Naproxen inhibits CSD-evoked meningeal afferent mechanosensitization but not prolonged activation: a role for cortical oligemia and hypoxia?

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Abstract

We have shown previously that cortical spreading depression (CSD), the underlying cause of the migraine aura, also promotes persistent activation and mechanical sensitization of trigeminal meningeal nociceptors - a likely source of the migrainous intracranial headache. CSD involves a prolonged phase of reduced cerebral blood flow (CBF) and reduced tissue oxygen (tpO2). Here we tested the hypothesis the COX-derived prostaglandins, which mediate the post CSD cortical oligemia and tissue hypoxia, also contribute to CSD-evoked meningeal afferent responses. We employed in-vivo extracellular recording from the trigeminal cell body of meningeal afferents to assess changes in their activity and responses to threshold and suprathreshold mechanical stimulation of the meninges. Single unit recordings were conducted in combination with measurements of cerebral blood flow, using laser Doppler flowmetry, and tissue partial pressure of oxygen (tpO_2), using a modified Clark-type Polarographic oxygen microelectrode. The role of COX-mediators was tested by infusing naproxen (10 mg/kg), 60 min before the induction of CSD using a pin prick stimulus made in the frontal cortex. Treatment with naproxen significantly inhibited the development of cortical oligemia, as expected, and also blocked the prolonged reduction in cortical tpO_2. Naproxen did not affect the propensity of meningeal afferents to become activated by CSD, or the activation characteristics, but significantly blocked the development of mechanical sensitization. Our data suggest that CSD-evoked release of COX-derived mediators play a key role in the mechanisms that link CSD and mechanical

sensitization of meningeal afferents either directly, or indirectly by promoting cortical oligemia and hypoxia.

Introduction

Migraine headache affects about 15% of the global adult population and is considered the third most prevalent and seventh most disabling disease in the world [1-3]. A key migraine concept implicates persistent activation and increased mechanosensitivity of trigeminal afferent neurons that innervate the intracranial meninges and their related large blood vessels as the origin of migraine headache [4-7]. However, endogenous factors that promote these headache-promoting neural processes remain incompletely understood. One endogenous factor that has been suggested to play a role in migraine pathophysiology is cortical spreading depression (CSD), a slowly moving wave of intense cortical neuronal and glial depolarization (followed by depression), which has been linked to the aura experienced in about third of patients with migraine [8, 9]. The temporal proximity between the aura and the headache as well as indirect evidence from preclinical studies led to the theory that CSD also promotes the headache by promoting the activation of the meningeal sensory system [10-12] }. Recently, we provided a critical missing piece of direct evidence by showing that CSD, initiated in different parts of the cortex, can lead to persistent activation and increased mechanosensitivity of meningeal afferents in rats [13-15]. How CSD influences meningeal afferents remains unknown although numerous factors, including activation of inflammatory signaling in cortical neurons and astrocytes as well as the development of dural neurogenic inflammation, have been proposed [11, 12].

Another process, which could influence indirectly the responses of meningeal afferents, relates to the vascular and metabolic cortical events that occur in the wake of CSD. During migraine with aura there is a biphasic change in cerebral blood flow (CBF) - an initial increase, followed by a sustained reduced CBF (i.e. oligemia) that lingers during the headache phase [16-19]. In animal studies, CSD also leads to a similar vascular response [20-23]. In animal studies, the CSD-related cortical oligemia phase is associated with an acute phase of cerebral hypoxia [24] and hemoglobin desaturation [25]. An ensuing reduction in cerebral tissue oxygen levels, however, can persist for many minutes following CSD [22] and 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 rCBF in the wake of CSD) [22, 26].

Reduced cerebral tissue oxygen has been suggested to play a role in migraine pathophysiology [27, 28]. Hypoxia resulting from acute exposure to high altitude can induce headache and other symptoms that fulfill the criteria for migraine [29, 30]. Normobaric hypoxia can also trigger migraine headache [31, 32]. The potential link between reduced CBF and cerebral oxygen levels and migraine led us to investigate whether in the wake of CSD, the development of cerebral oligmia and tissue hypoxia play a role in mediating the persistent meningeal afferent activation and mechanical sensitization. To address these questions, we initially examined whether inhibition of the CSD-evoked CBF oligemia using naproxen [33] also inhibits the related persistent cerebral hypoxia. Having found

such an effect, we tested whether naproxen could also block the responses of meningeal afferents following CSD.

