1.5 fold (0.9) [median

(IQR)]; U=51.0; p=0.18, Figure 5E]. In afferents deemed not-activated by CSD, the changes in ongoing activity following CSD were also not different between the naproxen and control-treated groups, in both the A-delta (U=41.0; p=0.8) and C afferent (U=35.0; p=0.2) populations (Figure 5F).

Naproxen inhibits CSD-evoked meningeal afferent mechanosensitization

In a previous study (Zhao and Levy, 2016) we proposed that the mechanisms underlying the CSD-evoked prolonged mechanical sensitization and activation of meningeal afferents are distinct. We therefore examined next in 15 afferents (8 A-delta and 7 C) whether the inhibition of the cortical oligemia and decreased tpO_2 by naproxen was associated with amelioration of the mechano-sensitization response. Because the characteristics of the CSD-evoked sensitizations of the A-delta and C afferents are similar (Zhao and Levy, 2016), we combined the data from the two afferent populations for comparisons with the data obtained from vehicle-treated animals (23 afferents; 10 A-delta and 13 C). At baseline, the number of dural mechanosensitive RFs and mechanical responsiveness of afferents recorded in naproxen-treated animals were similar to those in the control group (Table 1). Unlike its ability to inhibit the CSD-evoked activation of meningeal afferents, naproxen blocked the CSD-evoked increases in mechanical response, both at the threshold [(3/15 vs 13/23); $\chi(1) = 4.968$; p=0.026; Figure 6C] and suprathreshold levels [(2/15 vs 13/23); $\chi(1) = 7.088$; p=0.008; Figure 6]. When sensitization was defined as enhanced responsiveness at either stimulus

level, there were also fewer sensitized afferents in the naproxen group (4/15 vs 17/23; $\chi(1) = 9.084$; $p<0.003$). When the responses of all sensitized and non-sensitized units were combined, naproxen also had an overall inhibitory effect on the changes in threshold responses [Δ0 spike/sec (0.6) [median (IQR)] vs Δ0.393 spikes/sec (1.43) [median (IQR)]; U=90.5; p=0.013, Figure 6G] and suprathreshold responses [0.857 fold (0.5) [median (IQR)] vs 1.2 fold (0.5) [median (IQR)]; U=22.0; p=0.004, Figure 6H].

The K(ATP) channel opener Levcromakalim counteracts CSD-evoked cerebral oligemia and cerebral hypoxia.

Naproxen may inhibit the CSD-evoked meningeal afferent mechanosensitization indirectly, by blocking the post-CSD oligemia and reduced tpO_2. However, it is also possible that the inhibition of sensitization is independent of these metabolic effects, since prostanoids (elaborated by CSD) whose synthesis is blocked by naproxen, exert a direct sensitizing effect on nociceptors (independent of metabolic effects), and naproxen also inhibits sensitization induced by inflammatory mediators (Levy et al., 2008). We therefore asked next whether opposing the CSD-evoked cortical oligemia and its associated decrease in tpO_2 using an alternative, COX-independent approach, could also inhibit the afferent sensitization response. We postulated that local application of a potent cerebral vasodilator could serve this purpose and tested the effects of levcromakalim, a selective ATP-sensitive potassium channel (K(ATP) opener with strong cerebral

vasorelaxation properties (Reid et al., 1995). Prior to the elicitation of CSD, administration of levcromakalim (n=28, 500μM, topical meningeal bathing, beginning 30 min prior to CSD elicitation) led to a significant increase in basal CBF [196.10% (72.7) [median (IQR)]; W=231.0; p<0.0001, Figure 7A]. Levcromakalim treatment did not inhibit the elicitation of CSD (n=3, Figure 7C), which was associated with a significant cerebral hyperemic response, albeit smaller in magnitude when compared to the response in control animals [105.3% (1.9) [median (IQR)] vs 180.3% (61.6) [median (IQR)]; U=0.0; p<0.0001; Figure 7E]. In the presence of levcromakalim, the CSD-evoked cerebral oligemia phase was blunted; CBF values recorded at 15-45 min post-CSD period were not different than those observed during the pre-drug baseline period [99.56% (39.67 [median (IQR)]; W=-8.0; p>0.9999), and increased afterwards [119.1% (54.9) [median (IQR)] at 60 min post-CSD; W=366.0; p<0.0001). Overall, the post-CSD oligemic change in the levcromakalim-treated animals was significantly different from the oligemic changes observed in control animals (U=51.0; p<0.0001; Figure 7F).

We next tested the effect of levcromakalim treatment on the CSD-evoked changes in cerebral tpO_2 (n=8). Prior to CSD elicitation, levcromakalim administration increased basal tpO_2 [46.61 mmHg (16.16) [median (IQR)] to 90.24 mmHg (42.4) [median (IQR)]; W=36.0; p=0.008; Figure 8A]. As in control experiments, CSD was associated with a further brief increase in tpO_2 [120.5 mmHg (56.3) [median (IQR)]; W=36.0; p=0.0078; phase 1 in Figure 8A,]. The

magnitude of this increase was similar to that observed in control animals [18.22 mmHg (10.32) [median (IQR)] vs 14.18 mmHg (3.87) [median (IQR)]; U=24.0; p=0.109]. While the duration of this hyperoxic phase was shorter in levcromakalim-treated animals when compared to control animals [70.0 sec (30.0) [median (IQR)] vs 100 sec (50.0) [median (IQR)]; U=11.0; p=0.048], overall the hyperoxia (AUC) was not different between the two groups (U=33.0; p=0.395; Figure 8C). Following the brief increase, there was a transient decrease in tpO_2 with bottom values not different than those observed in the control group [18.5 mmHg (26.36)] [median (IQR)] vs 17.66 mmHg (18.64) [median (IQR)]; U=32.0; p=0.351). However, the hypoxic phase in levcromakalim-treated animals was significantly shorter [20 sec (27.5) [median (IQR)] vs 110 sec (70.0) [median (IQR)] in controls; U=3.5; p=0.0002], yielding overall a significantly reduced hypoxic response [246.6 mmHg*sec (676.0) [median (IQR)] vs 2001.0 mmHg*sec (4883.9) [median (IQR)] in controls; U=7.0; p=0.002; Figure 8D]. Furthermore, in levcromakalim-treated animals, following the brief decrease, tpO_2 increased and values were not different than baseline when sampled at 5-10 min [49.49 mmHg (43.2) [median (IQR)]; U=26.0; p=0.46] as well as at the 10-20 min time-point [47.16 mmHg, 44.51)] [median (IQR)]; U=31.0; p=0.64]. Overall, when compared to control animals, the prolonged decrease in tpO_2 phase was significantly reversed in animals treated with levcromakalim (U=15.0; p=0.001; Phase 3 in Figure 8B, Figure 8E).

Levcromakalim inhibits CSD-evoked acute and prolonged meningeal afferent activation

Given its ability to counteract the CSD-evoked cortical metabolic changes, we next asked whether levcromakalim treatment could also ameliorate the CSD evoked afferent responses, in particular the sensitization response similar to naproxen. We initially examined the effect of levcromakalim treatment on CSD-evoked activation of meningeal afferents in 20 afferents (9 A-delta and 11 C). In the presence of levcromakalim, baseline ongoing activity in the A-delta [0.13 spikes/sec (0.31)] [median (IQR)] and C afferents [0.28 (0.89)] [median (IQR)] were not different than in control animals (U=91.0; p=0.324 and U=156.0; p=0.413 respectively). When compared to the control group, levcromakalim treatment significantly inhibited the acute activation [A-delta; 0/9 vs 9/26 A-delta in the control group; ($\chi(1)$ = 4.194, p=0.04); C afferents; 1/11 vs 14/34 in the control group; ($\chi(1)$=3.85, p=0.0497); combined population 1/20 vs 23/60; ($\chi(1)$ = 7.937; p=0.005); Figure 9C]. A similar pattern of inhibition was also noted for the development of the CSD-evoked prolonged afferent activation [A-delta; 1/9 vs 13/26; ($\chi(1)$ = 4.213; p=0.04); C afferents; 3/11 vs 22/34; ($\chi(1)$ = 4.717; p=0.03); combined population; 4/20 vs 35/60; ($\chi(1)$=8.822; p=0.003); Figure 9D].

Levcromakalim does not inhibit CSD-evoked meningeal afferent mechanosensitization

Finally, we tested the effect of levcromakalim of the development of CSD evoked mechanical sensitization in 17 afferents (7 A-delta; 10 C), which had a similar number of identified dural mechanosensitive RFs and baseline mechanical responsiveness as the afferent tested in the control group (Table 1). Unlike its effect on the CSD-evoked afferent activation, levcromakalim treatment did not inhibit the sensitization response (see an example in Figure 10A). When compared to control experiments, there were no differences in the propensities to develop sensitization to threshold [(10/17 vs 13/23 affected afferents; $\chi(1) = 0.04$; p=0.85)] or suprathreshold [(6/17 vs 14/23 affected afferents; $\chi(1) = 1.1$, p=0.29)] stimuli. When sensitization was defined as enhanced responsiveness at either the threshold or suprathreshold levels, the ratio of sensitized/non-sensitized was also not significantly different when compared to that observed in the control group [11/17 vs 17/23; ($\chi(1) = 0.2$, p=0.66)]. When compared to control animals, levcromakalim also did not affect the magnitude of the threshold sensitization responses [Δ1.73 spikes/sec (3.36) [median (IQR)] vs Δ1.3 spikes/sec (2.18) [median (IQR)]; U=38.5; p=0.1042); Figure 10D], or the suprathreshold responses [1.39 fold (0.5) [median (IQR)] vs 1.37 fold (0.54) [median (IQR)]; U=34.0; p=0.54; Figure 10E].

Discussion

Spreading cerebral oligemia has been demonstrated during the early stage of migraine with aura attacks and likely reflects a CSD-related vascular event (Ayata and Lauritzen, 2015). In our CSD rodent model, the decrease in CBF coincided with the emergence of the prolonged activation of meningeal afferents (Zhao and Levy, 2015). Decreases in tissue perfusion were proposed to drive ischemically-sensitive afferents in various tissues, including cornea, skin and abdominal viscera (Longhurst et al., 1991; MacIver and Tanelian, 1992; Fuchs et al., 2010). We found, however, that naproxen treatment did not block the CSD-evoked afferent activation despite its powerful inhibitory effect on the protracted cortical oligemia, suggesting that the decrease in CBF or downstream processes do not contribute directly to the induction or maintenance of the increase in meningeal afferent activity. That naproxen, a powerful anti-inflammatory agent, did not ameliorate the CSD-evoked activation of meningeal afferents was somehow surprising in light of its ability to inhibit the persistent activation of these afferents in response to the application of a mixture of inflammatory mediators to their meningeal receptive field (Levy et al., 2008) – a model of enhanced meningeal nociception (Strassman et al., 1996) and migraine pain (Oshinsky and Gomonchareonsiri, 2007; Edelmayer et al., 2009; De Felice et al., 2013). This discrepancy suggests that the prolonged activation of meningeal afferents in the wake of CSD occurs via a distinct mechanism, one that does not involve the local nociceptive actions of these classical inflammatory mediators (Strassman et al.,

1996) or vasoconstricting prostanoids that mediate the cerebral oligemia following CSD (Shibata et al., 1992; Gariepy et al., 2017).

