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ORIGINAL ARTICLE

Evaluation of c-Fos immunoreactivity in the rat brainstem nuclei relevant in migraine pathogenesis after electrical stimulation of the trigeminal ganglion

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Abstract Migraine is a common neurological condition, causing high disability, but the pathomechanism of the disease is not yet fully understood. Activation of the trigeminovascular system could play a crucial role in the manifestation of the symptoms, but initial step of this activation remains unknown. Functional imaging studies have revealed that certain brainstem areas, referred to as migraine generators, are activated during a migraine attack, including the dorsal raphe, the periaqueductal gray, the locus coeruleus, and the nucleus raphe magnus. However, the studies performed to date have not demonstrated whether this activation is a trigger or a consequence of the migraine attack. With the aim of evaluating the functional relationship between activation of the trigeminal system and migraine generators, we examined the changes in c-Fos immunoreactivity in the above-mentioned nuclei after stimulation of the trigeminal ganglion, an animal model for trigeminovascular activation. The stimulation led to significant increases in the number of c-Fos immunoreactive cells in the nucleus raphe magnus and in the caudal part of the spinal trigeminal nucleus, 2 and 4 h after the stimulation. Activation of the trigeminal system failed to exhibit uniform activation of the brain stem nuclei related to migraine. Our results suggest that the activation of the trigeminal system in the rat by electrical stimulation of the trigeminal ganglion leads to the activation of the descending pain modulatory system, but not to the activation of "migraine generator" nuclei. Therefore, the activity pattern seen in functional studies may reflect a unique feature, exclusively present in migraine.

Keywords Migraine generators · C-Fos · Electrical stimulation of the trigeminal ganglion · Rat

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Introduction

Migraine is a neurological disorder, with an unrevealed pathomechanism. Over the years, many theories have emerged as the cause of the disease; vascular, neurogenic, and genetic processes have been proposed, but none of these could provide a reliable explanation of all the events related to this disorder [1].

During a migraine attack, the trigeminovascular system is activated, neuropeptides and inflammatory mediators are released, leading to neurogenic inflammation with vasodilation and plasma extravasation which can activate the trigeminal nociceptors and cause pain [2].

The activation and sensitization of the trigeminal system play a crucial role in migraine pathomechanism. These activation processes affect the caudal part of the spinal trigeminal nucleus (TNC); therefore, numerous studies have examined the activation pattern of this nucleus in different animal models [3], but the very first step, which leads to these phenomena, remains unexplained.

Functional imaging studies have revealed activation of the dorsolateral pons and the dorsal midbrain during a

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migraine attack, this activation persisting even after successful treatment with antimigraine agents [4], which suggests that this brainstem activation is not related to the perception of pain itself. The experimental results tend to support this view since cranial pain elicited by capsaicin did not give rise to brainstem activation [5]. The data suggest that the dorsal midbrain and dorsolateral pons, including the periaqueductal gray (PAG), the dorsal raphe (DR), the nucleus raphe magnus (NRM), and the locus coeruleus (LC), are involved in migraine headache. Since most of the reported functional imaging studies were carried out in the course of a migraine attack, it has not been ascertained whether the activation of this region is a cause or an effect of the attack.

The NRM comprises part of the descending pain modulatory system receiving strong input from the cerebral cortex [6]. Stimulation of the NRM can modify the nociceptive input at the level of the spinal trigeminal nucleus [7], and the activity of the NRM can be modulated by agents effective in migraine therapy, suggesting a crucial role in the pathomechanism of migraine [8].

The DR, one of the largest serotonergic nuclei in the central nervous system, plays an important part in nociceptive processing and antinociception [9]. This nucleus is activated during trigeminal nociception, thus it is possibly involved in the pathomechanism of migraine [10].

The LC is the main source of norepinephrine in the central nervous system, sending projections both to higher brain structures and to the spinal cord dorsal horn [11]. The LC has been suggested to possess a function parallel to that of NRM in pain control [12]. It has strong projection to the TNC [13], with a possible regulatory role, and sends direct projections to intracranial and extracranial vessels, suggesting an involvement in the vascular events of migraine [14].