Materials and Methods

Ethics statement

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 and anesthesia

The current study employed male Sprague-Dawley rats (250–350 g). 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 [15]. 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 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 [4]. 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 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.

Assessment of changes in meningeal afferent mechanosesnitivity

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 evoked 1-3-Hz responses) followed by a suprathreshold stimulus (STH, usually ×2 of the threshold; 8-10 Hz responses). To minimize response desensitization, stimulus trials were delivered every 15 min throughout the experiment [15, 34]. Ongoing afferent discharge rate was recorded continuously between the stimulation trials. Baseline ongoing activity and 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 [15].

Measurements of changes in cerebral blood flow

CSD-related CBF changes induced by a cortical pinprick made in the frontal cortex [14] were documented using a needle probe (0.8 mm diameter) connected to a laser

Doppler flowmeter (Vasamedic, St Paul MN) was positioned just above the intact dura, about 2 mm from the receptive field of the recorded afferent in an area devoid of large blood vessels (>100 μm). Such LDF 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 [35]. LDF recordings were obtained with the ambient lights turned off. Baseline values were collected for at least 30 min prior to treatments and data from animals with CBF varying by more than 10% were excluded.

Measurements of cortical O_2 levels (tPO_2)

Changes in tissue partial pressure of O_2 were recorded using a modified Clark-type Polarographic oxygen microelectrode (OX-10; Unisense A/S, Aarhus, Denmark) with a guard cathode for tpO_2 measurements as indicated previously [22]. The oxygen electrode was connected to a high-impedance Pico-amperometer (PA 2000; Unisense A/S) that sensed the currents of the oxygen electrodes. Signals were A/D converted and recorded at 100 Hz (Power 1401 and Spike 6.0; CED). 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 Na-ascorbate and 0.1 M NaOH dissolved in saline. Changes in tpO_2 values were recorded at a superficial cortical depth of (100-200 μm) and about 1 mm from the LDF probe.

Data analysis

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 300-sec prior to the induction of CSD. Offline analyses for afferent responses were conducted using template matching in Spike 2. All data were analyzed 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 [14, 15]. 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 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 [15]). Group differences were analyzed using two-tailed, Fisher's exact test. Statistical differences were analyzed using two-tailed 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

The effect of systemic naproxen on CSD-related change in CBF

We have shown previously that local COX inhibition with naproxen blocks the cerebral oligemia that develops following CSD. Here we validated the effect of naproxen delivered via a systemic intravenous route (10 mg/kg), to ensure that in studies of meningeal afferents, neurons with unexposed receptive field could also be affected by the treatment. In control, vehicle-treated animals (n=24), CSD gave rise to the typical acute hyperemia followed by persistent oligemia [33]. Immediately after the hyperemia, CBF was significantly reduced to 56.9±1.8% of baseline values. At 45 min post-CSD, CBF demonstrated a slight recovery, but values remained significantly below baseline (-71.9±2.7% of baseline). In animals systemically administered with naproxen, 1 hr prior to the induction of CSD (n=19, Fig. 1A), the initial CSD-related increase in CBF was enhanced when compared to the control group (237.7±9.0 % vs 189.5±9.0 %; p=0.0001, two tailed t-test). In addition, naproxen completely blocked the CSD-evoked persistent oligemia with values not statistically different those seen at baseline and statistically higher than those observed in vehicle treated animals, at the 4 time points tested (5, 15, 30 and 45 min post CSD; $F_{1,41}$=87.1, p<0.0001). This local effect of systemic naproxen treatment corroborates the previous data of Shibata et al. [36] in rabbits, which employed systemic administration of the COX inhibitor indomethacin.

The effect of naproxen on CSD-related changes in cerebral tpO_2.