CSD has been shown to promote persistent vasodilatation of the middle meningeal artery. This meningeal vascular response has been hypothesized to depend upon the prolonged activation of meningeal afferents and downstream signaling via a central parasympathetic reflex (Bolay et al., 2002). Importantly, a recent study demonstrated that this CSD-evoked meningeal vasodilatory response could be blocked by treatment with naproxen (Karatas et al., 2013). That naproxen did not inhibit the CSD-evoked activation of meningeal afferents together with previous data showing its inability to block parasympathetic outflow to the cranium (Akerman et al., 2012) suggests therefore that CSD-evoked dural vasodilatation is not driven by a neurogenic mechanism involving enhanced meningeal afferent ongoing activity. Our present results call into question the use of dural (or pial) artery vasodilation as a surrogate of increased meningeal afferent activity and the activation of the migraine pain pathway in preclinical studies.

We found that counteracting the protracted CSD-evoked cerebral oligemia and its related decrease in tpO_2, by preemptively increasing CBF using levcromakalim, was associated with the inhibition of the CSD-evoked acute and prolonged activation of meningeal afferents. We propose however that the inhibition of these afferent responses did not involve the normalization of the CSD-evoked changes in CBF and cortical tpO_2 given that naproxen, which also

abrogated these cortical metabolic perturbations, failed to inhibit the afferent activation. At present, we cannot exclude the possibility that the K(ATP)-mediated cerebral vasodilation we induced prior to the onset of CSD contributed somehow to the blockade of the post-CSD afferent activation. However, the findings that levcromakalim did not affect the baseline ongoing activity of the afferents and that other cerebral vasodilators, including nitric oxide and CGRP also do not affect the ongoing activity of meningeal afferents suggest that the cerebral vasodilation *per se* did not mediate the inhibition of the CSD-evoked afferent activation. It is also noteworthy that a previous study proposed that CSD leads to a brief endogenous activation of cerebral vascular K(ATP) (Shimizu et al., 2000), likely in response to the local release and ensuing action of CGRP on pial arteries (Colonna et al., 1994; Wahl et al., 1994; Gozalov et al., 2008). These findings further support the view that the inhibition of the CSD-evoked afferent activation by levcromakalim we observed in the current study did not involve its cerebral or potentially meningeal vascular action.

Cortical neurons also express K(ATP) channels (Karschin et al., 1997) and their prolonged activation by levcromakalim could have resulted in diminished cortical excitability (Soundarapandian et al., 2007), potentially affecting the elicitation of CSD. However, levcromakalim did not affect the induction on CSD and particularly not the initial strong depolarization prior to the prolonged suppression of cortical neural activity. K(ATP) opening in cortical neurons could, nonetheless, reduce the release of transmitters with pro-

nociceptive action such as glutamate (Zhao et al., 2013) that may contribute to the acute and potentially the prolonged activation of meningeal afferents. Cortical astrocytes also express K(ATP) channels (Thomzig et al., 2001) and their activation could potentially decrease the high extracellular K^+ level that is attained during CSD (Kraig and Nicholson, 1978) by enhancing K^+ "spatial buffering" (Kofuji and Newman, 2004). In response to CSD, some of the elaborated K^+ in the cortical extracellular space has been suggested to diffuse into the leptomeninges, leading directly to the acute activation of meningeal afferent and potentially also to their prolonged activation, at least in part (Pietrobon and Moskowitz, 2012; Zhao and Levy, 2017). Activation of astrocytic K(ATP) channels leading to a decrease in parenchymal K^+ thus could have potentially inhibited the CSD-evoked afferent activation. Our recent finding that K^+-evoked acute activation of meningeal afferents did not promote mechanical sensitization (Zhao and Levy, 2017) is also in agreement with our observation that levcromakalim did not inhibit the CSD-evoked mechanical sensitization response. Finally, activation of K(ATP) channels expressed at the peripheral nerve endings of meningeal afferents could have also potentially contributed to the inhibition of the afferent activation by reducing their overall excitability (Chi et al., 2007). However, the finding that levcromakalim did not inhibit the mechanical sensitization argues against such a mechanism.

Cerebral hypoxia has also been suggested as a turning point in the pathogenesis of a migraine attack (Amery, 1982). Low oxygen levels during

acute exposure to high altitude has been proposed to promote headache and trigger migraine (Appenzeller, 1994; Broessner et al., 2016). Several hours of normobaric hypoxia can also trigger a migraine attack in susceptible individuals (Schoonman et al., 2006; Arngrim et al., 2016). We also observed that naproxen, in addition to blocking the CSD-evoked cerebral oligemia, also abrogated the decrease in cortical tpO_2 and these effects were associated with a significant and distinct inhibition of the afferents' mechanical sensitization. That levcromakalim also counteracted the cortical oligemia, greatly reduced the acute hypoxia phase, and normalized the prolonged decrease in tpO_2 values but did not inhibit the enhanced mechanical responsiveness suggests though that the prolonged reduction in CBF and cerebral tpO_2 *per se* also do not contribute to the mechanical sensitization. The finding that while levcromakalim treatment normalized the CBF and tpO_2 (to values not different than those observed before CSD) it did not inhibit the actual decrease in CBF and tpO_2 values following CSD suggests that the mediators that contribute to these metabolic changes (likely COX-derived; see below), rather than reduced CBF and tpO_2 values are responsible for the sensitization response. Finally, it is possible that the acute hypoxic phase, which occurred in levcromakalim-treated animals (albeit for only a fraction of the time noted in control animals) may have contributed somehow to the afferent sensitization process. However, we also observed a brief acute decrease in cortical tpO_2 in some naproxen-treated animals, further suggesting a dissociation between the acute cortical hypoxic phase and the afferent changes.

The discrepancy between the effects of naproxen and levcromakalim on the CSD-evoked afferent activation and mechanical sensitization responses supports the view that these two migraine-related nociceptive responses are mediated by distinct mechanisms (Levy and Strassman, 2004; Zhang et al., 2010b; Zhang et al., 2013; Zhao and Levy, 2016). Naproxen's site of action and the nature of the COX-derived factors that distinctly promote the sensitization of meningeal afferents following CSD require further studies. The two major COX isoforms, COX-1 and COX-2, are constitutively expressed in vascular and immune dural cells, as well as in peptidergic dural afferents (Zhang et al., 2009), and COX-derived mediators have been suggested to promote the mechanical sensitization of meningeal afferents evoked by local action of tumor necrosis alpha (Zhang et al., 2010b). CSD-evoked release of COX-derived sensitizing mediators from cortical neurons and glia, and particularly from the glial limitans that interface with the meninges (Xu et al., 2004), could also contribute to the afferent sensitization. The mode of cortex-to-meninges transport of COX-derived prostanoids could involve the glymphatic system (Iliff et al., 2012; Schain et al., 2017). In particular, the transport of sensitizing factors from the parenchymal interstitial space into the CSF, where they could interact with meningeal afferents that terminate in the subarachnoid space and arachnoid granulations (von During and Andres, 1991; Fricke et al., 1997). Additional flow of sensitizing mediators from the subarachnoid CSF into the dural lymphatics system (Raper et al., 2016; Louveau et al., 2017) may also affect the responses of dural afferents.

In summary, our findings suggest that in the wake of CSD, reduced CBF and decreased cerebral tpO_2 do not contribute directly to the prolonged activation and mechanical sensitization of meningeal afferent neurons, and thus may not contribute to the onset of headache pain, at least not in migraine with aura. The findings that K(ATP) activation and COX inhibition have distinct effects on the CSD-evoked activation and sensitization of meningeal afferents may explain the ineffectiveness of COX inhibitors in many cases of migraine (Suthisisang et al., 2010). A combination therapy, which includes a COX inhibitor and a K(ATP) channel opener, could be explored for treating headache in migraine with aura.

References

Akerman S, Holland PR, Summ O, Lasalandra MP, Goadsby PJ (2012) A translational in vivo model of trigeminal autonomic cephalalgias: therapeutic characterization. Brain 135:3664-3675.

Amery WK (1982) Brain hypoxia: the turning-point in the genesis of the migraine attack? Cephalalgia 2:83-109.

Appenzeller O (1994) High-altitude headache. Cephalalgia 14:317-318.

Arngrim N, Schytz HW, Britze J, Amin FM, Vestergaard MB, Hougaard A, Wolfram F, de Koning PJ, Olsen KS, Secher NH, Larsson HB, Olesen J, Ashina M (2016) Migraine induced by hypoxia: an MRI spectroscopy and angiography study. Brain 139:723-737.

Ayata C, Lauritzen M (2015) Spreading Depression, Spreading Depolarizations, and the Cerebral Vasculature. Physiol Rev 95:953-993.

Bolay H, Reuter U, Dunn AK, Huang Z, Boas DA, Moskowitz MA (2002) Intrinsic brain activity triggers trigeminal meningeal afferents in a migraine model. Nat Med 8:136-142.

Broessner G, Rohregger J, Wille M, Lackner P, Ndayisaba JP, Burtscher M (2016) Hypoxia triggers high-altitude headache with migraine features: A prospective trial. Cephalalgia 36:765-771.

Chen SP, Qin T, Seidel JL, Zheng Y, Eikermann M, Ferrari MD, van den Maagdenberg A, Moskowitz MA, Ayata C, Eikermann-Haerter K (2017) Inhibition of the P2X7-PANX1 complex suppresses spreading depolarization and neuroinflammation. Brain.

Chi XX, Jiang X, Nicol GD (2007) ATP-sensitive potassium currents reduce the PGE2-mediated enhancement of excitability in adult rat sensory neurons. Brain Res 1145:28-40.

Colonna DM, Meng W, Deal DD, Busija DW (1994) Calcitonin gene-related peptide promotes cerebrovascular dilation during cortical spreading depression in rabbits. Am J Physiol 266:H1095-1102.

De Felice M, Eyde N, Dodick D, Dussor GO, Ossipov MH, Fields HL, Porreca F (2013) Capturing the aversive state of cephalic pain preclinically. Ann Neurol 74:257-265.

Disease GBD, Injury I, Prevalence C (2017) Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet 390:1211-1259.

