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Short communication

Cortical spreading depression decreases Fos expression in rat periaqueductal gray matter

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HIGHLIGHTS

- Cortical spreading depression (CSD) likely underlies migraine aura.
- Periaqueductal gray matter (PAG) controls pain and autonomic functions.
- CSD provocation decreased neuronal Fos in PAG and Edinger–Westphal nucleus of rats.
- The numbers of CSDs correlated negatively with Fos-immunoreactive counts.

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ABSTRACT

The migraine headache involves activation and central sensitization of the trigeminovascular pain pathway. The migraine aura is likely due to cortical spreading depression (CSD), a propagating wave of brief neuronal depolarization followed by prolonged inhibition. The precise link between CSD and headache remains controversial. Our objectives were to study the effect of CSD on neuronal activation in the periaqueductal grey matter (PAG), an area known to control pain and autonomic functions, and to be involved in migraine pathogenesis. Fos-immunoreactive nuclei were counted in rostral PAG and Edinger–Westphal nuclei (PAG–EWn bregma −6.5 mm), and caudal PAG (bregma −8 mm) of 17 adult male Sprague–Dawley rats after KCl-induced CSD under chloral hydrate anesthesia. Being part of a pharmacological study, six animals had received, for the preceding 4 weeks daily, intraperitoneal injections of lamotrigine (15 mg/kg), six others had been treated with saline, while five sham-operated animals served as controls. We found that the number of Fos-immunoreactive nuclei in the PAG decreased after CSD provocation. There was no difference between lamotrigine- and saline-treated animals. The number of CSDs correlated negatively with Fos-immunoreactive counts. CSD-linked inhibition of neuronal activity in the PAG might play a role in central sensitization during migraine attacks and contribute to a better understanding of the link between the aura and the headache.

1. Introduction

In about 20% of subjects, the migraine headache is preceded by neurological aura symptoms [42]. Cortical spreading depression (CSD) is thought to be the underlying mechanism of the migraine aura [19]. The migraine headache involves activation and central sensitization of the trigeminovascular pain pathway. The precise link between CSD and headache remains controversial [18], as does the possibility that CSD-like events may also occur during attacks of migraine without aura [49].

In rat CSD can activate second order trigeminovascular projections of the spinal trigeminal nucleus [23] and provoke behavioral changes comparable to those seen during the migraine attack [2]. Migraine attacks are associated with activation in an upper brain

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stem area that includes periaqueductal grey matter (PAG) [1]. On magnetic resonance imaging (MRI), iron deposition [47], and tissue density [41] were found increased in the PAG of episodic and chronic migraine patients. Lesions in the PAG can provoke migraine-like headaches [17] as can electrodes implanted for neurostimulation therapy of chronic pain [40]. In functional MRI studies the connectivity of the PAG in migraine patients was increased with somatosensory and nociceptive circuits which correlated with disease duration [33]. Specific anti-migraine drugs, like triptans, when injected in the PAG of experimental animals, are able to inhibit trigeminal nociceptor responses induced by dural stimulation [4].

The PAG is a pivotal component of the descending pain inhibitory system but also of emotional behavior and autonomic control [5]. It receives afferents from the cortex [46], where CSD occurs [19], and projects to hypothalamus [46], and raphe nuclei [5] thought to be involved in migraine pathogenesis. Neuronal dysfunction in the PAG may, thus, be involved in various features that accompany the migraine attack [30].

We hypothesized that CSD would be able to modify neuronal activity in the PAG. Fos expression, a validated marker of neuronal activation, was used to estimate activity of PAG neurons after artificial provocation of CSDs over the occipital cortex of anesthetized rats. Since we previously shown that lamotrigine (LTG) is a potent inhibitor of KCl-induced CSD in rat cerebral cortex [6], we included LTG-treated animals in that study to verify if pharmacological suppression of CSDs abolishes the effect of CSDs on PAG.

2. Methods

2.1. Experimental animals

Seventeen adult male Sprague–Dawley rats were randomly chosen among those used in a previously published study on the effect of preventive anti-migraine drugs on CSD [6]. The animals weighed between 300 g and 480 g on the day of recordings. All animals were separated randomly onto three experimental groups during the therapeutic study [6]. Five rats were sham-operated and received no treatment. Two groups of six animals received, for 4 weeks daily, intraperitoneal (IP) injections of saline or lamotrigine (15 mg/kg, a gift by GSK, UK). The number of animals used was kept to a minimum and chosen according to the numbers used in other *c*-Fos studies in PAG [22]. The Ethics Committee of University of Liege approved the study and the guidelines for animal care were followed.