Electrical stimulation of the PAG in humans resulted in analgesia [15], but chronic implants, mainly situated in the ventrolateral PAG (VLPAG), caused migraine-like symptoms in some cases [16], implying a role in pain modulation and migraine generation. Experimental and clinical findings have revealed that the PAG is a member of the descending pain modulatory system, which receives input from the spinal cord dorsal horn and from higher brain structures [17]. The PAG can be divided into longitudinal columns, each with different functions, suggesting an integrative role in pain processing, emotional and autonomic regulation [17]. The VLPAG is activated by trigeminal pain in animals [18], indicating that this area has as an integrative role in trigeminal sensory modulation.

Electrical stimulation of the trigeminal ganglion causes structural alterations in calcitonin gene-related peptide positive sensory nerve terminals [19], furthermore it leads to the extravasation of albumin from the vessels of the dura mater [20]. These results indicate that electrical stimulation of the trigeminal ganglion could induce chemical and vascular changes similar to those during migraine pain.

It is clear that the above-mentioned nuclei play an important part during a migraine attack, but it is still unknown whether their role is active or passive. The aim of our study was to investigate their pattern of activation and to evaluate possible functional connections between them and the trigeminal system in an experimental trigeminal activation model, electrical stimulation of the trigeminal ganglion.

Materials and methods

The procedures of this study conformed to the guidelines of the International Association for the Study of Pain and the European Communities Council (86/609/ECC). They were approved by the Ethics Committee of the Faculty of Medicine, University of Szeged, and by the Committee of Animal Research at the University of Szeged (I-74-14-16/2008) and the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XI./15.1/02384/001/2007).

Adult male Sprague–Dawley rats (weighing between 280 and 340 g) were used. The animals were raised and maintained under standard laboratory conditions, with tap water and regular rat chow available ad libitum, on a 12:12 h dark–light cycle.

Stimulation

Twenty-six rats were divided into two groups. The animals in the first group ($n = 13$) were deeply anaesthetized with chloral hydrate (400 mg/kg) and placed in a stereotaxic instrument (Stoelting Co., Wood Dale, USA). A hole was drilled with a dental drill 3.2–3.4 mm posterior to and 2.8–3.2 mm laterally from the bregma. A concentric bipolar electrode (FHC Inc., Bowdoin, USA, CBBRE75) was lowered to the right trigeminal ganglion, and maintained in it for 30 min. After surgery, the animals were kept under deep anaesthesia, with additional doses of chloral hydrate, if needed, being given. They were returned back to their cages and were kept warm.

The animals in the second group ($n = 13$) underwent a similar surgical procedure, but were additionally stimulated for 30 min with square pulses at 10 Hz, 0.5 mA, with a pulse duration of 5 ms. The applied stimulation frequency induces depolarization, thus rapid firing of the pseudounipolar neurones of the trigeminal ganglion, and the intensity applied ensures that the area of the stimulation covers the entire trigeminal ganglion [21].

The correct placement of the electrode was checked via the twitching of the jaw when the electrode reached the ganglion, and was confirmed during the autopsy.

From both the groups, seven animals were perfused transcardially 2 h after the placement of the electrode, while six animals were perfused 4 h after the electrode placement, with 100 mL cold 0.1 M phosphate buffered saline (PBS), followed by 500 mL 4 % paraformaldehyde in 0.1 M phosphate buffer. The brains and cervical spinal cord were removed and postfixed overnight in the same fixative.

Immunohistochemistry

After cryoprotection 30 μm thick serial sections were cut 1 mm rostrally and 5 mm caudally from the obex, representing the TNC. The series were collected into 18 wells, and the distance between consecutive sections was 540 μm.

Similarly 30 μm thick serial sections were cut from the brainstem, the series started 1.5 mm rostrally from the obex and 30 series were cut from each animal. The series