Edelmayer RM, Vanderah TW, Majuta L, Zhang ET, Fioravanti B, De Felice M, Chichorro JG, Ossipov MH, King T, Lai J, Kori SH, Nelsen AC, Cannon KE, Heinricher MM, Porreca F (2009) Medullary pain facilitating neurons mediate allodynia in headache-related pain. Ann Neurol 65:184-193.

Fabricius M, Lauritzen M (1993) Transient hyperemia succeeds oligemia in the wake of cortical spreading depression. Brain Res 602:350-353.

Fabricius M, Akgoren N, Lauritzen M (1995) Arginine-nitric oxide pathway and cerebrovascular regulation in cortical spreading depression. Am J Physiol 269:H23-29.

Fabricius M, Akgoren N, Dirnagl U, Lauritzen M (1997) Laminar analysis of cerebral blood flow in cortex of rats by laser-Doppler flowmetry: a pilot study. J Cereb Blood Flow Metab 17:1326-1336.

Fordsmann JC, Ko RW, Choi HB, Thomsen K, Witgen BM, Mathiesen C, Lonstrup M, Piilgaard H, MacVicar BA, Lauritzen M (2013) Increased 20-HETE synthesis explains reduced cerebral blood flow but not impaired neurovascular coupling after cortical spreading depression in rat cerebral cortex. J Neurosci 33:2562-2570.

Fricke B, von During M, Andres KH (1997) Topography and immunocytochemical characterization of nerve fibers in the leptomeningeal compartments of the rat. A light- and electron-microscopical study. Cell Tissue Res 287:11-22.

Fuchs D, Birklein F, Reeh PW, Sauer SK (2010) Sensitized peripheral nociception in experimental diabetes of the rat. Pain 151:496-505.

Gariepy H, Zhao J, Levy D (2017) Differential contribution of COX-1 and COX-2 derived prostanoids to cortical spreading depression-Evoked cerebral oligemia. J Cereb Blood Flow Metab 37:1060-1068.

Gooch CL, Pracht E, Borenstein AR (2017) The burden of neurological disease in the United States: A summary report and call to action. Ann Neurol 81:479-484.

Gozalov A, Jansen-Olesen I, Klaerke D, Olesen J (2008) Role of K ATP channels in cephalic vasodilatation induced by calcitonin gene-related peptide, nitric

oxide, and transcranial electrical stimulation in the rat. Headache 48:1202-1213.
Hadjikhani N, Sanchez Del Rio M, Wu O, Schwartz D, Bakker D, Fischl B, Kwong KK, Cutrer FM, Rosen BR, Tootell RB, Sorensen AG, Moskowitz MA (2001) Mechanisms of migraine aura revealed by functional MRI in human visual cortex. Proc Natl Acad Sci U S A 98:4687-4692.
Hansen JM, Schankin CJ (2017) Cerebral hemodynamics in the different phases of migraine and cluster headache. J Cereb Blood Flow Metab:271678X17729783.
Iliff JJ, Wang M, Liao Y, Plogg BA, Peng W, Gundersen GA, Benveniste H, Vates GE, Deane R, Goldman SA, Nagelhus EA, Nedergaard M (2012) A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta. Sci Transl Med 4:147ra111.
Karatas H, Erdener SE, Gursoy-Ozdemir Y, Lule S, Eren-Kocak E, Sen ZD, Dalkara T (2013) Spreading depression triggers headache by activating neuronal Panx1 channels. Science 339:1092-1095.
Karschin C, Ecke C, Ashcroft FM, Karschin A (1997) Overlapping distribution of K(ATP) channel-forming Kir6.2 subunit and the sulfonylurea receptor SUR1 in rodent brain. FEBS Lett 401:59-64.
Kleppisch T, Nelson MT (1995) ATP-sensitive K+ currents in cerebral arterial smooth muscle: pharmacological and hormonal modulation. Am J Physiol 269:H1634-1640.
Kofuji P, Newman EA (2004) Potassium buffering in the central nervous system. Neuroscience 129:1045-1056.
Kraig RP, Nicholson C (1978) Extracellular ionic variations during spreading depression. Neuroscience 3:1045-1059.
Lauritzen M (1984) Long-lasting reduction of cortical blood flow of the brain after spreading depression with preserved autoregulation and impaired CO2 response. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 4:546-554.
Levy D (2012) Endogenous mechanisms underlying the activation and sensitization of meningeal nociceptors: the role of immuno-vascular interactions and cortical spreading depression. Curr Pain Headache Rep 16:270-277.
Levy D, Strassman AM (2004) Modulation of Dural Nociceptor Mechanosensitivity by the Nitric Oxide - Cyclic GMP Signaling Cascade. J Neurophysiol 92:766-772.
Levy D, Zhang XC, Jakubowski M, Burstein R (2008) Sensitization of meningeal nociceptors: inhibition by naproxen. Eur J Neurosci 27:917-922.
Longhurst JC, Rotto DM, Kaufman MP, Stahl GL (1991) Ischemically sensitive abdominal visceral afferents: response to cyclooxygenase blockade. Am J Physiol 261:H2075-2081.

Louveau A, Plog BA, Antila S, Alitalo K, Nedergaard M, Kipnis J (2017) Understanding the functions and relationships of the glymphatic system and meningeal lymphatics. J Clin Invest 127:3210-3219.
MacIver MB, Tanelian DL (1992) Activation of C fibers by metabolic perturbations associated with tourniquet ischemia. Anesthesiology 76:617-623.
Messlinger K (2009) Migraine: where and how does the pain originate? Exp Brain Res 196:179-193.
Olesen J, Larsen B, Lauritzen M (1981) Focal hyperemia followed by spreading oligemia and impaired activation of rCBF in classic migraine. Ann Neurol 9:344-352.
Olesen J, Burstein R, Ashina M, Tfelt-Hansen P (2009) Origin of pain in migraine: evidence for peripheral sensitisation. Lancet Neurol 8:679-690.
Olesen J, Lauritzen M, Tfelt-Hansen P, Henriksen L, Larsen B (1982) Spreading cerebral oligemia in classical- and normal cerebral blood flow in common migraine. Headache 22:242-248.
Oshinsky ML, Gomonchareonsiri S (2007) Episodic dural stimulation in awake rats: a model for recurrent headache. Headache 47:1026-1036.
Pietrobon D, Moskowitz MA (2012) Pathophysiology of Migraine. Annual review of physiology 75:365-391.
Pietrobon D, Moskowitz MA (2014) Chaos and commotion in the wake of cortical spreading depression and spreading depolarizations. Nat Rev Neurosci 15:379-393.
Piilgaard H, Lauritzen M (2009) Persistent increase in oxygen consumption and impaired neurovascular coupling after spreading depression in rat neocortex. J Cereb Blood Flow Metab 29:1517-1527.
Raper D, Louveau A, Kipnis J (2016) How Do Meningeal Lymphatic Vessels Drain the CNS? Trends Neurosci 39:581-586.
Reid JM, Davies AG, Ashcroft FM, Paterson DJ (1995) Effect of L-NMMA, cromakalim, and glibenclamide on cerebral blood flow in hypercapnia and hypoxia. Am J Physiol 269:H916-922.
Schain AJ, Melo-Carrillo A, Strassman AM, Burstein R (2017) Cortical Spreading Depression Closes Paravascular Space and Impairs Glymphatic Flow: Implications for Migraine Headache. J Neurosci 37:2904-2915.
Schoonman GG, Sandor PS, Agosti RM, Siccoli M, Bartsch P, Ferrari MD, Baumgartner RW (2006) Normobaric hypoxia and nitroglycerin as trigger factors for migraine. Cephalalgia 26:816-819.
Shibata M, Leffler CW, Busija DW (1992) Pial arteriolar constriction following cortical spreading depression is mediated by prostanoids. Brain Res 572:190-197.
Shimizu K, Bari F, Busija DW (2000) Glibenclamide enhances cortical spreading depression-associated hyperemia in the rat. Neuroreport 11:2103-2106.
Shin HK, Lee JH, Kim CD, Kim YK, Hong JY, Hong KW (2003) Prevention of impairment of cerebral blood flow autoregulation during acute stage of

subarachnoid hemorrhage by gene transfer of Cu/Zn SOD-1 to cerebral vessels. J Cereb Blood Flow Metab 23:111-120.

Shinohara M, Dollinger B, Brown G, Rapoport S, Sokoloff L (1979) Cerebral glucose utilization: local changes during and after recovery from spreading cortical depression. Science 203:188-190.

Soundarapandian MM, Zhong X, Peng L, Wu D, Lu Y (2007) Role of K(ATP) channels in protection against neuronal excitatory insults. J Neurochem 103:1721-1729.

Steiner TJ, Stovner LJ, Birbeck GL (2013) Migraine: the seventh disabler. J Headache Pain 14:1.

Strassman AM, Raymond SA, Burstein R (1996) Sensitization of meningeal sensory neurons and the origin of headaches. Nature 384:560-564.

Suthisisang CC, Poolsup N, Suksomboon N, Lertpipopmetha V, Tepwitukgid B (2010) Meta-analysis of the efficacy and safety of naproxen sodium in the acute treatment of migraine. Headache 50:808-818.

Takano T, Tian GF, Peng W, Lou N, Lovatt D, Hansen AJ, Kasischke KA, Nedergaard M (2007) Cortical spreading depression causes and coincides with tissue hypoxia. Nat Neurosci 10:754-762.

Thomzig A, Wenzel M, Karschin C, Eaton MJ, Skatchkov SN, Karschin A, Veh RW (2001) Kir6.1 is the principal pore-forming subunit of astrocyte but not neuronal plasma membrane K-ATP channels. Mol Cell Neurosci 18:671-690.

Thrane AS, Takano T, Rangroo Thrane V, Wang F, Peng W, Ottersen OP, Nedergaard M, Nagelhus EA (2013) In vivo NADH fluorescence imaging indicates effect of aquaporin-4 deletion on oxygen microdistribution in cortical spreading depression. J Cereb Blood Flow Metab 33:996-999.

von During M, Andres KH (1991) Sensory nerve fiber terminals in the arachnoid granulations of non-human primates. Neurosci Lett 127:121-124.

Wahl M, Schilling L, Parsons AA, Kaumann A (1994) Involvement of calcitonin gene-related peptide (CGRP) and nitric oxide (NO) in the pial artery dilatation elicited by cortical spreading depression. Brain Res 637:204-210.

Xu HL, Koenig HM, Ye S, Feinstein DL, Pelligrino DA (2004) Influence of the glia limitans on pial arteriolar relaxation in the rat. Am J Physiol Heart Circ Physiol 287:H331-339.

Zhang X, Kainz V, Zhao J, Strassman AM, Levy D (2013) Vascular extracellular signal-regulated kinase mediates migraine-related sensitization of meningeal nociceptors. Ann Neurol 73:741-750.

