Activation of Central Trigeminovascular Neurons by Cortical Spreading Depression

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Objective: Cortical spreading depression (CSD) has long been implicated in migraine attacks that begin with visual aura. Having shown that a wave of CSD can trigger long-lasting activation of meningeal nociceptors—the first-order neurons of the trigeminovascular pathway thought to underlie migraine headache—we now report that CSD can activate central trigeminovascular neurons in the spinal trigeminal nucleus (C1–2).

Methods: Stimulation of the cortex with pinprick or KCl granule was used to induce CSD in anesthetized rats. Neuronal activity was monitored in C1–2 using single-unit recording.

Results: In 25 trigeminovascular neurons activated by CSD, mean firing rate (spikes/s) increased from 3.6 ± 1.2 before CSD (baseline) to 6.1 ± 1.8 after CSD ($p < 0.0001$) for a period >13 minutes. Neuronal activity returned to baseline level after 30.0 ± 3.1 minutes in 14 units, and remained elevated for 66.0 ± 8.3 (22–108) minutes through the entire recording period in the other 11 units. Neuronal activation began within 0.9 ± 0.4 (0–2.5) minutes after CSD in 7 neurons located in laminae I–II, or after a latency of 25.1 ± 4.0 (7–75) minutes in 9 neurons located in laminae I–II, and 9 neurons located in laminae III–V. In 27 trigeminovascular neurons not activated by CSD, mean firing rate was 2.0 ± 0.7 at baseline and 1.8 ± 0.7 after CSD.

Interpretation: We propose that CSD constitutes a nociceptive stimulus capable of activating peripheral and central trigeminovascular neurons that underlie the headache of migraine with aura.

Ann Neurol 2011;000:000–000

Approximately 30% of episodic migraine attacks are associated with one or more symptoms of transient cortical malfunction, collectively referred to as aura.1–3 Most common among those symptoms are visual aura (scintillating lights or scotoma4) sensory aura (tingling or numbness5), speech aura (expressive aphasia or dysarthria6), and motor aura (impaired coordination or paresis6). Symptoms of aura typically develop some 30 to 60 minutes ahead of the onset of migraine headache.7–9 Patients experiencing migraine with aura (especially women) were found to be at higher risk for impaired cognitive functions10 and prone to developing cerebral infarction11–13 and deep white-matter lesions in the cerebellum and the red nucleus.14–16

Aura has long been suggested to be caused by a biphasic electrophysiological phenomenon dubbed cortical spreading depression (CSD). Animal studies have documented the propagation of spreading depression through the cortex (especially the visual cortex) as a wave of cellular hyperexcitability (depolarization) followed by a prolonged phase of quiescence.17,18 The phenomenon has been replicated, using subdural electrocorticography, during craniotomy in humans with ischemic stroke or acute brain injury.19–21 Short of intracranial electrophysiology, neuroimaging studies during migraine with aura have documented a number of hemodynamic changes—arguably reflecting CSD—including local changes in cerebral blood flow, cortical hyperemia, spreading oligemia, and hypoperfusion.22,23 A near-continuous functional magnetic resonance imaging recording during migraine with visual aura detected an initial wave of hyperemia followed by a wave of hypoperfusion that progressed slowly through the visual cortex in lockstep with the developing symptoms of aura.23

It has been proposed that CSD can precipitate headache during migraine with aura by activating a trigeminovascular pathway that originates in meningeal nociceptors.24 Using single-unit recording in the rat trigeminal ganglion, we have provided the first direct evidence that meningeal nociceptors can be activated by a wave of spreading depression passing through the visual cortex underneath...
their dural receptive field. Such activation was manifested as a 2-fold increase in mean neuronal firing rate that began either immediately, or some 14 minutes after CSD, and persisted for 45 minutes or longer.\textsuperscript{25}