2.2. Anesthesia and surgery

Anesthesia was induced by 400 mg/kg chloral hydrate (8% in saline, 5 ml/kg) and maintained by smaller doses (80 mg/kg) added every hour. Level of anesthesia was monitored by tail pinch and electrocorticogram (ECoG) where applicable. Rectal temperature was maintained between 36.5 and 37.0 °C using a thermostatically controlled heating blanket (ATC 1000®, WPI Inc., USA). The rats were placed in a stereotaxic frame (David Kopf Instruments, USA). Three 1–2 mm wide burr holes were drilled 2 mm off the midline: 7 mm posterior to bregma (P-7; occipital cortex; stimulation site), 4 mm posterior to bregma (P-4; occipito–parietal junction; posterior recording site), and 1 mm anterior to bregma (A + 1; frontal cortex; anterior recording site) [39] to observe the propagation of CSD which was the aim of main study.

2.3. CSD provocation and recordings

The detailed stimulation and recording procedures were described previously [6]. Briefly, after surgery that lasted 30–40 min, in all animals except in the sham-operated group, we induced CSD with a cotton ball soaked with 1 M KCl placed over the occipital cortex (AP-7, R + 2). The cotton ball was kept moist by adding every 20 min a 1.5 μl drop of KCl and left in place for 2 h. For sham-operated animals the cotton ball was soaked with artificial CSF. CSDs and the electrocorticogram (DC-100 Hz) were recorded ipsilaterally from two cortical areas: frontal (bregma AP + 1, R + 2) and occipito-parietal (AP -4, R + 2) at a depth of 1 ± 0.2 mm. Animals were sacrificed immediately after the recordings, i.e., on average 2 h 30 min after induction of the first CSD by an IP nembutal injection (natrium pentobarbital 60 mg/ml:3 ml/kg BW according to the ethical guidelines of the University of Liege).

2.4. Histology

Rats were perfusion-fixed with 500 ml 4% paraformaldehyde in phosphate-buffered saline. After cryoprotection (30% sucrose for two days), 30 μm transverse serial cryostat sections of the brain were cut and serially collected in 12 wells containing cold PBS. Each well received sections at a 360 μm distance throughout the rostro-caudal extent of the brain. Fos expression was assessed using standard immunohistochemistry protocol [8]. Free-floating sections after pretreatment were kept overnight at room temperature in anti-*c*-Fos (Santa Cruz Biotechnology, sc-52-G) primary antiserum at a dilution of 1:1500. The immunocytochemical reaction product was visualized using the Vectastain (Vector Laboratories, Inc., PK-6101) avidin–biotin kit (ABC) with imidazole-intensified 3,3′-diaminobenzidine. Running during each staining procedure a series of sections without the primary antiserum served to control specificity of immunoreactivity.

Fos-immunoreactive (Fos-Ir) nuclei were counted automatically on a digital image analyzer in 2000 × 2000 μm areas of interest manually adjusted. The software package analySIS (Olympus soft imaging solutions GmbH) was used for the automatic counting. Counts were performed on three sections separated by 360 μm at two PAG levels: (1) PAG, bregma −8 mm or (2) PAG + Edinger–Westphal nucleus (EWn), bregma −6,5 mm [39].

As the PAG comprises different functional segments, we separately quantified Fos expression in ventro-lateral, dorso-lateral, and dorso-medial segments of the rostral PAG area and EWn (bregma −6.5 mm). For every image the aqueduct was located; we corrected rotation of sections according to the shape of the aqueduct. The periaqueductal area was then subdivided into six radial segments, as shown in Fig. 1 (top panel). The number of Fos-Ir particles was estimated separately for each of functional segments.

2.5. Statistical analyses

Statistica 9.0 (StatSoft Inc., Tulsa, OK, USA) was used for the statistical analysis. Group values were expressed as means ± 95% confidence intervals. The unit of analysis was a count of Fos-Ir in a single slice for histology and an animal for occurrence of electrophysiologically recorded CSDs during 2 h. Shapiro–Wilk test for normality used for each group separately did not detect violation of normal distribution of the dependent variables; we relied, therefore, on parametric statistics.

We used repeated measures two-way ANOVA to test for the group differences in Fos-Ir expression. Where ANOVA revealed a significant between-group difference, we used post-hoc Scheffe's test for pair-wise comparison of the group means.