Zhang X, Levy D, Noseda R, Kainz V, Jakubowski M, Burstein R (2010a) Activation of meningeal nociceptors by cortical spreading depression: implications for migraine with aura. J Neurosci 30:8807-8814.

Zhang X, Levy D, Kainz V, Noseda R, Jakubowski M, Burstein R (2011) Activation of central trigeminovascular neurons by cortical spreading depression. Ann Neurol 69:855-865.

Zhang XC, Kainz V, Burstein R, Levy D (2010b) Tumor necrosis factor-alpha induces sensitization of meningeal nociceptors mediated via local COX and p38 MAP kinase actions. Pain 152:140-149.
Zhang XC, Kainz V, Jakubowski M, Burstein R, Strassman A, Levy D (2009) Localization of COX-1 and COX-2 in the intracranial dura mater of the rat. Neurosci Lett 452:33-36.
Zhao J, Levy D (2014) The sensory innervation of the calvarial periosteum is nociceptive and contributes to headache-like behavior. Pain 155:1392-1400.
Zhao J, Levy D (2015) Modulation of intracranial meningeal nociceptor activity by cortical spreading depression: a reassessment. J Neurophysiol 113:2778-2785.
Zhao J, Levy D (2016) Cortical Spreading Depression Promotes Persistent Mechanical Sensitization of Intracranial Meningeal Afferents: Implications for the Intracranial Mechanosensitivity of Migraine. eNeuro 3:ENEURO.0287-0216.2016.
Zhao J, Levy D (2017) The CGRP receptor antagonist BIBN4096 inhibits prolonged meningeal afferent activation evoked by brief local K+ stimulation but not cortical spreading depression-induced afferent sensitization. PAIN Reports In Press:1.
Zhao J, Bree D, Harrington MG, Strassman AM, Levy D (2016) Cranial dural permeability of inflammatory nociceptive mediators: Potential implications for animal models of migraine. Cephalalgia.
Zhao K, Wen R, Wang X, Pei L, Yang Y, Shang Y, Bazan N, Zhu LQ, Tian Q, Lu Y (2013) EPAC inhibition of SUR1 receptor increases glutamate release and seizure vulnerability. J Neurosci 33:8861-8865.

FIGURE LEGENDS

Figure 1. ***A,*** Experimental setup: 3 skull openings (red ovals) were made. A small burr hole was made over the left frontal cortex to elicit a single cortical spreading depression (CSD) event using a pinprick (PP) stimulation. Meningeal afferent activity was recorded in the left trigeminal ganglion (TG) using a tungsten microelectrode inserted through a craniotomy made over the contralateral hemisphere. An ipsilateral craniotomy was made to expose a part of the left transverse sinus (TS) and superior sagittal sinus (SSS) and their vicinity to

search for mechanosensitive meningeal afferents. Quantitative mechanical stimuli were delivered to the afferents receptive field using a feedback-controlled mechanical stimulator. Laser Doppler flowmetry (LDF) probe was placed over the cortex near the afferent's receptive field to record changes in cerebral blood flow (CBF) and validate the induction of CSD non-invasively. In some animals, we also recorded the induction of CSD by monitoring multi-unit activity (MUA) of cortical neurons. An oxygen microelectrode was place in the superficial cortex to record CSD-evoked changes in cortical tissue partial pressure of oxygen (tpO_2). ***B***, Example of raw data recording depicting the activation of a C-afferent meningeal afferent (with an overdrawn spike waveform used for data analysis) following the elicitation of CSD with a pinprick (PP) during the prolonged reduction in cerebral blood flow (CBF) and cortical tpO_2. Afferent recording trace represents a peristimulus time histogram (PSTH, 1 second bin-size). Average ongoing activity rate (spikes/s) are denoted in parentheses. The activation phase and associated oligemia and reduced tpO_2 are colored red. LDF values are in arbitrary units (AU).

.

Figure 2. The effect of systemic naproxen on CSD-induced changes in CBF. ***A***, Changes in CBF following induction of CSD in vehicle- and naproxen-treated animals. **C**, CBF changes during the hyperemia stage, showing the enhancing effect of naproxen. **D,** CBF changes during the oligemic phase, 10-60 min following CSD, depicting the inhibitory effect of naproxen. P values based on a Mann Whitney U-test.

Figure 3. Naproxen inhibits CSD-induced decreases in cortical tpO_2. Time–course of changes in tpO_2 following CSD induction in control (***A***) and naproxen-treated animals (***B***). Comparisons between CSD-evoked changes in control and naproxen-treated animals during the hyperoxia phase (phase 1), the acute hypoxia phase (phase 2, in control animals) and at 15-25 min post-CSD (phase 3) (***C, D, E***). P values based on a Mann Whitney U-test.

Figure 4. Naproxen treatment does not inhibit CSD-evoked acute activation of meningeal afferents. Examples of experimental trials depicting the acute activation of an A-delta afferent (***A***) and a C afferent (***B***) following the induction of CSD using a pinprick (PP) stimulus in animals treated with naproxen. Traces represent post-stimulus time histogram (1 second bin-size). Average ongoing activity rate (spikes/sec) is denoted in parentheses. Red areas represent the acute phase of afferent activation during CSD. Raw LDF traces are provided below each recording showing the CSD-evoked increase in CBF. Comparisons of the magnitude of change in ongoing activity between afferents recorded in control and naproxen-treated animals depicting activated (***C***) and non-activated afferents (***D***). P values based on a Mann Whitney U-test.

Figure 5. Naproxen treatment does not inhibit CSD-evoked prolonged activation of meningeal afferents. Experimental trials depicting the CSD-evoked prolonged activation of an A-delta afferent (***A***) and a C afferent (***B***) in animals treated with

naproxen. Naproxen treatment did not affect the onset latency of the afferent activation (***C***), its duration (***D***), or magnitude (**E**). Changes in ongoing activity rate in non-activated afferents in naproxen-treated animals were not different than those in controls (***F***). P values based on a Mann Whitney U-test.

Figure 6. Naproxen inhibits CSD-induced mechanical sensitization of meningeal afferents. Examples of experimental trials depicting the responses (post-stimulus time histogram; 0.1 sec) of two C afferents to threshold (TH) and suprathreshold (STH) mechanical stimuli (green traces) applied to the their dural receptive field during baseline recording and then at 30 minutes following CSD elicitation in control (***A***) and naproxen-treated animals (***B***). Note the lack of sensitization following naproxen treatment. Individual threshold and suprathreshold responses of sensitized and non-sensitized afferents in control and naproxen-treated animals (***C-F***). Magnitudes of CSD-related threshold (***G***) and suprathreshold (***H***) changes in mechanosensitivity depicting the inhibition of sensitization by naproxen. P values based on a Mann Whitney U-test.

Figure 7. Effects of the K(ATP) channel opener levcromakalim on CSD elicitation and CSD-evoked CBF changes. **A**. Levcromakalim increased basal CBF. Examples of combined recording of multiunit recording of cortical neuronal activity (post-stimulus time histogram; 1 sec in middle panels) and CBF (bottom panels) showing a similar induction of CSD in control (***B***) and levcromakalim-

treated animals (***C***). Note the initial depolarization (increased cortical activity; arrows) followed by the suppression and gradual recovery of neural activity. Also note the CSD-evoked cortical hyperemic response in the control animal and the diminished response in levcromakalim-treated animal (arrowheads). ***D***. Time-course changes of CBF in control and levcromakalim-treated animals following PP-induced CSD. Note that despite the decrease in CBF in levcromakalim-treated animals, values remained near pre-drug baseline values (denoted as 100%) unlike the marked decrease in control animals. ***E***. CBF changes in control and levcromakalim-treated animals during the hyperemia stage. ***F***. CBF changes during the oligemic phase, 10-60 min following CSD. P values based on a Mann Whitney U-test.

Figure 8. Levcromakalim inhibits CSD-evoked cortical hypoxia. Time-course changes (median and IQR) in cortical tpO_2 values at baseline (BL) and during the acute hyperoxia and hypoxia phases in response to CSD induction with PP (phases 1 and 2 respectively) in control and levcromakalim-treated animals (***A***). Note the shorter duration of the hypoxia phase following levcromakalim treatment. The prolonged, post-CSD decrease in tpO_2 (phase 3) in control animals and its amelioration by levcromakalim are shown in ***B***. Dashed line represents pre-drug baseline values. Differences between control and levcromakalim-treated animals in the hyperoxic response (Area under the Curve, AUC, phase 1; ***C***), acute hypoxia (AUC, phase 2; ***D***), and the prolonged

decrease in tpO2 at 5-25 min post-CSD (AUC, phase 3; ***E***). P values based on a Mann Whitney U-test.