The baseline (pre-CSD) levels of cerebral tpO_2 in the vehicle control group (n=12), was 40.1±4.3 mmHg, which was not significantly different than that observed in animals treated systemically with naproxen (49.9±6.1 mmHg; n=6; p=0.2). During the post-CSD hyperemic stage, there was a short-lasting (~30-120 sec) increase in tpO_2 values in the control group to peak values of 62.8±7.2 mmHg, which were similar to those observed in the naproxen treated group (62.9±3.9 mmHg; p=0.47; unpaired t-test). Following this brief increase, and during the 60 min post-CSD recording period, the changes in tpO_2 values between the control and naproxen treated group were statistically significant ($F_{1,16}$=18.8; p=0.0005, see an example trace in Fig 1B). In both treatment groups, tpO_2 values were acutely reduced as shown previously [22, 24]. However, the decrease in tpO_2 values in the control group (hypoxic values:10.9±1.6 mmHg; 70.6±5.3% decrease) were significantly larger than those observed in the naproxen treated group (hypoxic values 31.5±7.1 mmHg; 28.1±20.3% decrease; p=0.02, Fig 1C). The duration of the acute decrease in tpO2 in the control group (54.4±6.6 sec) however, was not statistically different than that observed in the naproxen-treated animals (vs 36.0±2.5 sec; p=0.07). Following the recovery from the acute hypoxic event, control animals exhibit a milder but persistent reduction in their tpO_2 values, lasting on average 37.1±4.6 min (range 5.6-48.7 min; Fig 1B), with maximum reduction observed at 5-10 min (-18.7±6 %). In the naproxen treated group, however, in all but one animal (in which reduced tpO2 levels lasted for ~10 min), tpO_2 values were markedly increased by 109.4±29.6% during the next 10 min (compared to a 19.4±5.6% decrease in the control group;

p<0.0001, Fig 1C) and then either remained elevated or returned to baseline values by the end of the recording period.

Naproxen has no effect on CSD-evoked persistent activation of meningeal afferents.

The effect of naproxen on CSD-evoked activation of meningeal afferents was tested in 31 units (14 A-delta and 17 C-units). At baseline, prior to the induction of CSD, the ongoing activity level of meningeal afferents was similar in the naproxen-treated and control group (table 1). Following the induction of CSD, ~45% of the afferents recorded in naproxen treated animals (6/14 A-delta and 8/17 C-units) developed a persistent increase in their ongoing activity level that occur concomitantly with the cerebral oligemia and prolonged hypoxic state (Fig 2). The propensity for developing CSD-related afferent activation after naproxen treatment was not different than that observed in the control vehicle group (12/22 A-delta; 9/17 C; p>0.05 overall and for each afferent population). For further analyses of the CSD-related activations parameters, we pooled the data from the A-delta and C afferents given their similarities in the control group. Overall, the CSD-evoked afferent activation characteristics were not different between the two treatment groups. In naproxen-treated animals, the average latency for the development of persistent afferent activation (12.4±3.1 min; range 0-40) was not statistically different when compared to the control group (17.5±4.3 min; range 0-45, p>0.05, Fig 3, top). The duration of the CSD-evoked persistent afferent activation in naproxen treated animals (23.2±3.5 min; range 10-45) was also not

different than that observed in the control group (31.7±3.4 min; range 10-55; p>0.05, Fig. 3 middle). Finally, the magnitude of the increase in afferent discharge rate following CSD in the naproxen-treated group (2.2±0.4 fold; range 1.1-6.4) was not different than that observed in the control group (3.0±0.5 fold; range 1.2-10.4, p>0.05, Fig.3 bottom).

Naproxen inhibits CSD-evoked meningeal afferent mechanosensitization. To examine the effect of Naproxen pretreatment on the CSD-evoked meningeal afferent mechanosensitization we analyzed data from 15 afferents (8 A-delta and 7 C), all of which have shown consistent mechanically-evoked responses at baseline prior to the elicitation of CSD. These afferent responses were compared to those observed the saline control group (10 A-delta and 11 C). Because the CSD-evoked sensitization observed in the A-delta and C afferent populations were similar in the control group (see also [15]), data was pooled from the two afferent populations. There were no differences between the number of meningeal mechanosensitive receptive fields located in each group (2.36±0.23; range 1-5 for the naproxen group vs 2.04±0.14; range 1-5 for the control group). Baseline mechanical thresholds were also similar (0.56±0.1 gr; range 0.04-2 for the naproxen group vs 0.67±0.1 gr; range 0.04-4 in the control group). In contrast to its lack of effect on the development of the CSD evoked persistent meningeal afferent activation, naproxen treatment significantly inhibited the sensitization response, resulting in a significant lower rate of sensitization (4/15; 1 afferent with TH and STH affected, 2 afferents with only TH affected and 1 afferent with