Spearman's rank order correlations (*R*) where computed in each CSD group to explore the links between the number of CSDs and of Fos-Ir nuclei in individual animals.

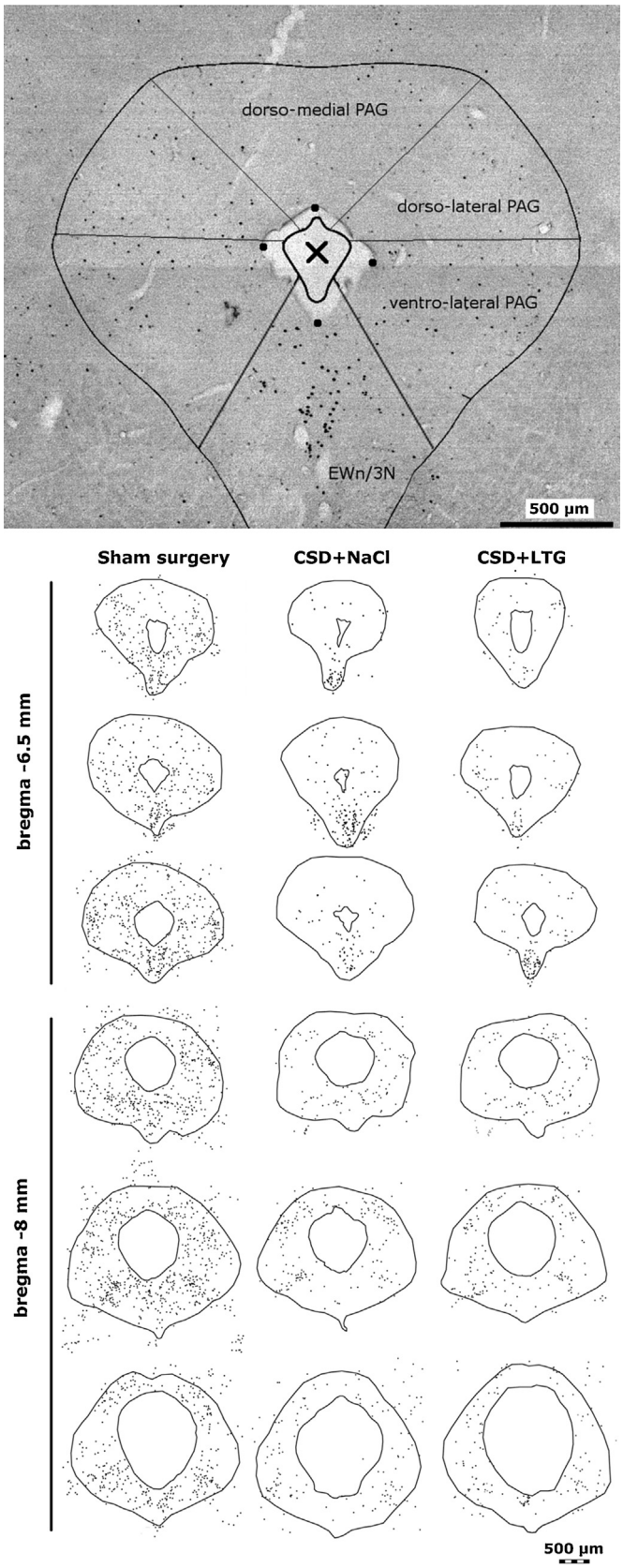

Fig. 1. Top: scheme of segmentation into dorso-medial PAG, dorso-lateral PAG, and ventro-lateral PAG (periaqueductal gray matter) segments and Edinger–Westphal nucleus, EWn/3N areas on a Fos-Ir transverse section of the region of interest (bregma −6.5 mm). Bottom: illustrative camera lucida drawings of Fos-Ir nuclei identified in transverse sections of rostral (bregma −6.5 mm) and caudal parts (bregma −8 mm) of the PAG in an animal belonging to the sham-operated, CSD-stimulated and NaCl-treated, or CSD-stimulated and lamotrigine (LTG)-treated groups. Three sections separated by 360 μm are shown for the two PAG levels.

3. Results

3.1. CSD numbers

The results on CSD numbers and propagation were reported and discussed previously [6] and will not be described in detail here. Briefly, in lamotrigine treated group frequencies of occipito–parietal and frontal CSDs (4.4 ± 3.1 and 1.6 ± 0.8, respectively) were significantly reduced comparatively to saline treated animals (6.7 ± 0.9 and 3.4 ± 0.8, respectively).