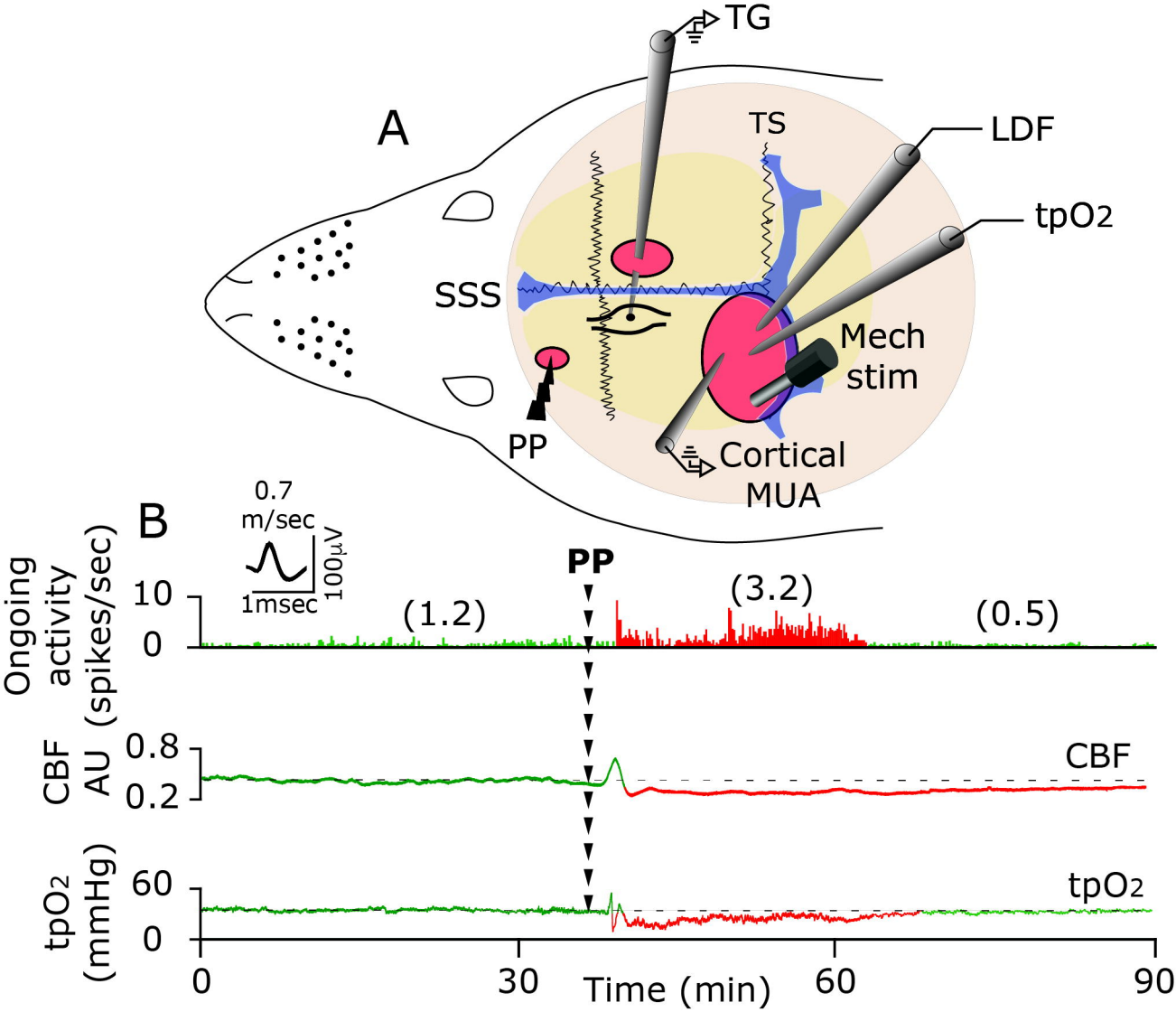
Figure 9. Levcromakalim inhibits CSD-evoked acute and prolonged meningeal afferent activation. Examples of single-unit recording of an A-delta afferent (***A***) and a C afferent (***B***), combined with cortical laser Doppler flowmetry, depicting the lack of acute and prolonged afferent following the elicitation of CSD with PP in animals treated with levcromakalim. In the presence of levcromakalim, only one C afferent became acutely activated (open square outside the min/max box in ***C***.). Prolonged activation was noted only in 1 A-delta and 3 C afferents (open circle and squares outside the min/max box in ***D***.)

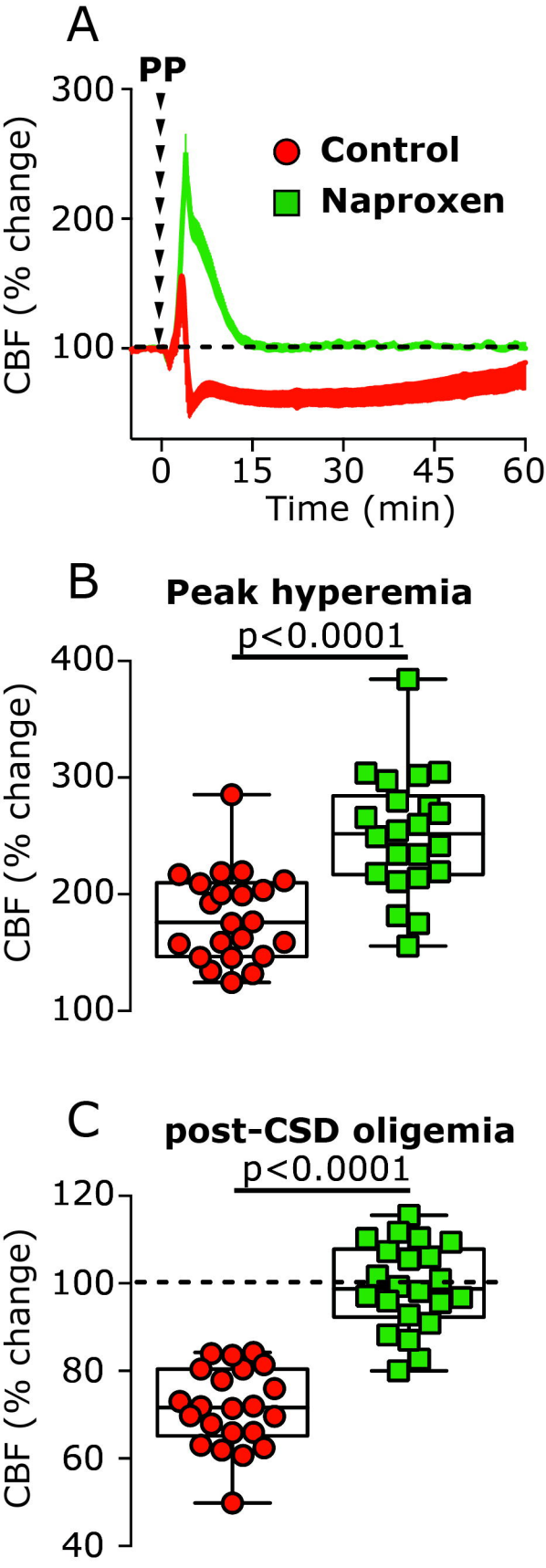
Figure 10. Levcromakalim does not inhibit CSD-evoked mechanical sensitization of meningeal afferents. ***A***. An example of an experimental trial depicting the CSD-evoked increase in mechanical responsiveness of a C afferent recorded from a levcromakalim-treated animal. Note that CSD-evoked sensitization at both the threshold and suprathreshold levels (post-stimulus time histogram; 0.1 sec). Individual threshold (***B***) and suprathreshold (***C***) responses of sensitized and non-sensitized afferents. Comparisons between the magnitudes of CSD-related changes in mechanosensitivity at the threshold (***D***) and suprathreshold levels (***E***) depicting similar CSD-evoked sensitization responses in control and levcromakalim animals. P values based on a Mann Whitney U-test.

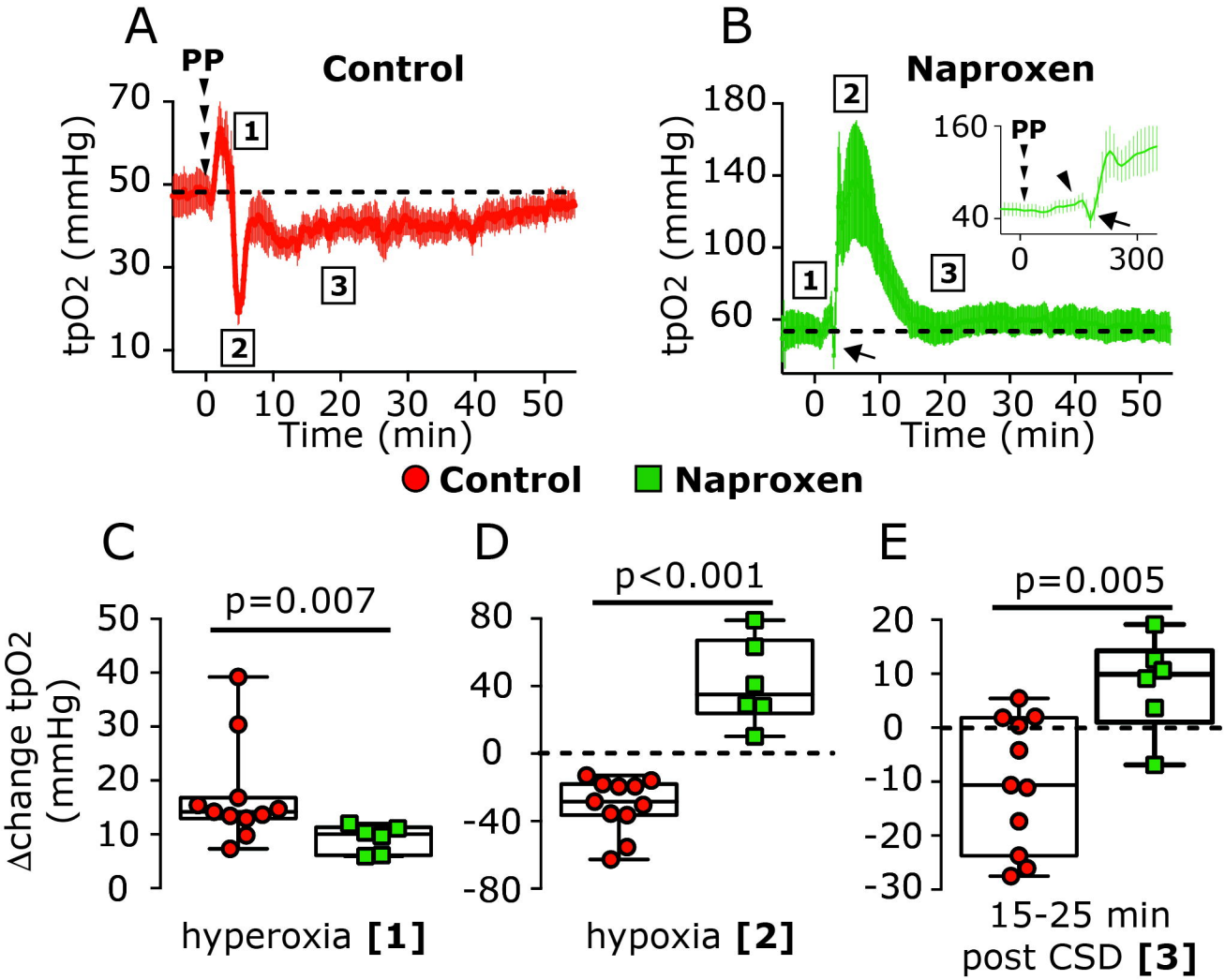
Table 1: Baseline mechanical response properties of meningeal afferents in animals treated with vehicle, naproxen, or levcromakalim.