only STH affected) compared to that observed in the control group (14/21, 10 afferents with TH and STH affected, 3 afferents with only TH affected and 1 afferent with only STH affected; $p<0.05$, two-tailed Fisher's exact test, Fig. 4).

Discussion

The main findings of this study are: 1) naproxen inhibits the development of cortical hypoxia following CSD, 2) naproxen does not inhibit the propensity of meningeal afferents to develop prolonged activation following CSD or the activation characteristics, 3) naproxen strongly inhibits the development of CSD-evoked mechanical sensitization of meningeal afferents.

A recent mouse study, which assessed the mechanism underlying CSD-evoked dural vasodilatation - a proposed signature of dural afferent activation, suggested an inhibitory effect of naproxen on this vascular response [12]. Our data showing no effect on the CSD-evoked prolonged activation of meningeal afferents by naproxen, suggests that the dural vasodilatation observed following CSD, which was also shown in rats [11], may not be directly mediated by the increased firing of trigeminal dural afferents. It should be noted however that our earlier findings suggest that naproxen can inhibit the prolonged activation of meningeal afferents, induced by local administration of inflammatory mediators to their receptive field [37]. That naproxen was ineffective in inhibiting the CSD-evoked prolonged activation of meningeal afferents thus suggests that this afferent

response is mediated by a mechanism different than the one responsible for the persistent response evoked by the mediators we and others tested previously [4].

The cellular source of COX, its isoform and related prostaglandins (PGs) that participate in the mechanisms responsible for the mechanical sensitization of meningeal afferents following CSD remain to be determined. The two main COX isoforms are constitutively expressed in the dura mater [38]; COX-1 is present in most dural mast cells, while COX-2 is mainly expressed in resident dural macrophages. Release of COX-derived PGs from activated mast cells could potentially lead to the activation and sensitization of meningeal afferents following CSD [12, 39]. However, CSD-evoked prolonged meningeal afferent activation and sensitization in vivo occurs in a preparation that involves a prior craniotomy, which degranulates dural mast cells [40] already before the induction of CSD. Future studies would be required to determine whether COX-2 derived PGs from dural macrophages, or other meningeal constituents, could explain the inhibitory effect of naproxen we observed in the current study.

The sensitization of meningeal afferents following CSD may be mediated also by release of COX-derived PGs from cortical constituents, including neurons, vascular cells and glia and their circulation into the CSF at the subarachnoid space, and subsequently through the glymphatic system into the dural vasculature [15, 41-43]. Of particular interest is the COX-2 isoform, which has

been shown to be upregulate in cortical astrocytes shortly after CSD [12] Our previous study also indicate COX-2 involvement in the mechanism responsible for the CSD-evoked prolonged cortical oligemia [44].

The finding that naproxen blocks the cortical oligemia and the prolonged reduction in tPO_2, points also to the possibility that release of cortical PGs as well as other factors, related to metabolic stress, contribute to the effect of CSD on meningeal afferents. Of particular interest are ATP [45] and lactate [46], which can activate and sensitize nociceptors [14, 47-51]. Whether the cortical oligemia and hypoxia, directly contributes to the nociceptive effects of CSD, remains to be determined. We are currently conducting further studies to examine whether inhibition of this cortical vascular and metabolic responses, using vasodilating agents that do not block COX, can also inhibit the CSD-evoked enhanced responses of meningeal afferents.

Conflict of Interest: Authors report no conflict of interest

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Table 1: Baseline ongoing activity of meningeal afferents in naproxen-treated and control group.