3.2. Numbers of Fos-Ir neurons in PAG and EWn

There was no difference in Fos-Ir between right and left PAG in any animal group (Fig. 1, bottom panel). ANOVA showed between-group differences for both rostral ($p < 0.001$) and caudal ($p < 0.001$) PAG areas. CSD provocation resulted in reduction in the number of Fos-positive cells both in the rostral PAG + EWn area and in the caudal PAG area in animals treated either with NaCl (respectively, 53% and 61% decrease, $p < 0.01$) or lamotrigine (respectively, 68% and 73% decrease, $p < 0.001$), as compared to animals that underwent sham craniotomy and anesthesia of equivalent duration (Fig. 2, top panel). The difference between NaCl- and lamotrigine-treated rats was not significant.

When we analyzed counts of Fos-Ir nuclei separately in dorso-medial, dorso-lateral, and ventro-lateral segments of the PAG, the between-group differences were similar to those found for the total PAG area (Table 1). Both NaCl and lamotrigine groups had decreased Fos-Ir expression in all PAG segments compared to the sham group with a trend towards the least Fos expression in the lamotrigine group. In the EWn segment, the difference in Fos expression was significant only between sham and lamotrigine groups, while NaCl-treated animals had an intermediate level of Fos-Ir nuclei.

3.3. Correlation between PAG and EWn Fos-Ir neuron counts and CSD numbers

Using Spearman's rank correlation test, the number of Fos-Ir nuclei in the PAG overall correlated negatively with that of CSDs. In lamotrigine-treated animals, in particular, there was a significant inverse correlation between Fos-Ir expression in both rostral and caudal PAG areas and number of occipito–parietal CSDs ($R = -0.82$, $p = 0.05$, and -0.84, $p = 0.036$, respectively) (Fig. 2, bottom panel). Fos-Ir in the EWn segment tended strongly to correlate negatively with number of frontal CSDs in the NaCl-treated group ($R = -0.79$, $p = 0.059$) and with occipito-parietal CSDs in the lamotrigine-treated group ($R = -0.75$, $p = 0.084$). In the global rostral PAG/EWn area we found the same inverse correlation with a strong trend for significance between number of Fos-Ir nuclei and frontal CSDs in NaCl-treated rats ($R = -0.79$, $p = 0.059$) (Fig. 2, bottom panel).

4. Discussion

This study shows that waves of CSDs induced by unilateral KCl application on the occipital cortex result in a reduction of the number of Fos-positive cells in the PAG of anesthetized rats. The reduction is proportional to the number of induced CSDs, and thus, not due to KCl itself; it is not found in sham-operated animals.

The neuronal inactivation in PAG is likely due to the CSD-induced changes in cortical activity. Whether activation of inhibitory cortical projections to the PAG by the initial depolarization associated with CSDs or inactivation of excitatory projections during the subsequent prolonged neuronal inhibition is the culprit cannot be determined here. Given, however, that most known projections from cortical areas to the PAG are excitatory [10,15,27,38]