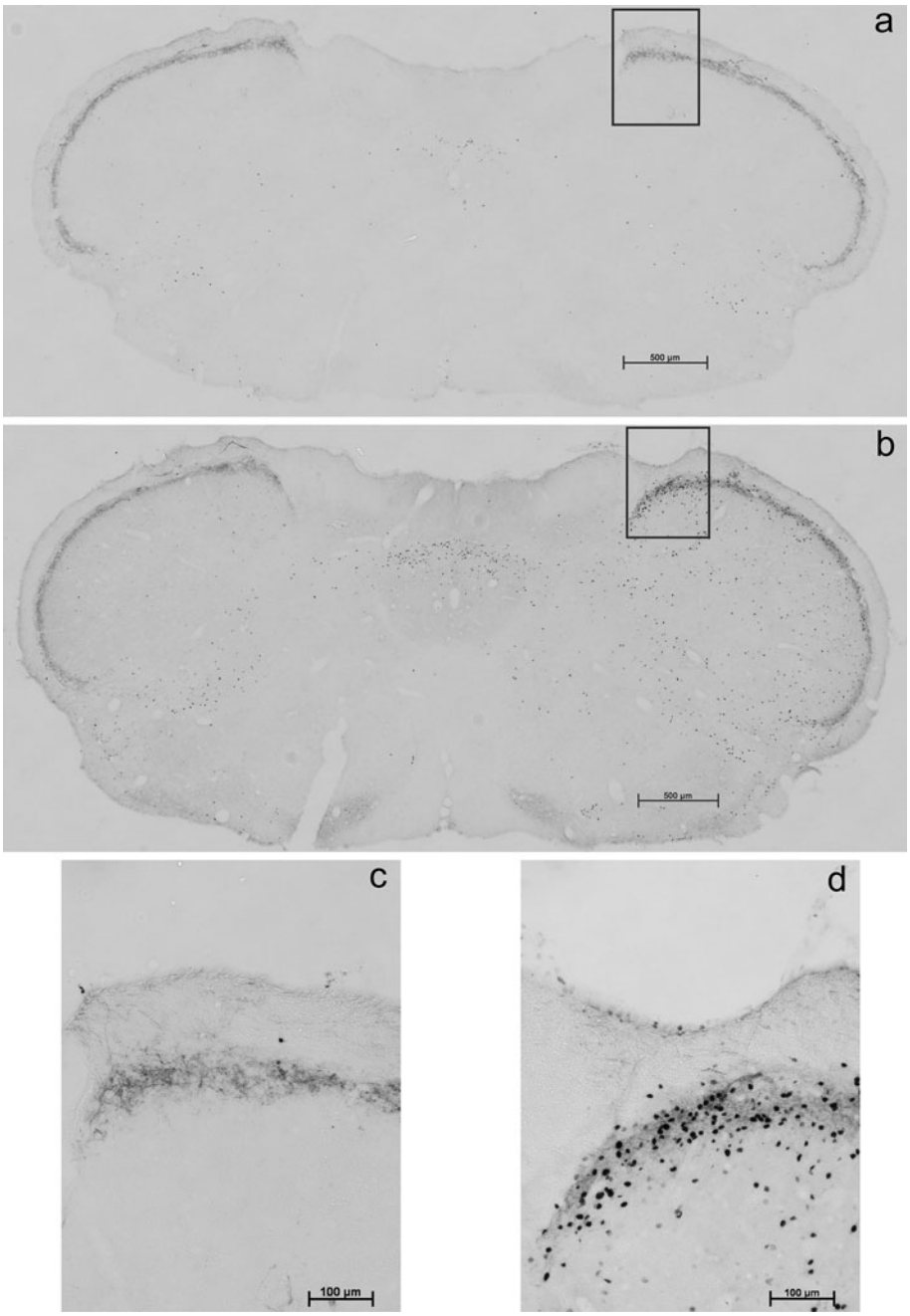

Fig. 1 Representative photos of the caudal part of the spinal trigeminal nucleus from a sham animal (**a**, **c**) and from a stimulated one (**b**, **d**) approximately 0.5 mm caudal from the obex. In sham animals, there was no noticeable difference between the control and the electrode side regarding the number of c-Fos IR cells (**a**). However, in stimulated animals a remarkable increase in the number of c-Fos IR cells could be observed at the stimulated side (**d**) compared to the control side of the same animal (**b**) and also compared to the electrode side of sham animals (**c**). *Rectangles* on picture **a** and **b** indicate the origin of pictures **c** and **d**, both from the sides of animals associated with the electrode. *Scale bars* indicate 500 μm on **a** and **b**, and 100 μm on **c** and **d**

were collected into 10 wells, the distance between consecutive sections therefore being 300 μm.