	A-delta	**C**	**A, C combined**
Saline	0.24±0.13; 0-3.08	0.99±0.33; 0.02-2.65	0.71±0.19
Naproxen	0.16±0.04, 0.01-3.72	1.15±0.29; 0-4.6	0.58±0.16

Figure 1: A. The effects of systemic administration of naproxen on the CSD-evoked hyperemia (peak), immediate decrease in CBF (dip) and persistent oligemia. B. An example of raw recording traces demonstrating the immediate hypoxia and prolonged decrease in cortical tPO_2 in saline- and naproxen-treated animals. C. Naproxen had a significant effect on the CSD-evoked persistent reduction in tPO_2. * $p<0.05$ t-test.

Figure 2: Development of delayed meningeal afferent activation (bottom) concomitant with the prolonged decrease in cerebral tPO_2(arrow head, top) and reduced CBF (arrow head, middle). Arrows indicate the acute hypoxic and hyperemic responses to CSD.

Figure 3: Naproxen treatment had no effect on the latency, duration or magnitude of the prolonged meningeal afferent activation following CSD.

Figure 4: Naproxen had a significant inhibitory effect on the development of meningeal afferent sensitization to punctate mechanical stimulation of the dura following CSD, on both the threshold and suprathreshold responses (top). Numbers in parentheses indicate the number of sensitized/total tested afferents. Bottom figures illustrate the pre- and post CSD mechanically evoked responses in meningeal afferents that were not sensitized by CSD.