Pursuant to the activation of meningeal nociceptors by CSD, we sought to examine the effects of CSD on the activity of second-order trigeminovascular neurons in spinal trigeminal nucleus. Such activation has been deduced from evidence that CSD can induce neuronal c-fos immunoreactivity in the superficial laminae of the trigeminal nucleus caudalis at the level of spinal segments C1–2.\textsuperscript{26}

### Materials and Methods

#### Surgical Preparation

Experiments were approved by the Beth Israel Deaconess Medical Center and Harvard Medical School standing committees on animal care, and in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (250–350g) were anesthetized with urethane (1.2g/kg intraperitoneally [i.p.]), fitted with an intratracheal tube for artificial ventilation of the lungs. The rat's head was then mounted in a stereotaxic apparatus, and core body temperature was maintained at 37°C using a heating blanket. Rats were paralyzed with gallamine triethiodide (0.5g/kg i.p.) and ventilated. End-tidal CO\textsubscript{2} was continuously monitored and kept within a physiological range of 3.5 to 4.5%.

For stimulation of the cranial dura mater in the experiment, a 5 × 5mm opening was carefully carved in the parietal and occipital bones in front and behind the lambda suture, directly above the left transverse sinus. The exposed dura was kept moist using a modified synthetic interstitial fluid (135mM NaCl, 10mM Hepes, pH 7.2). For single-unit recording in the spinal trigeminal nucleus at the level of spinal segments C1–2,\textsuperscript{26}

A tungsten microelectrode (impedance 0.8–1.2MΩ) was lowered repeatedly into the medullary dorsal horn in search for a trigeminovascular unit. A neuron was selected for the study if it exhibited discrete firing bouts in response to ipsilateral electrical and mechanical stimulation of the exposed cranial dura mater and to mechanical stimulation of the facial skin. Stimulation of the dura mater with electric pulses (0.1–4.0mA, 0.5 milliseconds, 0.5Hz pulses) and mechanical stimulation of the dura mater with calibrated von Frey monofilaments (3.63g) were associated with a simultaneous bout of activity. At the end of each experiment, the neuron under study was also challenged with chemical stimulation of the dura mater (50 or 100mM KCl), resulting in increased neuronal firing over ~30 to 60 seconds. Stimulation of the facial skin consisted of brush, pressure, and pinch, delivered sequentially (10 seconds each, 10-second interstimulus interval) using an artist paint brush, loose arterial clip, and forceps, respectively. Three classes of neurons were thus identified: wide dynamic range (WDR) neurons (incrementally responsive to brush, pressure, and pinch), low threshold (LT) neurons (equally responsive to all stimuli), and high threshold (HT) neurons (unresponsive to brush). A real-time waveform discriminator was used to create and store a template for the action potential evoked in the neuron under study by electrical pulses on the dura mater; spikes of activity matching the template waveform were acquired and analyzed online and offline using Spike 2 software (CED, Cambridge, UK).

#### Data Analysis

Data were analyzed by nonparametric statistics, using two-tailed level of significance set at \( p = 0.05 \). Mean firing rate (spikes/s) before cortical stimulation (baseline) was calculated in each trial over a period of 30 minutes. A given neuron was considered activated when its mean firing rate after CSD exceeded its mean baseline activity by 2 standard deviations of that mean for a period >10 minutes, which translated to a \( \geq 33\% \) increase in the final analysis. Latency to onset of neuronal activation and the duration of neuronal activation were compared across WDR, LT, and HT neurons using Kruskal-Wallis one-way analysis of variance. Mean firing rates before and after the induction of CSD were compared using Wilcoxon matched-pairs signed-ranks test.
Results