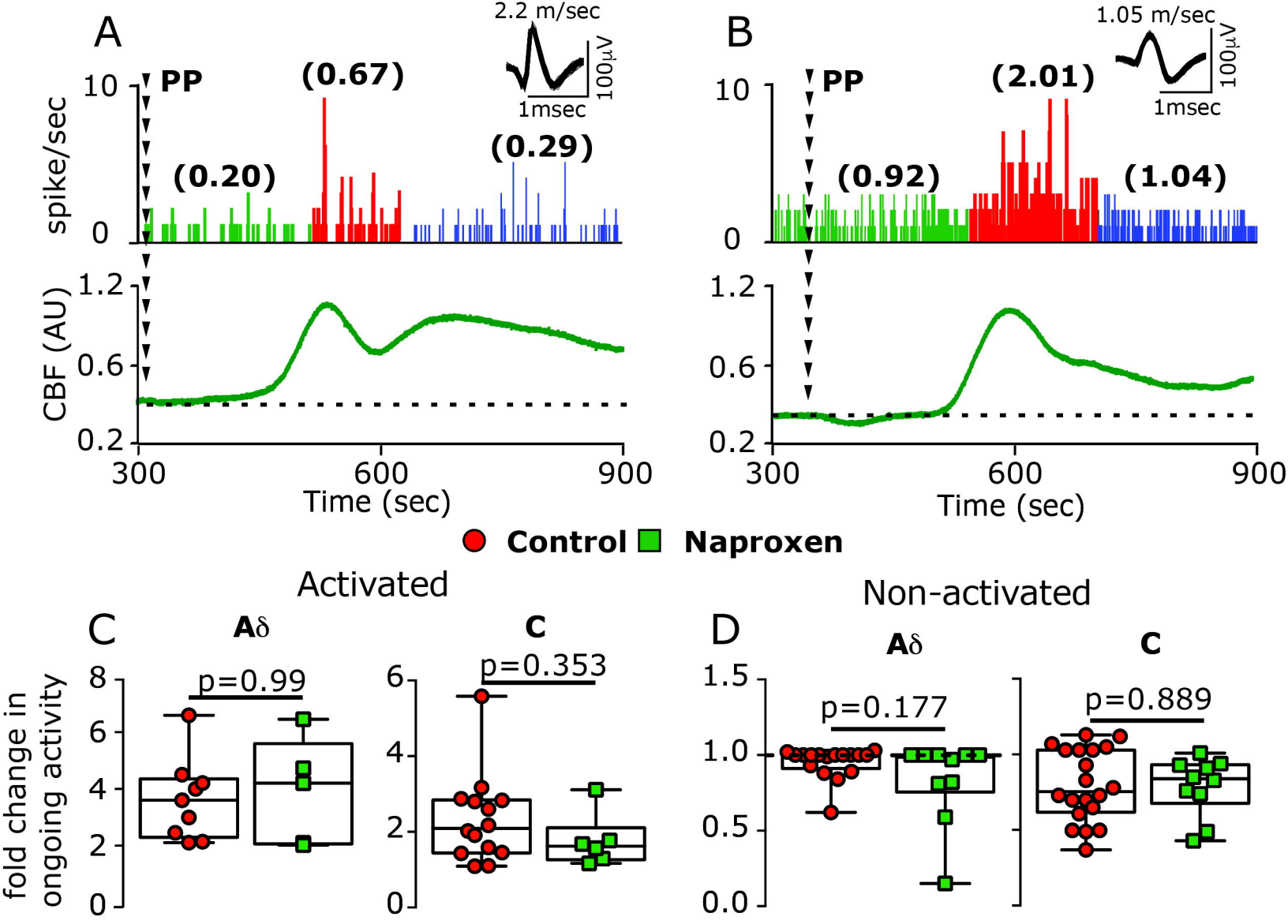
	Receptive fields [a]	Threshold [b]	Suprathreshold [c]
Control	[**2** (1)]	[**1.3** (0.7)]	[**7.3** (7.0)]
Naproxen	[**2** (1)]	[**0.5** (1.8)]	[**8.0** (6.5)]
Levcromakalim	[**2** (1)]	[**1.7** (1.5)]	[**9.0** (4.0)]

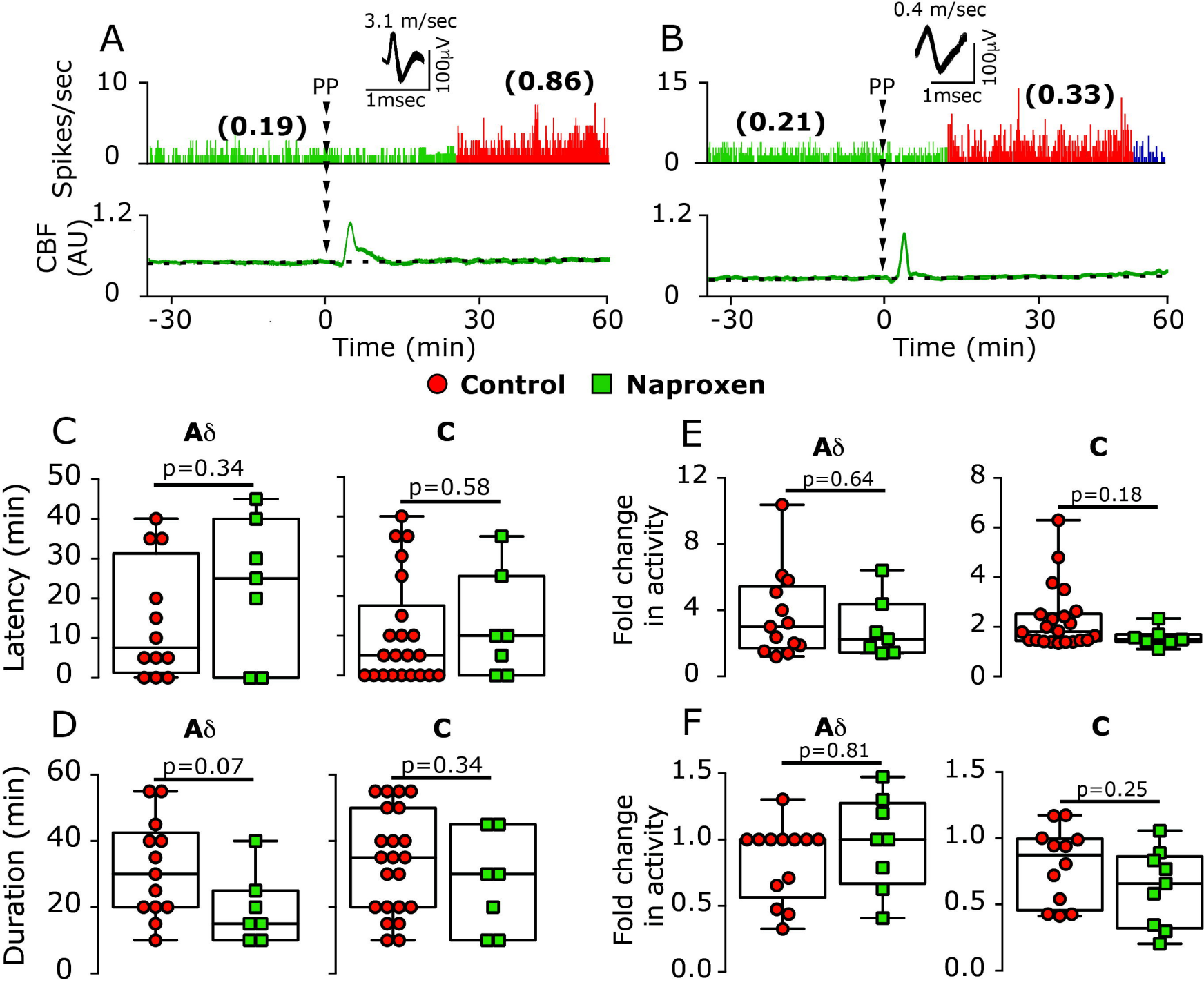
Data are expressed as the [**median** (IQR)]. Threshold and suprathreshold neural responses (spikes/sec) are based on activity recorded during a 2-second mechanical stimulus, and represent the average of 3-4 trials conducted prior to the elicitation of CSD in the presence of the vehicle, naproxen or levcromakalim. Group differences were analyzed using Kruskal-Wallis and were not statistically significant for the 3 parameters examined. ***a*** $H(2)=3.85$, $p=0.145$; ***b*** $H(2)=4.70$, $p=0.095$; ***c*** $H(2)=3.24$, $p=0.198$.

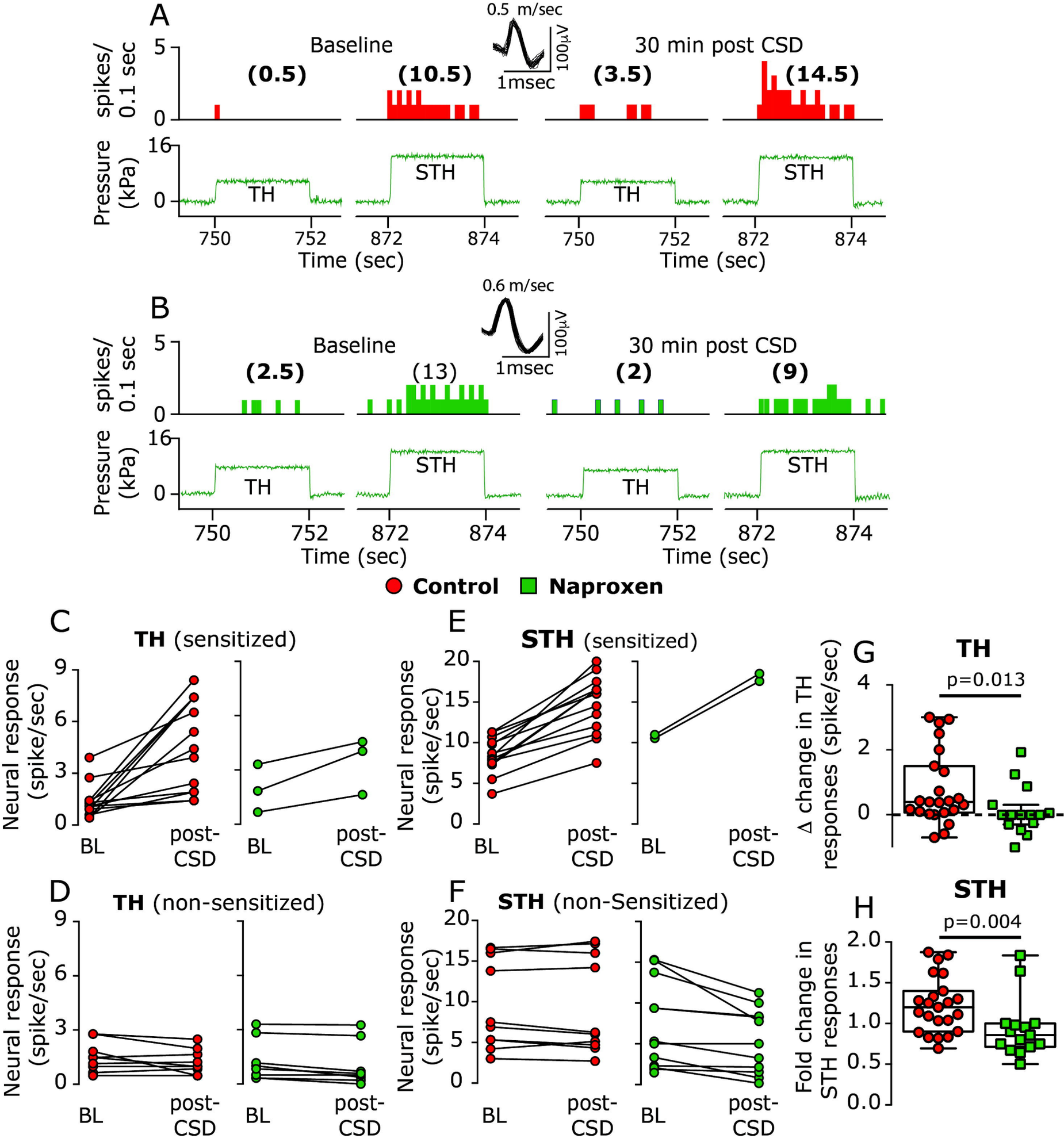

A
TG
TS
LDF
tpO2
SSS
Mech
stim
PP
Cortical
MUA
B
0.7
m/sec
1msec
100µV
PP
(1.2)
(3.2)
(0.5)
Ongoing
activity
(spikes/sec)
10
0
CBF
AU
0.8
0.2
CBF
tpO2
(mmHg)
60
0
tpO2
0
30
60
90
Time (min)