Figure 1

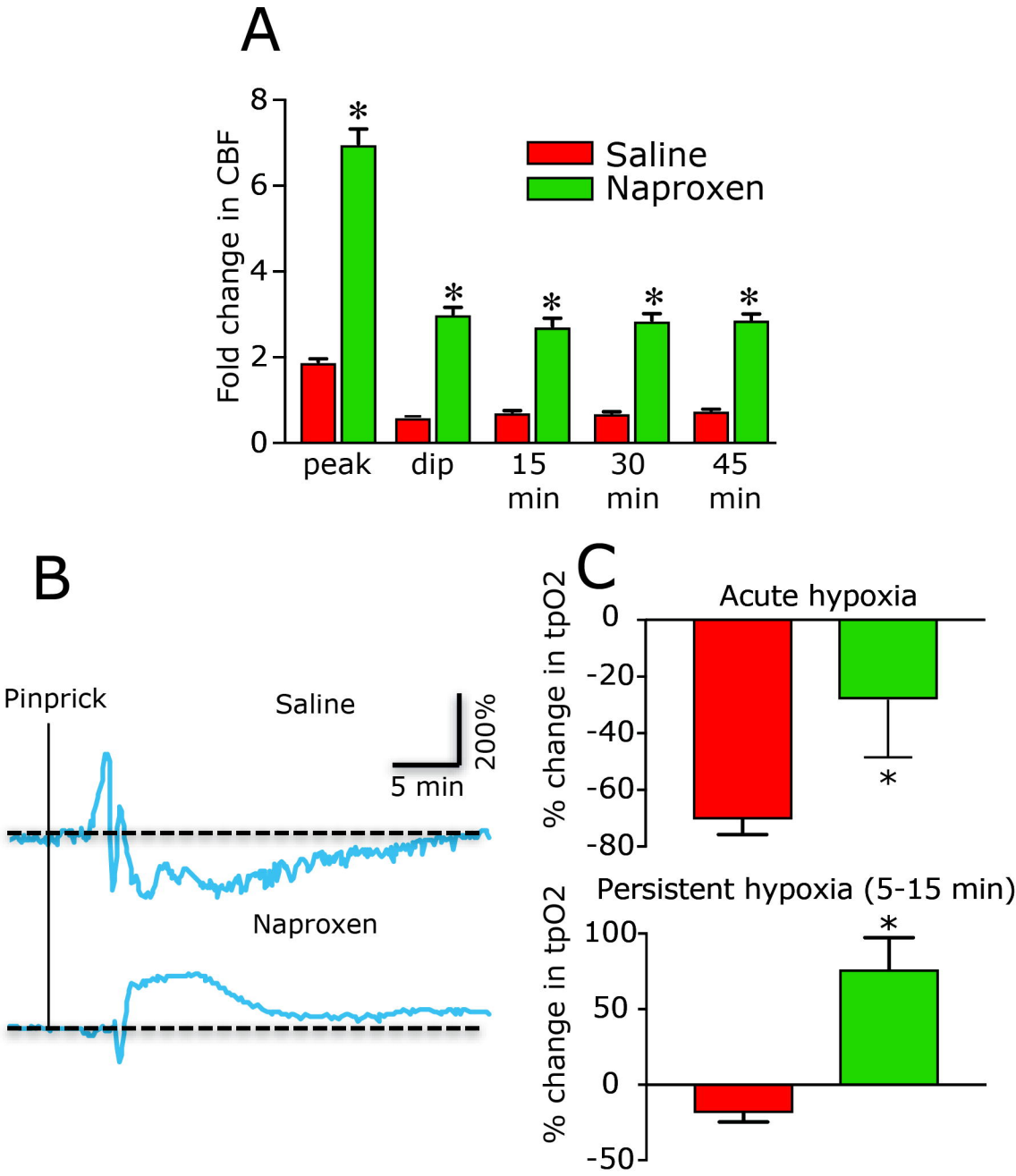

A
Fold change in CBF
8
6
4
2
0
Saline
Naproxen
peak
dip
15 min
30 min
45 min
B
Pinprick
Saline
200%
5 min
Naproxen
C
Acute hypoxia
% change in tpO2
0
-20
-40
-60
-80
Persistent hypoxia (5-15 min)
% change in tpO2
100
50
0
-50

Figure 2

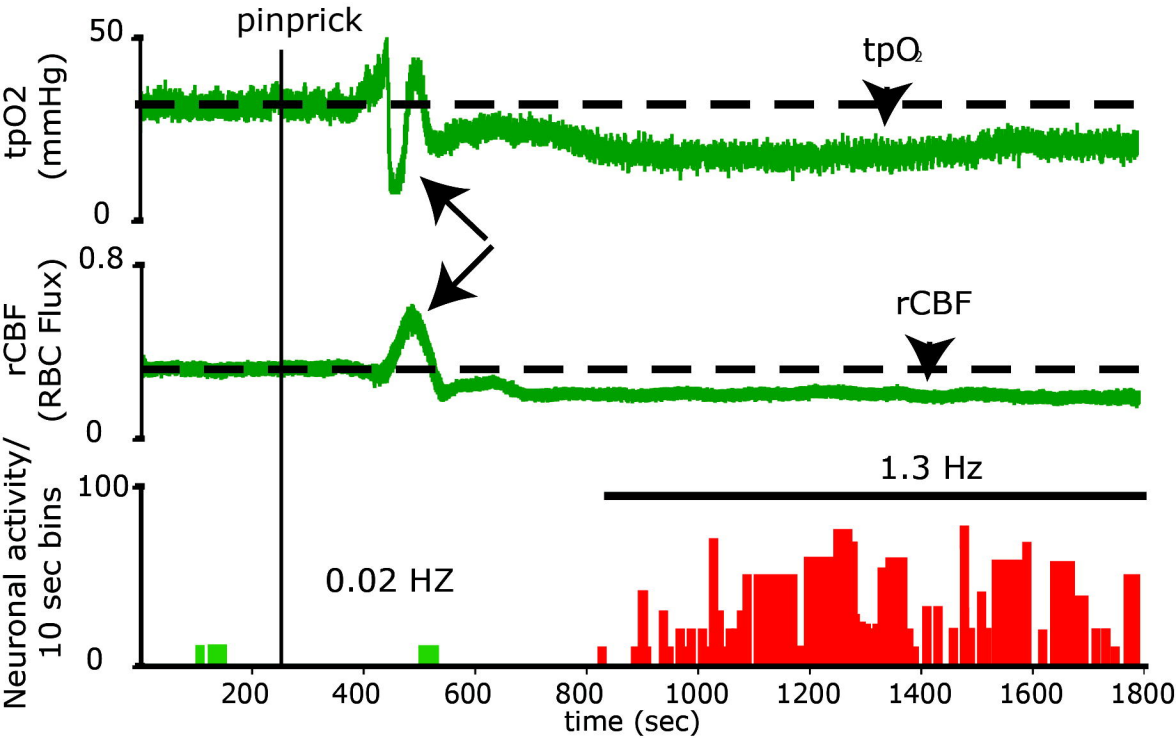

pinprick
50
0
tpO2 (mmHg)
tpO2
0.8
0
rCBF (RBC Flux)
rCBF
100
0
Neuronal activity/ 10 sec bins
1.3 Hz
0.02 HZ
200
400
600
800
1000
1200
1400
1600
1800
time (sec)