Using single-unit recording in the medullary dorsal horn (C1–2), we identified 52 trigeminovascular neurons (1 neuron/rat) that exhibited discrete bouts of activity in response to electrical, mechanical, and chemical stimulation of the cranial dura (Fig 1A–C). These units were functionally classified as HT, WDR, or LT according to the relative magnitude of their responses to ipsilateral stimulation of the facial skin with brush, pressure, and pinch (see Fig 1D–F). Histological localization of the neurons (Fig 2) showed that 30 units were located superficially in the dorsal horn (lamina I or II) and 22 in the deeper laminae (III, IV, or V). Excluding 5 unclassified units, the proportions of the different cell types were similar ($p > 0.73$) between the superficial and deep laminae (HT, 43 vs 47%; WDR, 40 vs 29%; LT, 17 vs 24%). Neuronal receptive fields at the dura (mostly at the transverse sinus above the visual cortex) and facial skin (mostly periorbital/ophthalmic) were consistently similar between superficial units (Fig 3A, B) and units located in deeper layers (see Fig 3C, D). Baseline ongoing activity was significantly different across HT, WDR, and LT units in the superficial laminae ($0.3 \pm 0.1, 2.2 \pm 0.9,$ and $3.0 \pm 1.7$ spikes/s, respectively; $p = 0.007$), but not in the deep laminae ($1.9 \pm 1.0, 2.9 \pm 2.5,$ and $4.4 \pm 2.9$ spikes/s, respectively; $p > 0.5$).

Waves of CSD lasting $72.8 \pm 4.9$ seconds (mean ± standard error of the mean) were registered within $33.4 \pm 3.0$ seconds of stimulating the visual cortex in 52 trials (41 with pinprick, 7 with crystalline KCl). Mean firing rate of all 52 tested neurons increased significantly ($p = 0.0009$) from $2.7 \pm 0.7$ at baseline to $3.9 \pm 1.0$ after CSD. Twenty-seven of those units, however, did not pass the criteria for neuronal activation (Fig 4A); their ongoing firing rate was $2.0 \pm 0.7$ at baseline and $1.8 \pm 0.7$ spikes/s after CSD (Fig 5). The remaining 25 neurons increased their firing rate for an extended duration (≥13 minutes) from $3.6 \pm 1.2$ spikes/s at baseline to $6.1 \pm 1.8$ after CSD ($p < 0.0001$). Firing rate increased after CSD by $33$ to $76\%$ in 8 activated units (see Fig 4D, Fig 5A, B, green plots), and by >106% in the 17 remaining units (see Fig 4B, C; Fig 5A–C, black plots). The fold increase in firing rate was most pronounced in units with mean baseline activity <0.4 spikes/s compared to those with baseline activity >0.6 spikes/s. Five units (all located in the superficial laminae) became activated during CSD or immediately after (0.2 ± 0.2; range, 0–1.0 minutes). The remaining 20 units (equally divided between superficial and deep laminae) became activated as late as $22.8 \pm 3.9$ (2–75) minutes after CSD. Neuronal activity returned back to baseline level after $30.1 \pm 3.1$ minutes in 14 units, and remained elevated for $66.0 \pm 8.3$ (22–108) minutes through the end of the recording period in the other 11 units. The pattern of neuronal activation (latency, duration, increased firing rate)
was unrelated to whether CSD was induced by pinprick (n = 18) or KCl (n = 7). The mean number of spikes recorded over the period of activation in all 25 neurons was $14,867 \pm 4,317$ compared with $7,592 \pm 2,164$ calculated for a corresponding interval at baseline.

The prospects for neuronal activation by CSD appeared to be unrelated to the functional classification of the neurons, their laminar position in the dorsal horn, or their cutaneous receptive field territories. The response profiles of activated HT, WDR, and LT neurons were similar.
to the corresponding profiles of their nonactivated counterparts (Fig 6). Neuronal activation occurred in 59% of WDR units (10 of 17), 56% of LT units (5 of 9), and 33% of HT units (7 of 21). Activated and nonactivated neurons were present in similar numbers in superficial laminae (16 and 14, respectively) and deep laminae (9 and 13, respectively) of the dorsal horn (see Fig 2). Dural and cutaneous receptive fields did not appear to vary between activated (see Fig 3A, C) and nonactivated neurons (see Fig 3B, D).