The free-floating sections were rinsed in PBS and immersed in methanol containing 0.3 % H_2O_2 for 30 min. After several washes in PBS containing 1 % Triton X-100 (PBS-T), the sections were blocked for 1 h in PBS-T containing 10 % normal goat serum (NGS). Sections from the TNC were incubated for one night at room temperature in PBS-T containing 2 % NGS and 1:2,000 anti c-Fos primary antibody (Santa Cruz Biotechnology, Santa Cruz, USA, sc-52), while sections from the brainstem were incubated for two nights at 4 °C in PBS-T containing 2 % NGS and 1:1,000 anti c-Fos primary antibody (Santa Cruz Biotechnology, Santa Cruz, USA, sc-52). The immunohistochemical reaction was visualized using the avidin–biotin kit of Vectastain (Vector Laboratories Inc., Burlingame, USA, PK-6101) and stained with nickel ammonium sulfate-intensified 3,3′-diaminobenzidine for sections of the TNC and 3,3′-diaminobenzidine without intensification for sections of the brainstem. The specificity of the immune reaction was checked by omitting the primary antiserum. On brainstem sections toluidine blue counterstaining was applied to facilitate cell counting.

Cell counting

The c-Fos-immunopositive cells were counted according to Paxinos and Watson [22] in the TNC, NRM, DR, VLPAG, lateral periaqueductal gray (LPAG), dorsolateral periaqueductal gray (DLPAG), dorsomedial periaqueductal gray (DMPAG), and LC separately. Sections were examined under a Zeiss Axio Imager M2 Upright Microscope (Carl Zeiss MicroImaging, Göttingen, Germany) supplied with an AxioCam MRc camera (Carl Zeiss MicroImaging, Göttingen, Germany). Photographs were taken on 20× magnification, using the MosaiX program feature of the AxioVision programme. Cells were counted and area of the different nuclei was delineated and measured on one series of sections from the TNC and from the brainstem in each animal. Cell counting was made by an investigator blinded to the stimulation procedure, furthermore stimulated and unstimulated sides of the sections were counted separately.

Statistical analysis

For the different nuclei, cell counts per side and per series were summed and divided by the corresponding area. In this way, the cell count per μm^2 was calculated for each side and for each series in each animal.

In case of the TNC counts were averaged by animal and statistical comparison was made using ANOVA (SPSS Statistics 17.0 for Windows, Games–Howell post hoc test).

In case of other paired nuclei of the brainstem, the first step was to determine if there is any difference between the sham and stimulated sides using Student's *t* test. There was no significant difference in any of the nuclei; therefore, data from separate sides were pooled. These data were than compared using ANOVA (SPSS Statistics 17.0 for Windows, Scheffe post hoc test).

Statistical comparison in the NRM and DR was also conducted by ANOVA (SPSS Statistics 17.0 for Windows, Scheffe post hoc test).

Results

The surgical procedure and lowering of the electrode to the trigeminal ganglion did not cause significant increase in the number of c-Fos immunoreactive (IR) cells on the ipsilateral side of the TNC neither in the 2 h nor in the 4 h survival group (Figs. 1c, 2). On the contralateral (control) side, no changes could be observed (Figs. 1a, 2). After electrical stimulation, a marked increase was observed on the ipsilateral and a slight increase on the contralateral side of the TNC at both survival times (Fig. 1b, d), from which only the changes on the ipsilateral side proved to be significant (Fig. 2). There was no difference in the activation of the TNC between the two stimulated groups.

It is interesting that we found differences in lateralization only in the TNC, in the other nuclei namely the VL, L, DL, DM PAG, and LC, there was no difference between the stimulated and unstimulated sides (data not shown).

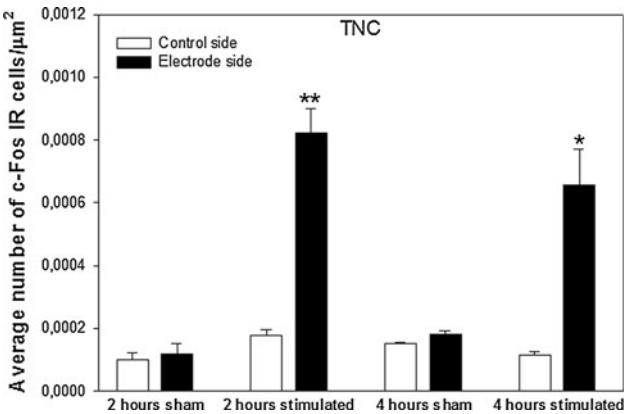

Fig. 2 Diagram showing the average number of c-Fos IR cells per μm^2 in the caudal part of the spinal trigeminal nucleus (TNC, group means per side + SEM). After sham surgery only a slight non-significant increase can be detected, while after electrical stimulation of the trigeminal ganglion a significant increase is present in the TNC both at 2 and 4 h of survival (*$p < 0.05$; **$p < 0.01$) compared to sham stimulated animals. There is no significant difference between the stimulated sides of the two distinct survival times

In the NRM, stimulation produced significant ($p < 0.05$) increase in the number of c-Fos IR cells, mainly in the rostral part of the nucleus at both survival times (Fig. 3c, e) compared to the animals with sham stimulation (Fig. 3b, d).