Figure 3

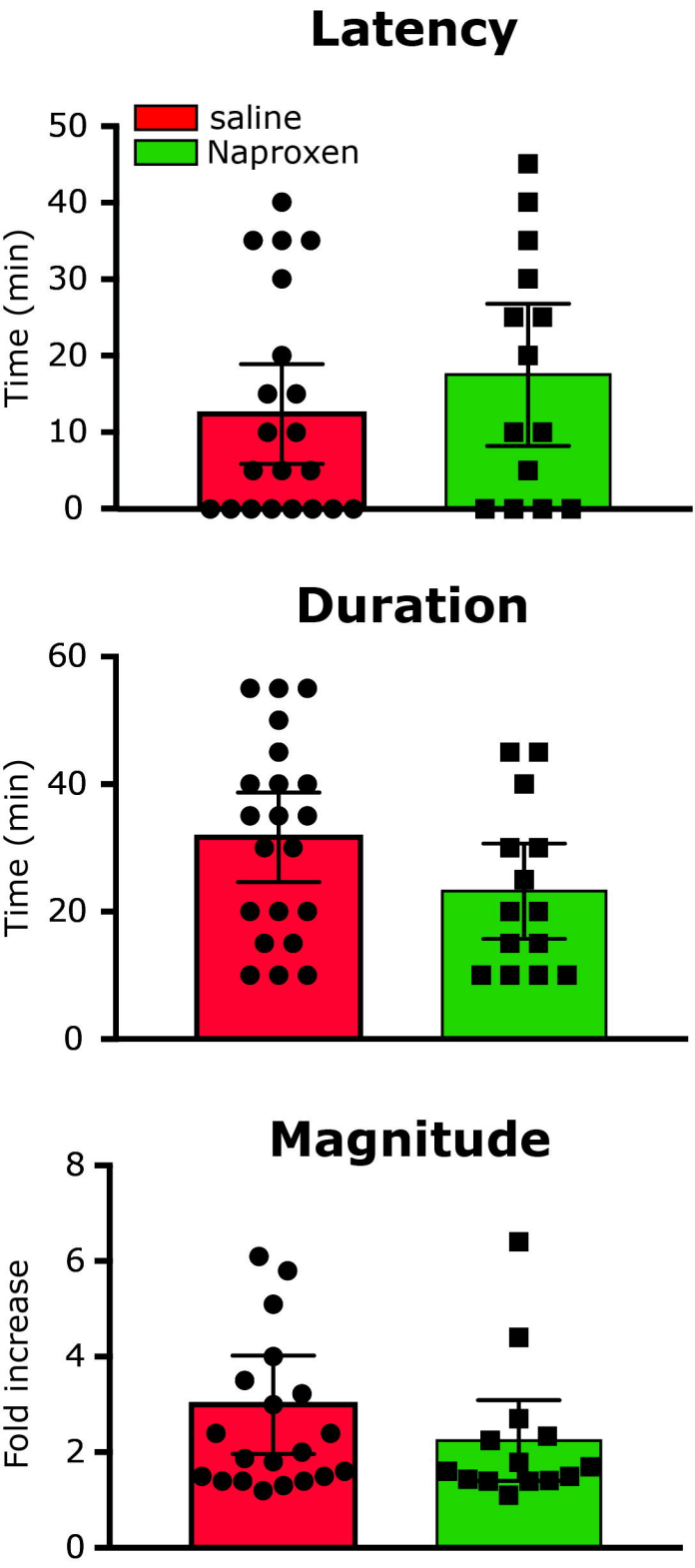
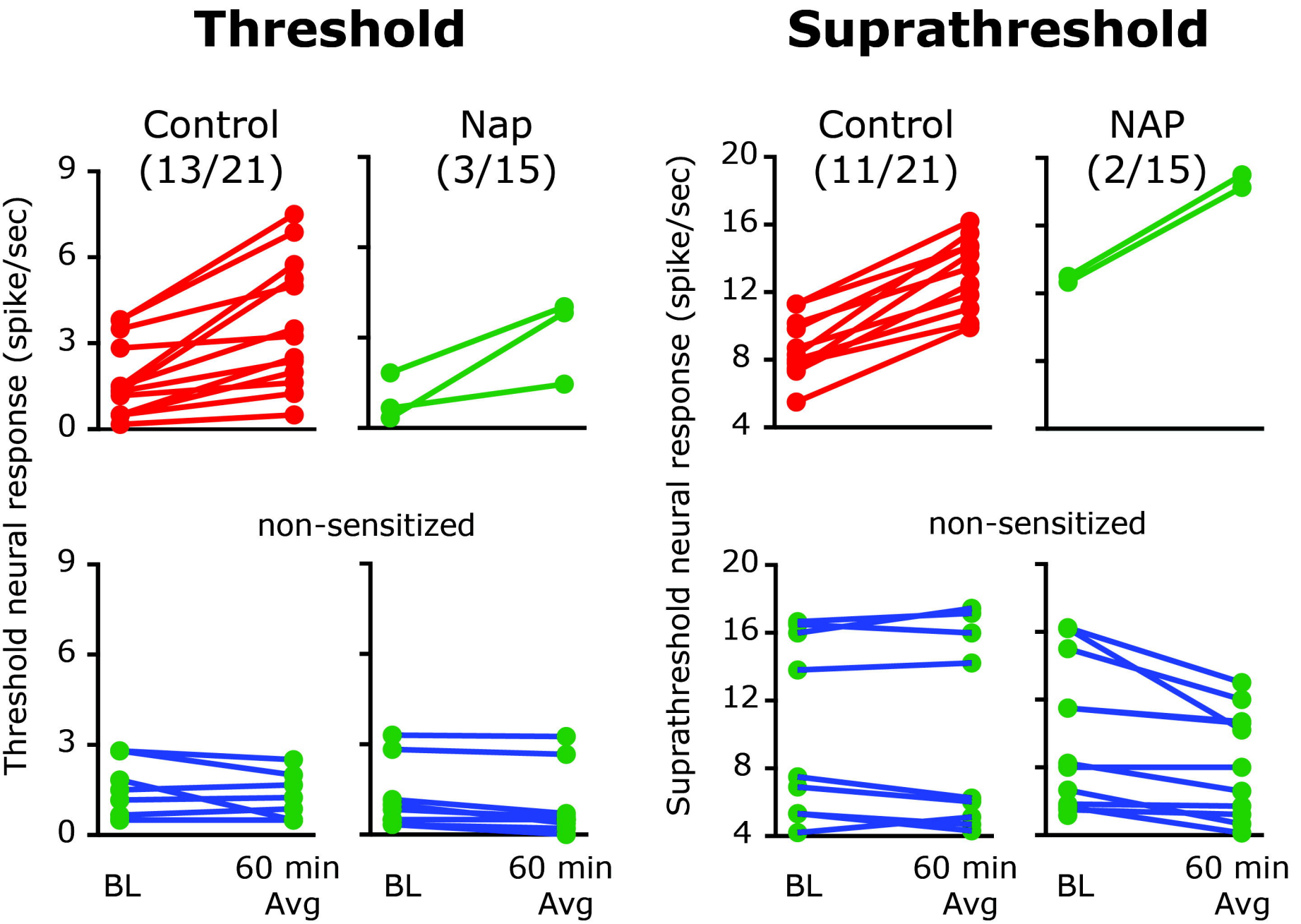

Latency
saline
Naproxen
Time (min)
50
40
30
20
10
0
Duration
Time (min)
60
40
20
0
Magnitude
Fold increase
8
6
4
2
0

Figure 4


Threshold
Suprathreshold
Control
(13/21)
Nap
(3/15)
Control
(11/21)
NAP
(2/15)
non-sensitized
non-sensitized
Threshold neural response (spike/sec)
Suprathreshold neural response (spike/sec)
9
6
3
0
20
16
12
8
4
BL
60 min
Avg
BL
60 min
Avg
BL
60 min
Avg
BL
60 min
Avg