**Discussion**

This is the first study to show that induction of CSD by focal stimulation of the rat visual cortex can lead to increased ongoing activity in central trigeminovascular neurons in the spinal trigeminal nucleus. Together with our evidence for a similar effect of CSD on the activity of meningeal nociceptors, the findings strongly support the proposition that a wave of spreading depression in the visual cortex—the presumed mechanism of visual aura—can induce nociceptive signals in the overlaying meninges, resulting in sequential activation of peripheral (first-order) and central (second-order) neurons of the trigeminovascular pathway—a presumed mechanism of migraine headache.

Our earlier evidence for the activation of meningeal nociceptors by CSD should be taken as essential—but not necessarily sufficient—for to the activation of second-order trigeminovascular neurons, because the absolute increase in the ongoing activity of the nociceptors

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**Figure 3**: Localization of dural (red) and cutaneous (blue) receptive fields. (A) Activated neurons in the superficial dorsal horn. (B) Nonactivated neurons in the superficial dorsal horn. (C) Activated neurons in the deep dorsal horn. (D) Nonactivated neurons in the deep dorsal horn. (E) Detailed view of drawing illustrating dural receptive field. Arrow points forward.
FIGURE 4: Individual examples of neuronal firing before and after cortical spreading depression (CSD). Waves of CSD induced by a single stimulation of the cortex are shown in green (cortical activity was monitored continuously throughout the experiment, and no other CSD waves were registered). Neuronal activation (red bars) was determined for each neuron when its firing rate after CSD exceeded its mean baseline activity (bottom of yellow box) by 2 standard deviations of that mean (top of yellow box) for a period of >10 minutes. Black line curves shown across the histograms describe the pattern of neuronal activity after applying a moving-average smoothing function. (A) Example of a neuron that was not activated by CSD. (B) Example of a neuron that became activated immediately after CSD and remained activated for 78 minutes, through the end of the recording session. (C) Example of a neuron that became activated 30 minutes after CSD and remained activated for 90 minutes through the end of the recording session. (D) Example of a neuron that became activated 30 minutes after CSD and remained activated for 32 minutes. Note the transient (6 minutes) surge of activation immediately after CSD. The laminar location of each neuron and its dural and facial receptive fields are illustrated on the right.
was rather small (<1 spike/s) relative to the magnitude of neuronal discharge elicited by skin stimulation. That a single wave of CSD induced a modest yet persistent increase in the ongoing neuronal activity in the spinal trigeminal nucleus (present study) implies that small rises in activity of the primary afferents should suffice to induce action potentials in the central neurons (rather

FIGURE 5: Firing rate before and after cortical spreading depression (CSD) in activated and nonactivated central trigeminovascular neurons. Individual plots and mean ± standard error of the mean values are shown for activated (left, red circles) and nonactivated units (right, blue circles). A neuron was considered activated when its mean firing rate after CSD exceeded its mean baseline activity by 2 standard deviations of that mean over a period of >10 minutes as shown in Figure 4. (A) Neurons exhibiting a high rate of baseline activity (12.09 ± 3.09; range, 5–26 spikes/s) increased their firing rate to 19.85 ± 3.19 spikes/s (65%) after CSD. (B) Neurons exhibiting a medium rate of baseline activity (2.3 ± 0.46; range, 0.6–4 spikes/s) increased their firing rate to 3.92 ± 0.49 spikes/s (75%) after CSD. (C) Neurons exhibiting a low rate of baseline activity (0.08 ± 0.03; range, 0.0–0.4 spikes/s) increased their firing rate to 0.49 ± 0.07 spikes/s (600%) after CSD.