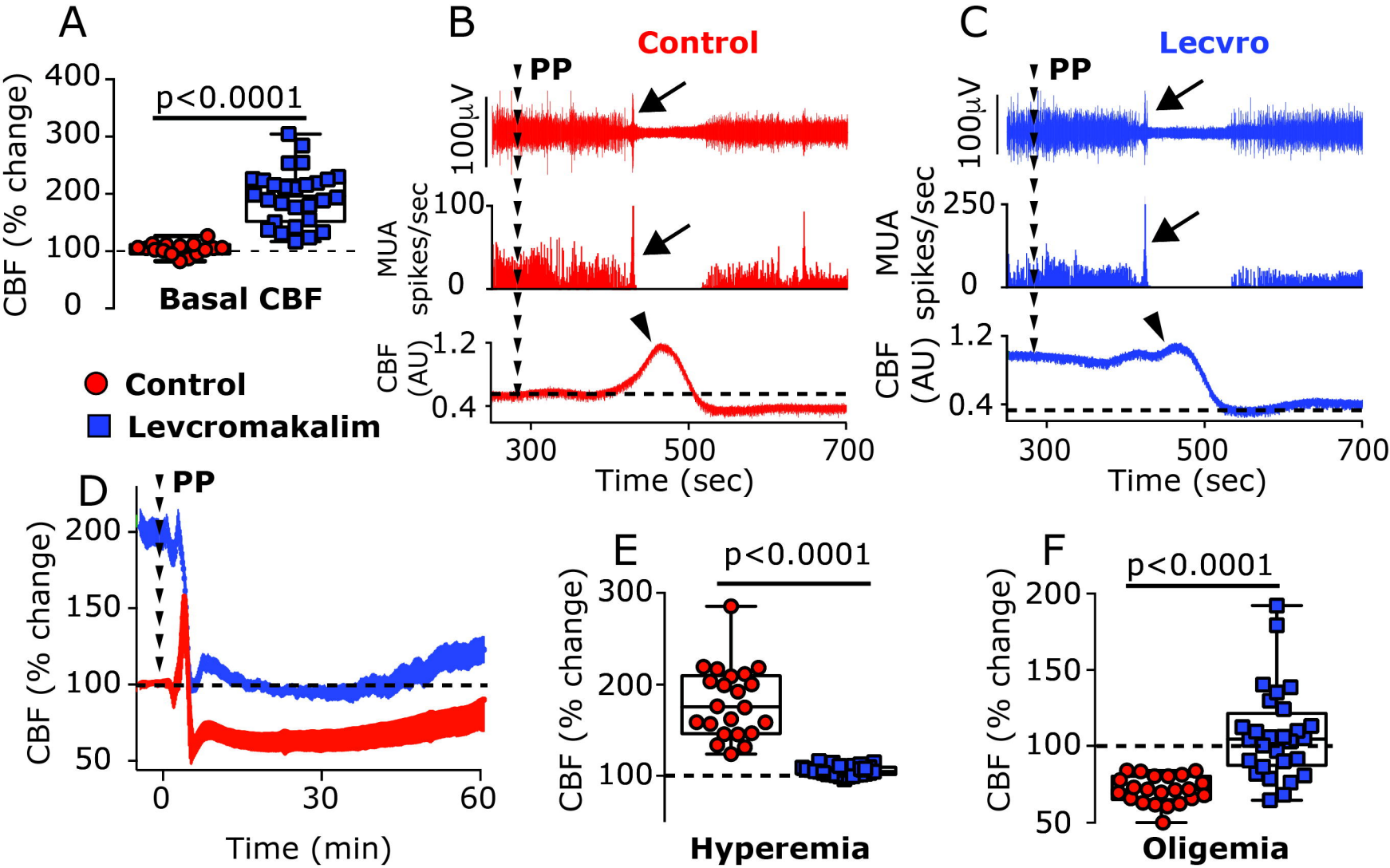

A
PP
300
200
100
CBF (% change)
Control
Naproxen
0
15
30
45
60
Time (min)
B
Peak hyperemia
p<0.0001
400
300
200
100
CBF (% change)
C
post-CSD oligemia
p<0.0001
120
100
80
60
40
CBF (% change)

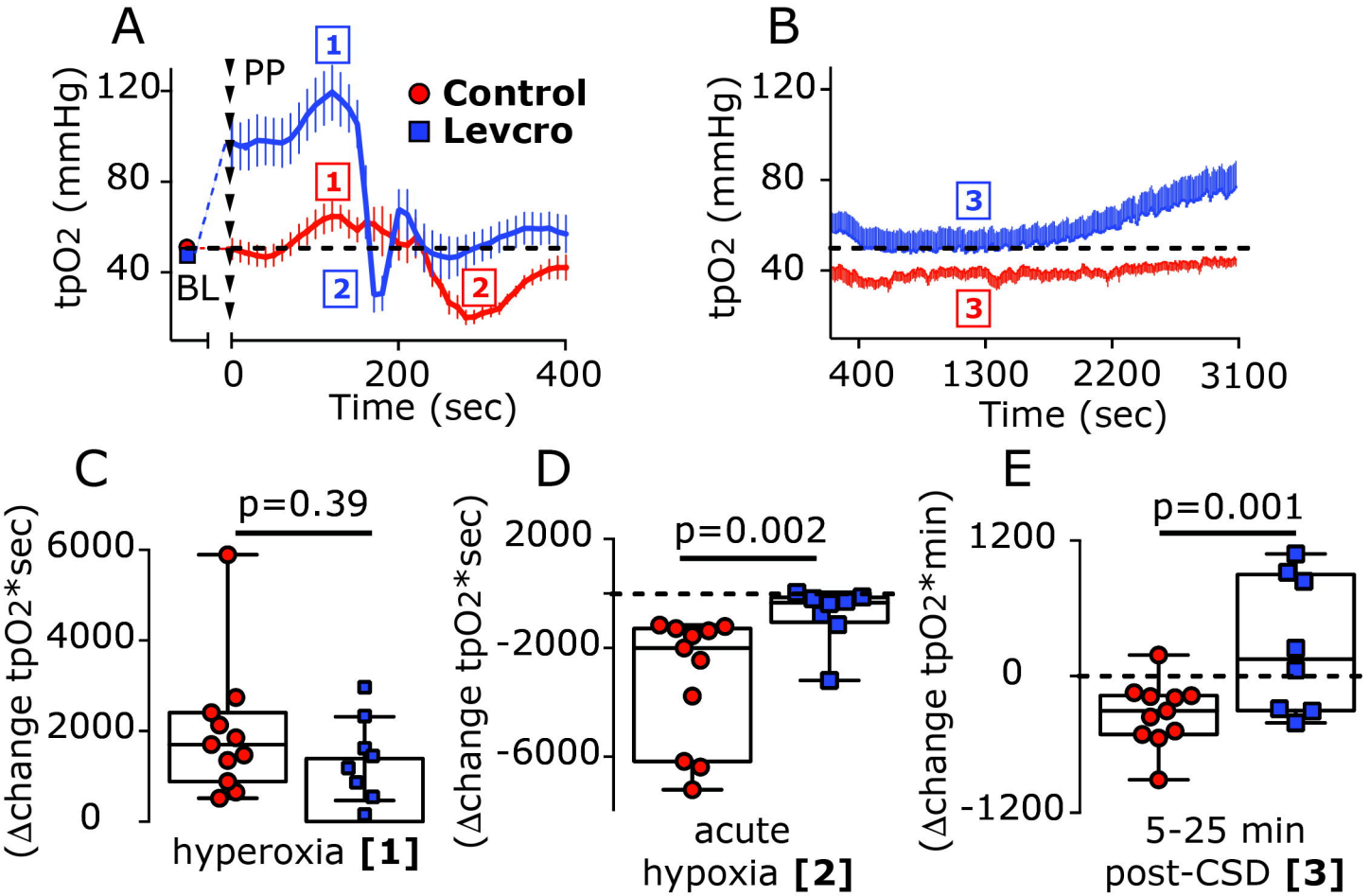

A
PP
Control
70
50
30
10
tpO2 (mmHg)
1
2
3
0
10
20
30
40
50
Time (min)
B
Naproxen
180
140
100
60
tpO2 (mmHg)
2
1
3
160
PP
40
0
300
0
10
20
30
40
50
Time (min)
Control
Naproxen
C
p=0.007
50
40
30
20
10
0
Δchange tpO2 (mmHg)
hyperoxia [1]
D
p<0.001
80
40
0
-40
-80
hypoxia [2]
E
p=0.005
20
10
0
-10
-20
-30
15-25 min post CSD [3]