In case of the other nuclei, no statistically significant alteration could be observed between the sham and stimulated groups, although a tendency of increase in the DR and LC could be noticed (Fig. 4).

To evaluate if there is any relationship between the TNC and the NRM regarding the increase in the cell numbers, linear regression analysis was performed (SPSS Statistics 17.0 for Windows). No correlation was found between the two nuclei ($R^2 = 0.252$; $F = 3.708$; $p = 0.80$).

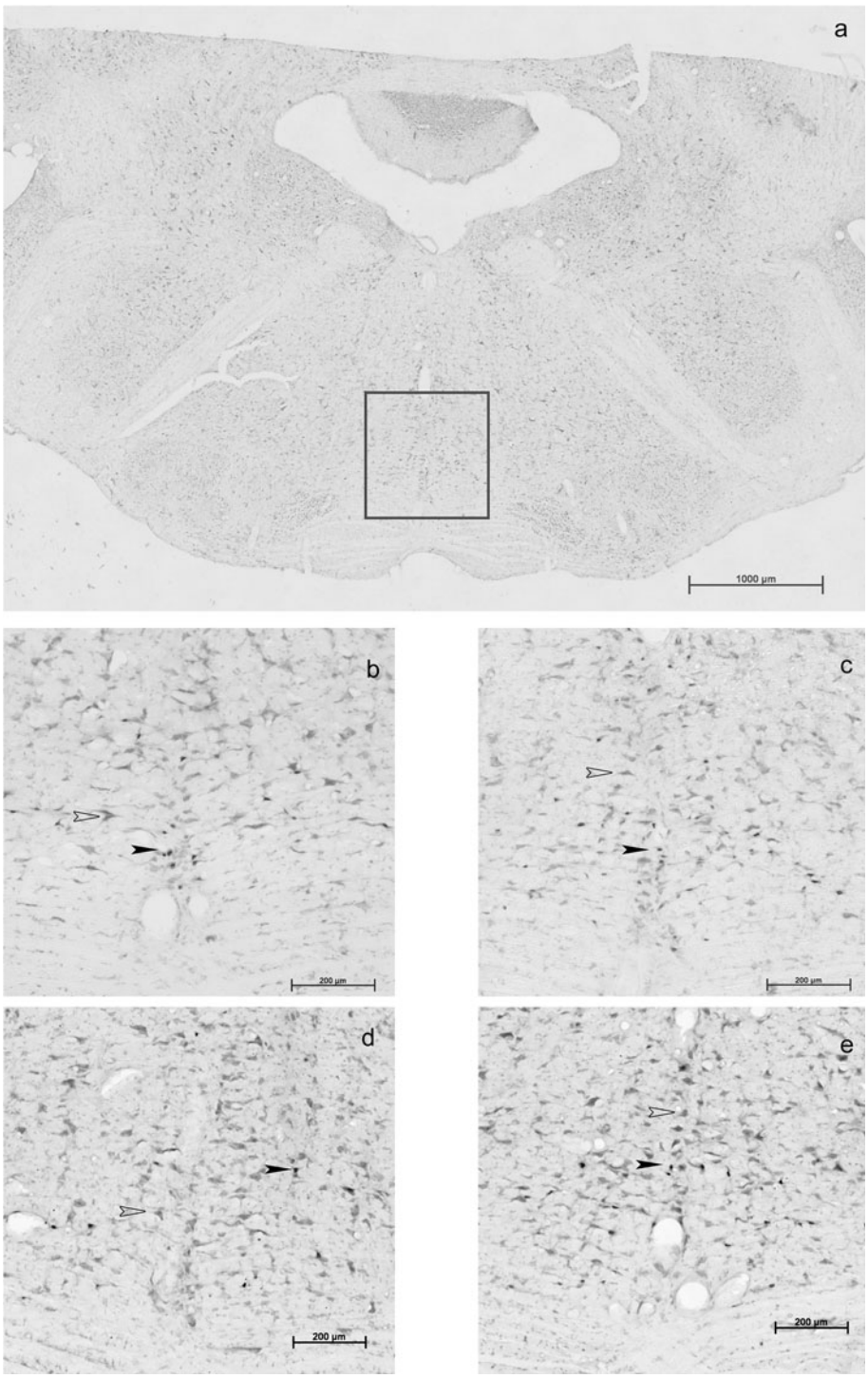

Fig. 3 Representative photos of the nucleus raphe magnus from a stimulated (**a**, **c**, **e**) and a sham animal (**b**, **d**) 2 (**a**, **b**, **c**) and 4 h (**d**, **e**) after stimulation approximately 4 mm rostral from the obex. *Rectangle* in **a** indicates the location where **c**; **b**, **d** and **e** were taken. *Filled arrowheads* indicate c-Fos positive cells, while *unfilled arrowheads* indicate c-Fos negative cells, counterstained with toluidine blue. After 2 h of survival, c-Fos immunoreactive cells are more numerous in the stimulated group (**c**). 4 h after the stimulation the activation of the nucleus raphe magnus is still present (**e**). *Scale bars* indicate 1,000 µm on **a**; and 200 µm on **b**, **c**, **d** and **e**

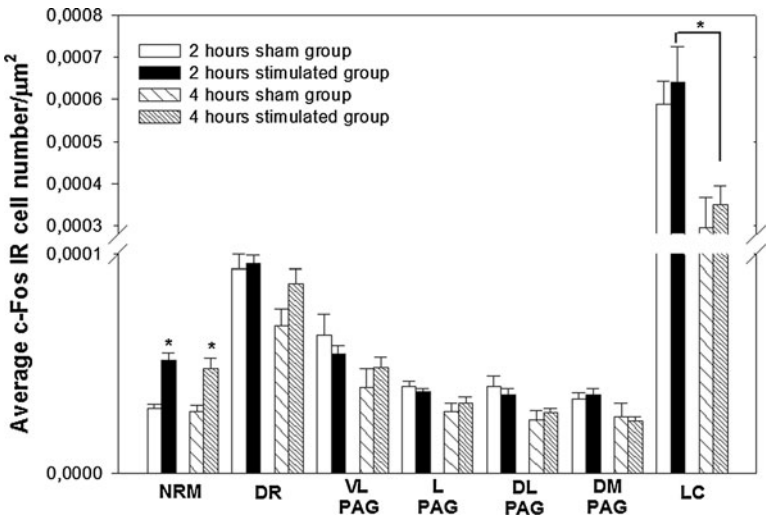