FIGURE 6: Response magnitude to innocuous and noxious stimulation of the cutaneous receptive fields in activated and nonactivated neurons. (A) High threshold neurons. (B) Wide dynamic range neurons. (C) Low threshold neurons. Note that the relative response magnitude to brush (Br), pressure (Pr), and pinch (Pi) were similar between activated and nonactivated neurons in each category.
than mere subthreshold excitatory postsynaptic potentials or inhibitory postsynaptic potentials. For example, for a neuron whose activity increased from 0.06 spikes/s at baseline to 0.73 spikes/s after CSD (see Fig 2B), the increase in the total number of spikes over the recording period of activation of this neuron was 3,575. Taken as a group, the 25 activated neurons sampled in the study generated 182,000 additional spikes along the trigeminovascular pathway. Assuming these neurons project to the thalamus, we propose that a relatively small rise in their firing rate after CSD can become over time sufficient to activate trigeminovascular neurons in the thalamus. The cumulative magnitude of such an effect provides a novel perspective on the potential power of CSD as a trigger of migraine headache.

The magnitude and duration of neuronal activation induced by CSD, as well as the latency to its onset, were quite similar between the peripheral25 and the central trigeminovascular neurons (present study), suggesting that CSD-evoked activation of meningeal nociceptors is sufficient to drive the central trigeminovascular neurons into an activity state. Both peripheral and central neuronal activation were manifested as a 2-fold increase in mean ongoing firing rate. Neuronal activity returned to baseline after a mean period of 30 minutes in 65% of the peripheral units and 37 minutes in 56% of the central units, which is by far shorter than the typical duration of migraine headache. This duration appears to correlate with the duration of the second wave of altered neurovascular function shown recently to be associated with negative current shift, arterial constriction, and hemoglobin desaturation. However, the remaining trigeminovascular units (35% peripheral; 44% central) maintained their elevated firing rate throughout the recording period (interrupted after nearly 2 hours), which would be longer than the reported duration of altered neurovascular functions following CSD. This raises the possibility that the initiation of headache during migraine with aura depends on CSD and its accompanied neurovascular changes, whereas the maintenance phase of the headache depends on long-lasting activation of the neural component of the trigeminovascular system.

Consistent with the typical delay between the onset of visual aura and the onset of migraine headache, neuronal activation started with a mean delay of 14 and 25 minutes after CSD in 78% and 72% of the peripheral25 and central units, respectively. A gradual increase in the firing rate of meningeal nociceptors25 may be due to a progressive recruitment of ramifying collateral branches31 during the propagation of the CSD wave underneath them. A gradual increase in the firing rate of the central neurons may reflect increased input from a growing number of nociceptors impacted by the propagating wave of CSD. Activation of the remaining units (22% peripheral; 28% central) coincided with the wave of CSD. Such immediate activation may be related to the direct current shift (and the accompanying arterial constriction, desaturation of hemoglobin, and marked increase in extracellular potassium concentration) recorded while the CSD wave was still propagating.29 It is tempting to propose that meningeal nociceptors whose axons extended into the pia32 were activated by increased extracellular level of potassium at the surface of the cortex during the wave of CSD, resulting in immediate activation of the central neurons.

In agreement with a c-fos study,26 we found that CSD can activate trigeminovascular neurons in the ventrolateral region of the superficial dorsal horn of C1–2, which constitutes a main termination zone of meningeal nociceptors.33,34 Histological examination of lesions performed at the end of each experiment to mark the recording site revealed that activated units were present not only in lamina I as previously reported using c-fos but, for the first time, also in laminae II, III, IV, and V, adjacent (within 200 μm) to the medial tip of the ventrolateral region we targeted originally. After sampling additional units in the deeper laminae, we found that the likelihood of inducing neuronal activation by CSD was similar between the superficial and the deep laminae of C1–2 (53 and 41%, respectively). Is it possible, therefore, that nociceptive signals are processed differentially between trigeminovascular neurons of the superficial dorsal horn compared to their deeper counterparts?