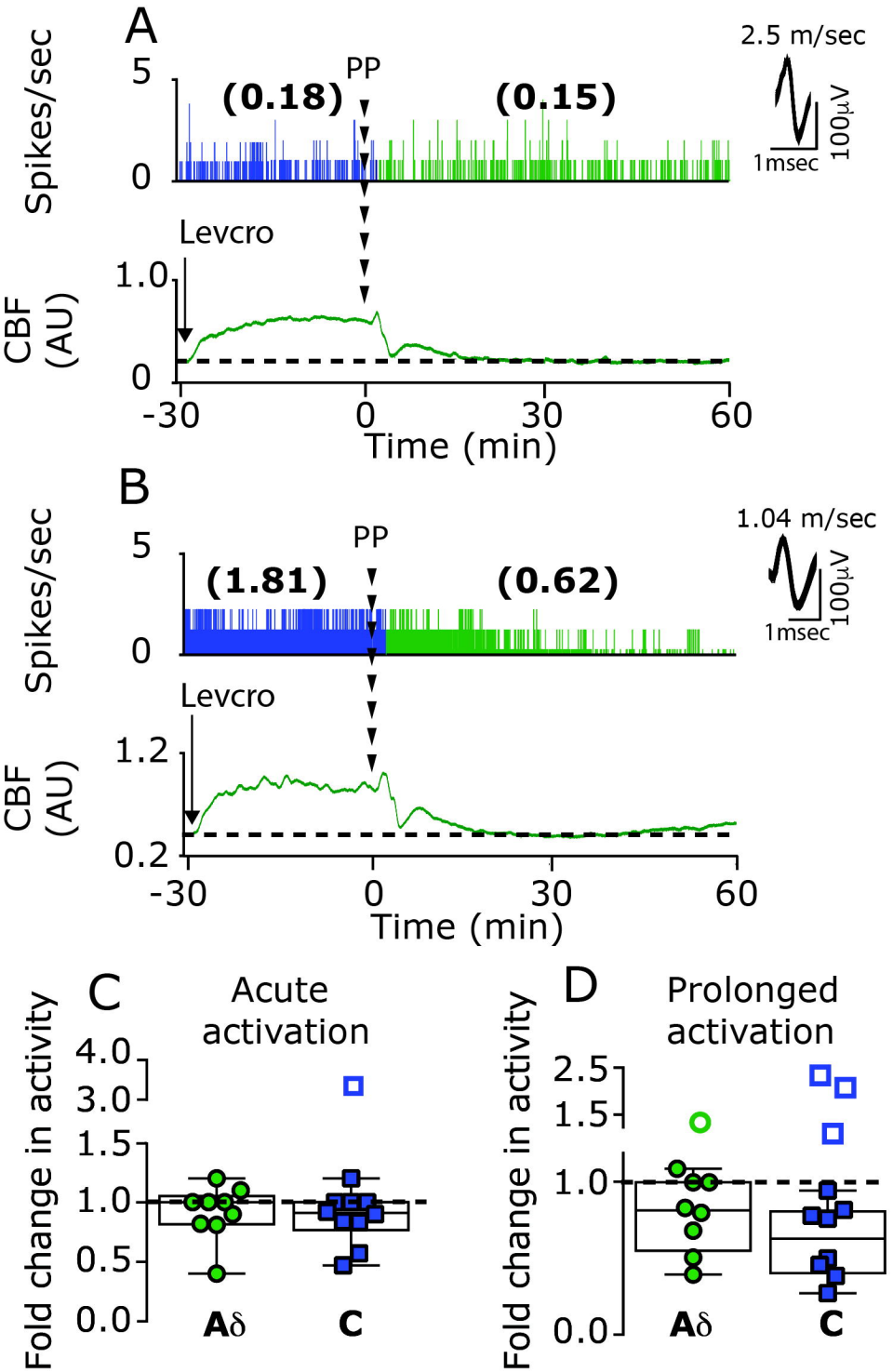

A
2.2 m/sec
100µV
1msec
PP
(0.67)
(0.20)
(0.29)
spike/sec
10
0
CBF (AU)
1.2
0.6
0.2
300
600
900
Time (sec)
B
1.05 m/sec
100µV
1msec
PP
(2.01)
(0.92)
(1.04)
10
0
1.2
0.6
0.2
300
600
900
Time (sec)
Control
Naproxen
Activated
Non-activated
C
Aδ
C
p=0.99
p=0.353
fold change in ongoing activity
8
6
4
2
0
6
4
2
0
D
Aδ
C
p=0.177
p=0.889
1.5
1.0
0.5
0.0

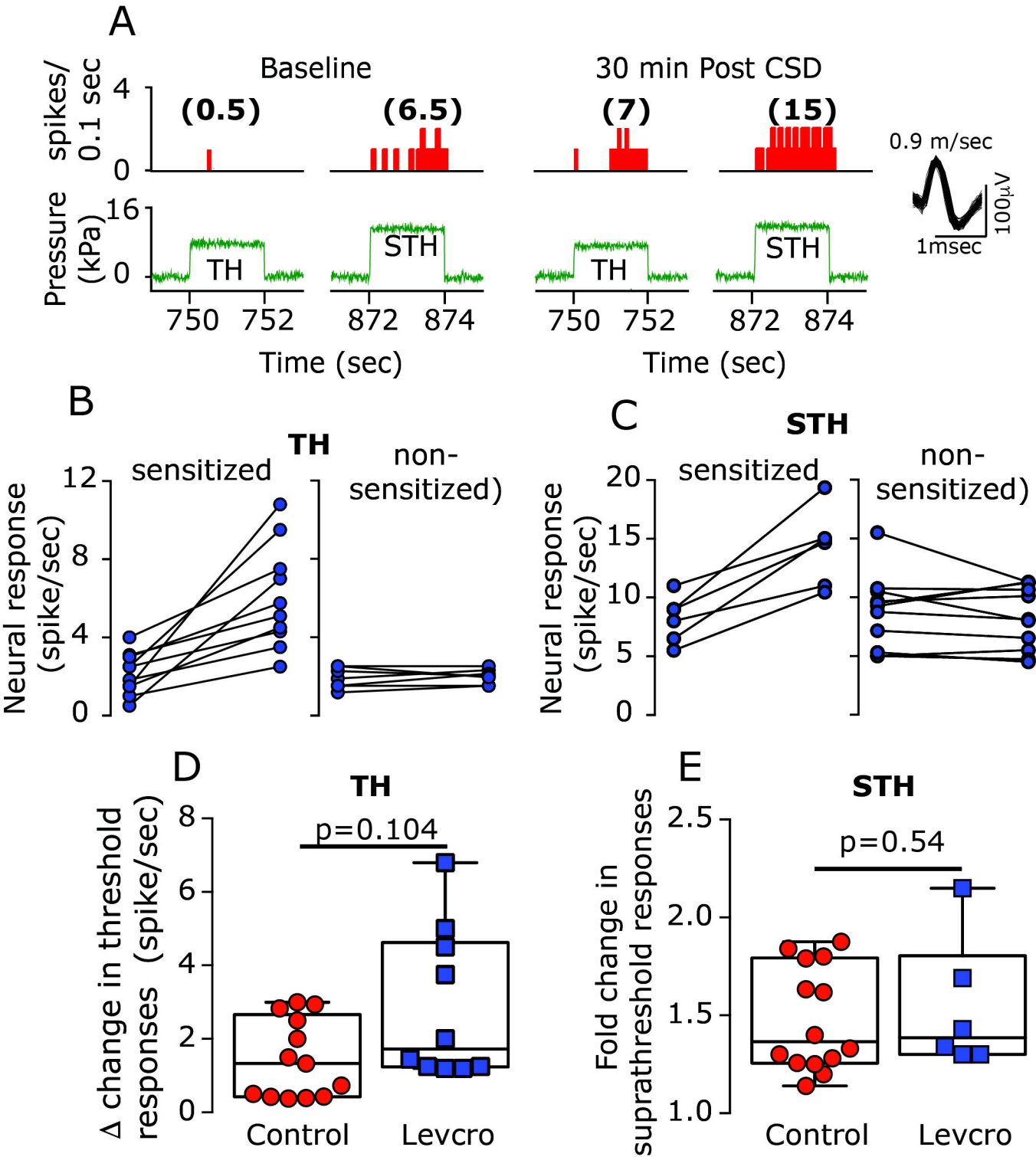

A
3.1 m/sec
100µV
1msec
PP
(0.19)
(0.86)
Spikes/sec
CBF (AU)
Time (min)
B
0.4 m/sec
100µV
1msec
PP
(0.21)
(0.33)
Time (min)
Control
Naproxen
C
Aδ
C
p=0.34
p=0.58
Latency (min)
E
Aδ
C
p=0.64
p=0.18
Fold change in activity
D
Aδ
C
p=0.07
p=0.34
Duration (min)
F
Aδ
C
p=0.81
p=0.25
Fold change in activity


A
0.5 m/sec
100µV
1msec
Baseline
30 min post CSD
(0.5)
(10.5)
(3.5)
(14.5)
spikes/ 0.1 sec
Pressure (kPa)
TH
STH
Time (sec)
B
0.6 m/sec
(2.5)
(13)
(2)
(9)
Control
Naproxen
C
TH (sensitized)
Neural response (spike/sec)
BL
post-CSD
D
TH (non-sensitized)
E
STH (sensitized)
F
STH (non-Sensitized)
G
TH
p=0.013
Δ change in TH responses (spike/sec)
H
STH
p=0.004
Fold change in STH responses


A
p<0.0001
CBF (% change)
400
300
200
100
0
Basal CBF
Control
Levcromakalim
B
Control
PP
100μV
MUA spikes/sec
100
0
CBF (AU)
1.2
0.4
300
500
700
Time (sec)
C
Lecvro
PP
100μV
MUA spikes/sec
250
0
CBF (AU)
1.2
0.4
300
500
700
Time (sec)
D
PP
CBF (% change)
200
150
100
50
0
30
60
Time (min)
E
p<0.0001
CBF (% change)
300
200
100
Hyperemia
F
p<0.0001
CBF (% change)
200
150
100
50
Oligemia


A
PP
1
Control
Levcro
1
2
2
BL
tpO2 (mmHg)
120
80
40
0
200
400
Time (sec)
B
3
3
tpO2 (mmHg)
120
80
40
400
1300
2200
3100
Time (sec)
C
p=0.39
(Δchange tpO2*sec)
6000
4000
2000
0
hyperoxia [1]
D
p=0.002
(Δchange tpO2*sec)
2000
-2000
-6000
acute
hypoxia [2]
E
p=0.001
(Δchange tpO2*min)
1200
0
-1200
5-25 min
post-CSD [3]


A
Spikes/sec
5
0
(0.18)
PP
(0.15)
2.5 m/sec
1msec
100µV
Levcro
CBF (AU)
1.0
0
-30
0
30
60
Time (min)
B
Spikes/sec
5
0
(1.81)
PP
(0.62)
1.04 m/sec
1msec
100µV
Levcro
CBF (AU)
1.2
0.2
-30
0
30
60
Time (min)
C
Acute activation
Fold change in activity
4.0
3.0
1.5
1.0
0.5
0.0
Aδ
C
D
Prolonged activation
Fold change in activity
2.5
1.5
1.0
0.0
Aδ
C


A
Baseline
30 min Post CSD
spikes/ 0.1 sec
4
0
(0.5)
(6.5)
(7)
(15)
0.9 m/sec
100µV
1msec
Pressure (kPa)
16
0
TH
STH
TH
STH
750 752
872 874
750 752
872 874
Time (sec)
Time (sec)
B
TH
sensitized
non-sensitized)
Neural response (spike/sec)
12
8
4
0
C
STH
sensitized
non-sensitized)
Neural response (spike/sec)
20
15
10
5
0
D
TH
p=0.104
Δ change in threshold responses (spike/sec)
8
6
4
2
0
Control
Levcro
E
STH
p=0.54
Fold change in suprathreshold responses
2.5
2.0
1.5
1.0
Control
Levcro