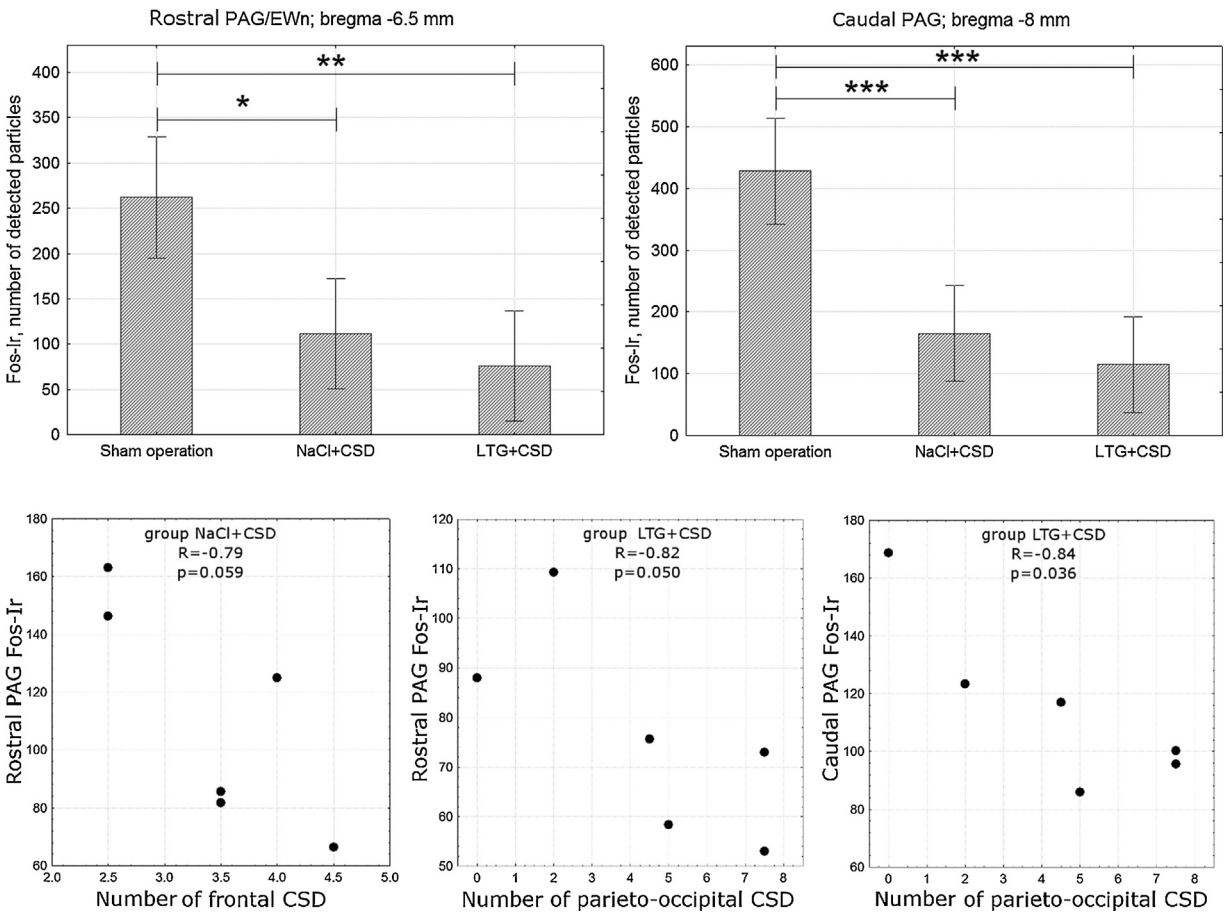

Fig. 2. Top: histogram of the number (mean ± 95% confidence intervals) of Fos-immunoreactive (Fos-Ir) nuclei in sham-operated animals ($n = 5$), in animals that had CSDs induced and were treated preventively either with NaCl ($n = 6$) or with lamotrigine (LTG-15 mg/kg; $n = 6$). Upper left panel: rostral PAG–Ewn portion (bregma −6.5 mm). Upper right panel: caudal PAG portion (bregma −8 mm). ANOVA group difference ($p < 0.001$) both for rostral PAG + EWn and caudal PAG. Scheffe's post-hoc test: $^{*}p < 0.01$, $^{**}p < 0.001$, compared to the sham-operated group. Bottom: scatterplots of the number of Fos-Ir nuclei (ordinate) and the number of CSDs per hour showing the negative correlation between the two. Left panel: frontal CSDs and rostral PAG Fos-Ir nuclei in NaCl-treated animals. Middle panel: occipito–parietal CSDs and rostral PAG Fos-Ir nuclei in LTG-treated animals. Right panel: occipito–parietal CSDs and caudal PAG Fos-Ir nuclei in LTG-treated animals.

decreased excitation of PAG neurons during the depression phase of CSDs is a plausible explanation. The observed bilateral reduction in Fos-Ir PAG neurons, despite unilateral CSDs, and unilateral cortical Fos activation [6], can be due to the fact that projections from the cerebral cortex to the PAG are both ipsi- and contra-lateral [15].

We cannot exclude the possibility that subcortical areas in part mediate the CSD-induced reduction of neuronal activity in PAG. CSD can indeed propagate to the amygdala where it significantly augments long-term potentiation [13]. The central nucleus of the amygdala has both excitatory and inhibitory connections with ventro-lateral PAG [11], an area receiving also projections from the trigeminal nucleus. Freezing induced by CSDs in awake rats has been attributed to synergic amygdala–PAG activity [2]. Another area projecting to PAG is the hypothalamus [5] and CSDs increase Fos expression bilaterally in the magnocellular part of hypothalamic paraventricular nulei while decreasing it in the parvocellular portion [24]. Interestingly, the early premonitory phase of migraine attacks is associated with activation of the hypothalamus in addition to midbrain and pons [34].