Fig. 4 Diagram showing the average number of c-Fos IR cells per μm^2 in brainstem nuclei (group means + SEM). The highest cell number per area ratio was present in the locus coeruleus, which is not surprising, considering the small extent of this nucleus. Statistically significant alteration between the sham and stimulated group could only be detected in the nucleus raphe magnus ($*p < 0.05$). There is a significant difference ($*p < 0.05$) between the two stimulated groups in case of the locus coeruleus, but no such difference could be observed in the other nuclei. In the nucleus raphe magnus the stimulation increased the number of c-Fos IR cells. *LC* locus coeruleus, *DR* dorsal raphe, *NRM* nucleus raphe magnus, *VL PAG* ventrolateral periaqueductal gray, *L PAG* lateral periaqueductal gray, *DL PAG* dorsolateral periaqueductal gray, *DM PAG* dorsomedial periaqueductal gray

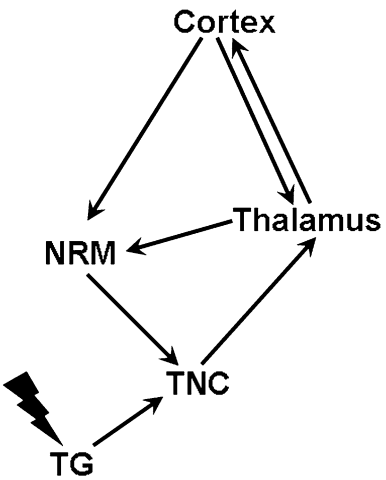

Fig. 5 Schematic route of activation based on our findings. Electrical stimulation activates the secondary trigeminal sensory nuclei in the TNC, the activation travels to the thalamus and the cortex. Activation of the NRM is probably secondarily related to the thalamus

Discussion

In this study, we first demonstrated that electrical stimulation of the trigeminal ganglion, i.e., activation of the trigeminal system, in the rat does not lead to the uniform activation of "migraine generator nuclei".