Delayed neuronal activation by CSD was documented in meningeal Aδ and C nociceptors25 and in dura-sensitive neurons located in laminae I–II and laminae III–V of the dorsal horn (present study). In contrast, immediate neuronal activation occurred mostly with C nociceptors25 and selectively in trigeminovascular neurons located in laminae I–II (present study). Although the role of laminae I and V in pain sensation remains controversial,35–38 anatomical evidence suggests that their afferent connections are different. Lamina I receives direct input from peptidergic C nociceptors (releasing calcitonin gene-related peptide and substance P) and Aδ nociceptors, whereas lamina V receives direct input from Aδ nociceptors and LT Aβ fibers, and only indirect input from C nociceptors.41 Thus, the activation of trigeminovascular neurons in the superficial dorsal horn immediately after CSD might be mediated by peptidergic meningeal nociceptors42 that project directly to lamina I. Activation of peptidergic meningeal nociceptors by CSD might lead to the initiation of neurogenic
inflammation, a process that has been proposed to involve (1) release of ATP, glutamate, K\(^+\), and H\(^+\) from cortical neurons, glia, and vascular cells during the depolarization phase of CSD, and (2) diffusion of those agents to the adjacent cerebral meninges where they presumably reach the nociceptors.

Due to the size of the lesions marking the recording sites, we were unable to determine the precise localization of the neurons between the outer and inner sublayers of lamina II. The outer layer of lamina II receives direct input from peptidergic C-nociceptors, whereas the inner layer receives direct input from nonpeptidergic C-nociceptors and from LT, myelinated A\(\beta\) fibers. Furthermore, the outer layer contains inhibitory interneurons, whereas the inner layer contains excitatory interneurons implicated in the transition from acute to persistent pain.

The activation of lamina II neurons by CSD leads us to propose that activity along the pathway ascending from the spinal trigeminal nucleus to the brainstem and thalamus can be modulated already at the level of lamina II of the dorsal horn by inhibitory interneurons in the outer layer and facilitatory interneurons in the inner layer. Malfunction of such a local modulatory circuit (presumably due to cumulative damage from repeated migraine attacks) may contribute to a transformation from episodic migraine to chronic migraine.

Eighty percent of the activated neurons were classified as HT or WDR. The exact role HT and WDR neurons play in nociception is controversial, but they both lie at the origin of the so-called labeled pain pathways, and their activation is believed to produce the complex human experience of pain. The remaining 20% of the activated neurons were LT. As their activation can produce tactile but not pain sensation, such LT neurons may be positioned to mediate nonpainful symptoms of migraine such as facial tingling or numbness that some patients experience throughout the attack. This speculation may be more appropriate than the notion that the numbness and tingling are manifestations of sensory aura, as auras are not believed to last for hours or days.

The relevance of CSD-induced activation of the trigeminovascular pathway to the precipitation of headache during migraine with aura remains to be determined. Although the endogenous triggers of CSD are largely unknown, recent data have suggested altered cerebrovascular function, potentially related to vasculopathy, as the culprit. For example, induction of cerebral vasoconstriction by endothelin-1, or brief hypoxic–ischemic episodes by cerebral microembolism, have been shown to trigger CSD.

The main conclusion drawn from our study—that CSD is a nociceptive stimulus capable of activating peripheral and central trigeminovascular neurons—appears to be at odds with studies that attempted and failed to induce activation of central trigeminovascular neurons by CSD. Those negative results were obtained in the transition zone between subnuclei caudalis and interpolaris, or in laminae III and IV of C1–2, whereas our positive results were observed in other laminae (I, II, and V) of C1–2. In view of these anatomical differences, we call for revising the categorical exclusion of CSD as a trigger for headache in migraine with aura, which has been based entirely on the aforementioned negative studies. Rather, we suggest that trigeminovascular neurons susceptible to CSD are located specifically in layers I, II, and V of the spinal trigeminal nucleus at the level of C1–2. Future studies will be necessary to document the effects of CSD on higher-order trigeminovascular neurons in the thalamus and brainstem to explain the full duration and fluctuating intensity of migraine headache, and its spread from one side to the other, or from the periorbital to occipital areas.

Acknowledgment

This research was supported by NIH National Institutes of Neurological Disorders and Stroke grants NS069847 and NS035611 (R.B.).

Potential Conflicts of Interest

R.B.: consultancy, Allergan; grants/grants pending, Allergan, Endo, GlaxoSmithKline, Merck; speaking fees, Merck.

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