Our findings are in line with those of Lambert et al, [30] showing in cat CSDs or flashing light stimulation, both considered to be migraine triggers, reduce the inhibition of second order trigeminovascular neurons induced by PAG stimulation (unpublished results [30]), and inhibit neurons in nucleus raphe magnus [29].

Suppression of PAG also might play a role in the cutaneous allodynia that frequently accompanies migraine attacks and predicts migraine chronification [32]. Migraineurs with severe allodynia have stronger functional connectivity between PAG and other cerebral areas involved in pain processing [32] while descending

Table 1
Number of Fos-immunoreactive cells (means ± 95% confidence intervals) in the different segments of the rostral PAG/EWn area at bregma −6.5 mm in sham-operated animals and in animals that had CSDs induced for 2 h after a preventive daily treatment for 1 month with NaCl or lamotrigine (LTG, 15 mg/kg).

	Sham surgery ($n = 5$)	NaCl treatment ($n = 6$)	LTG treatment ($n = 6$)	ANOVA group effect, p
Dorso-medial PAG	34 ± 8	14 ± 8*	7 ± 8**	0.001
Dorso-lateral PAG	56 ± 16	15 ± 15*	13 ± 15*	0.001
Ventro-lateral PAG	67 ± 17	23 ± 15*	16 ± 15*	0.001
EWn	83 ± 15	59 ± 13	41 ± 13*	0.002

* −0.01.
** −0.001; for post-hoc Scheffe's test, in comparison to the sham-surgery group.

analgesia areas are disconnected from PAG in patients with allodynia [33].

PAG inhibition might also be responsible for other migraine symptoms. Besides increasing perception of any pain stimulus, e.g., that coming from neurogenic inflammation [36], it could be involved in the behavioral and autonomic symptoms that accompany the migraine attack, as such functions are in part controlled by the ventro-lateral PAG [9] in particular freezing/quiescence, nausea, localized hyperalgesia during noxious stimulation, emotional irritability, and aggressive behavior [3].

The inhibitory effect of CSDs was not limited to the PAG, but extended to the Edinger–Westphal nucleus (EWn). The EWn is thought to be composed of at least two neuronal populations [44]. The cholinergic neurons of the preganglionic parasympathetic population innervate the pupillary constrictor and the ciliary muscles [16]. The second population of neurons uses peptides, such as urocortin-1 [45] and amphetamine-regulated transcript (CART) [14], as neurotransmitters and seems to be implicated in stress response and feeding behavior [28,43,48]. It remains to be determined whether the inhibition of these two neuronal populations by CSDs associated with the migraine aura could be implicated in the behavioral changes, nutritional disturbances, and pupillary abnormalities [12,20] that can be associated with the migraine attack.

There are no known direct cortical projections to the Edinger–Westphal nuclei. These nuclei receive projections from rostral PAG [26] and project reciprocally to all PAG columns that are themselves bilaterally interconnected [25]. In functional studies, however, the cerebral cortex is able to modulate pupil size via the Edinger–Westphal nuclei [21]. Transcranial magnetic activation of frontal, central, and parieto–occipital areas, for instance, produces dilatation of both pupils that is maximal after ±1.5 s [37]. Therefore, CSD-induced inhibition of EWn activity might be involved in the mydriasis accompanying certain migraine attacks.

We have previously shown that lamotrigine significantly lowers CSD frequency [6] and we expected, therefore, to find more Fos-Ir PAG neurons in lamotrigine-treated than in saline-treated animals. Surprisingly, there was, however, a similar decrease of Fos expression in both animal groups. A possible explanation for this finding is the fact that lamotrigine is known to block Ca^{2+} channels in PAG neurons [35]. This might decrease neuronal activity to a sufficient extent as to mask the potential increase of Fos expression related to the lamotrigine-induced reduction of CSD frequency.

5. Conclusion

Cortical spreading depressions, known to cause the aura symptoms of migraine, significantly decrease neuronal activity in the periaqueductal grey matter and Edinger–Westphal nucleus as assessed with Fos immunoreactivity in rats. In a translational perspective, CSD-induced reduction of neuronal activation in the pain-controlling upper brainstem centers during the migraine aura might disinhibit second order neurons in the trigemino-cervical complex, and thus, promote sensitization and headache.

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