Electrical stimulation of the trigeminal ganglion has a direct effect on the primary trigeminal sensory neuron causing alterations in both the peripheral and the central endings. In the periphery, mediators from the nerve endings around the meningeal vessels are released, which results in plasma protein extravasation and eventually neurogenic inflammation [20]. In the central arm, there is a marked activation of the second-order neurones in the TNC [23]. Our results are in accordance with these previous findings as we found marked increase in the number of c-Fos IR cells in the ipsilateral TNC both 2 and 4 h after stimulation. This pronounced increase may arise directly from the electrical stimulation or is a secondary phenomenon originating from the periphery due to the triggered dural extravasation and inflammation [20]. The activation of second-order neurones is followed by activation of the third-order thalamic and cortical neurones [24].

We observed a significant increase in neuronal activity in the NRM in the 2 h survival group, which persisted even 4 h after the stimulation, however a tendency of decrease could be noticed. Direct TNC projections to the NRM are sparse [25], and the superficial laminae, which are mainly activated in response to electrical stimulation, display even fewer connections to the NRM [26]; accordingly activation of the TNC may not evoke a direct NRM activation. This hypothesis is further supported by the fact that we did not find any correlation between the increased cell activities in the two nuclei neither after 2 nor after 4 h, suggesting that the activation of the NRM may not be a direct consequence of the activation of the TNC. Although there is no correlation between the activation patterns of these two nuclei, the increase in their activity is present simultaneously at both 2 and 4 h after stimulation.

After intracisternal administration of capsaicin, there is an elevation in the number of c-Fos immunoreactive cells in the NRM [10], suggesting a role in trigeminal nociceptive processing. The NRM is thought to be the main output of the descending pain modulatory system, providing dense innervations to the spinal cord dorsal horn. We assume that activation of the NRM may be a consequence of activation of the descending pain modulatory system. This activation could result from the cortical and thalamic input of the NRM, or it could originate from the PAG [25].

There is no evidence that the TNC projects directly to the DR [27]. The DR forms part of the pain modulatory system in both ascending and descending directions [9], and the DR receives dense afferents from the NRM, but cortical input can also modulate the activity of this nucleus [27]. We did not find significant change in the number of c-Fos IR cells in the DR after electrical stimulation of the TG. Our results suggests that the activation of the trigeminal system does not cause the activation of the DR, and thus in the rat, this nucleus may not participate in the short-term regulation of trigeminal nociceptive processing.

The LC is activated after painful stimuli [10, 28], suggesting an important role in nociceptive processing and modulation, however, activation due to stressful circumstances is also possible [29]. Our results indicate that after electrical stimulation of the TG, activity of the cells in the LC does not change, although it is noticeable that the activity of the LC is already pronounced in the sham group 2 h after the stimulation, suggesting that the stress and the possible pain caused by the surgical procedure may obscure the changes caused by the electrical stimulation. After 4 h of survival, the activity level of the LC is decreasing both in the sham and stimulated groups, further supporting our view that the activation of the LC is due to the invasive procedure.

The available information relating to TNC and LC direct inputs is controversial, some authors reporting that the TNC sends only a sparse direct input to the LC [30], and others describing the existence of neuronal input from lamina I to the LC. Our results indicate that the TNC may not have direct input to the LC [31]. It is possible that the contribution of LC to the descending pain modulatory system is via the NRM [32]; however, it is a well-known fact that LC has a descending projection to the spinal cord, which could modulate nociceptive processing directly.

No change in the number of c-Fos immunoreactive nuclei were noticed in any region of the PAG. There is a direct connection between the TNC and the PAG, thus it is surprising that a strong stimulus like we applied caused no alteration in this area. Previous studies indicated an increase in activity of the PAG after nociceptive stimulation [18], although the stimulation parameters and settings differed from those we applied.

If it is assumed that the changes seen in our experiments are secondary, the lack of direct projections from the TNC to most of the above-mentioned nuclei supports this hypothesis. In this conception, the stimulation activates the ascending nociceptive routes to the thalamus and to the cortex, and these structures react to the nociceptive input by activating the descending modulatory system (Fig. 5). After trigeminal stimulation, the examined nuclei failed to exhibit the uniform activation detected during a migraine attack suggesting that this activation pattern of the migraine generator nuclei may be exclusively present in migraine.

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Conflict of interest The authors declare that they have no conflict of interest.

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