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ORIGINAL

Melatonin treatment decreases c-fos expression in a headache model induced by capsaicin

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Abstract The aim of the present work was to analyze c-fos response within the trigeminal nucleus caudalis (TNC) of pinealectomized rats and animals that received intraperitoneal melatonin, after intracisternal infusion of capsaicin, used to induce intracranial trigeminovascular stimulation. Experimental groups consisted of animals that received vehicle solution (saline–ethanol–Tween 80, 8:1:1, diluted 1:50) only (VEI, $n = 5$); animals that received capsaicin solution (200 nM) only (CAP, $n = 6$); animals submitted to pinealectomy (PX, $n = 5$); sham-operated animals (SH, $n = 5$); animals submitted to pinealectomy followed by capsaicin stimulation (200 nM) after 15 days (PX + CAP, $n = 7$); and animals that received capsaicin solution (200 nM) and intraperitoneal melatonin (10 mg/kg) (CAP + MEL, $n = 5$). Control rats, receiving vehicle in the cisterna magna, showed a small number of c-fos-positive cells in the TNC (layer I/II) as well as the sham-operated and pinealectomized rats, when compared to animals stimulated by capsaicin. On the other hand, pinealectomized rats, which received capsaicin, presented the highest number of c-fos-positive cells. Animals receiving capsaicin and melatonin treatment had similar expression of the vehicle group. Our data provide experimental evidence to support the role of melatonin and pineal gland in the pathophysiology of neurovascular headaches.

Keywords Headache · Migraine · Pinealectomy · Melatonin · c-fos · Experimental models

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Introduction

Migraine is a common condition with significant impact in the general population [1–3]. In spite of its relevancy, migraine neurobiology has not been fully determined. Several mechanisms are important, including the trigeminovascular concept [4]. Neurovascular network is thought to be the anatomical substrate for neurovascular headaches such as migraine and cluster headaches [5, 6]. A-δ and unmyelinated C-fibers of the trigeminal nerve transmit painful stimuli from meninges to trigeminal nucleus caudalis (TNC) within the brain stem, via the trigeminal ganglion (TG) neurons [7]. The pathophysiology of neurovascular headaches is unclear but the involvement of trigeminovascular system is generally accepted [8].

c-fos expression within the TNC is induced experimentally after stimulation at various sites of the trigeminovascular system, such as meninges, TG and superior sagittal sinus [9]. Development of animal models and the use of direct electrical, mechanical or chemical

irritants stimulus (capsaicin, carrageenan and blood) have been used to mimic vascular head pain [10–13].

c-fos protein is a transcription factor expressed by immediate-early response of c-fos gene and has been accepted as a marker of functional activity in neurons [14, 15]. c-fos expression in layers I and II of the TNC is used to study the activity of the sensory part of the trigeminal system [16, 17]. This expression can be attenuated by pharmacological agents used for the treatment of migraine, including sumatriptan, dihydroergotamine and valproate [18, 19].

Melatonin, a hormone synthesized by the pineal gland with major influence on several circadian physiological activities, is maximally produced between midnight and dawn, with low levels during the light period [20]. Melatonin has been linked to migraine and other headaches, increasing evidence supports its role in mechanisms and treatment of headache disorders [21, 22]. Some headaches have a clear seasonal and circadian pattern, such as cluster and hypnic headaches. Melatonin levels have been found decreased in both migraine and cluster headaches [23–25]. Melatonin is related to headache pathophysiology in several ways. Due to its anti-inflammatory effect, free radical scavenging process, reduction of pro-inflammatory cytokine upregulation, nitric oxide synthase activity and dopamine release inhibition, membrane stabilization, GABA and opioid analgesia potentiation, protection against glutamate neurotoxicity, neurovascular regulation, 5-HT modulation and due to its similarity with the chemical structure of indomethacin. However, little is known about the putative effect of melatonin in experimental models of headache [26].

Thus, the aim of this study is to characterize the pattern of cerebral c-fos expression after noxious trigeminal stimulation in the group that received melatonin and in pinealectomized rats, using intracisternal administration of the capsaicin to appraise the pinealectomy and the melatonin effect in head pain.

Methods

Animals

All experimental protocols were approved by the ethical committee of the Universidade Federal de São Paulo (UNIFESP) and all efforts were made to minimize animal suffering following the proposal of International Ethical Guideline for Biomedical Research [27]. Wistar adult male rats (250–300 g) housed under environmentally controlled conditions in a 12/12 h light/dark and granted free access to food and water were used. These animals were separated into four groups.

Groups

VEI ($n = 5$): animals that received vehicle solution only; CAP ($n = 6$): animals that received capsaicin solution (200 nM) only; PX ($n = 5$): animals that were submitted to pinealectomy; SH ($n = 5$): animals that were sham-operated; PX + CAP ($n = 7$): animals submitted to pinealectomy followed by capsaicin stimulation (200 nM) after 15 days; and CAP + MEL ($n = 5$) animals that received capsaicin solution (200 nM) and intraperitoneal melatonin (Sigma, 10 mg/kg) 20 min after capsaicin injection.

Drugs

Capsaicin solution was prepared with 3.05 mg capsaicin (Merck) per 1 ml of vehicle (saline–ethanol–Tween 80, 8:1:1) and diluted 1:50 (200 nM) with saline. Vehicle was diluted 1:50 in saline.

Surgical procedures

Pinealectomy

Pinealectomy was performed following the method described by Siuciak and Dubocovich [28]. In brief, rats were anesthetized (pentobarbital, 40 mg/kg i.p.) and fixed in a stereotaxic frame. Using a pointed dental burr, a piece of bone was removed at the juncture of the lambda and the sagittal suture lines. The pineal was grasped with fine forceps and removed. The bone disk was returned to its original position and the skin flaps pulled together. In sham-operated rats, all procedures mentioned above were used, except for the fact that the pineal gland was not removed.

Capsaicin stimulation

For this procedure, all rats were anesthetized with pentobarbital (40 mg/kg i.p.) and a surgical opening in the region between the scalp and C1 (first cervical vertebra) was made. An amount of 10 μl of capsaicin solution (see "Drugs") was injected into the cisterna magna (over 15 min) using a Hamilton syringe with the aid of a stereotaxic frame [29]. To avoid capsaicin outflow, the needle was only removed 10 min after injection.

Perfusion and immuno-histochemistry

Two hours after infusion, the rats were anesthetized with pentobarbital overdose (120 mg/kg), followed by perfusion via the ascending aorta with 0.1 M phosphate saline buffer (PBS, 200 ml, pH 7.4) and 4% paraformaldehyde (200 ml) in 0.1 M phosphate buffer (PB, pH 7.4). Brain stem with attached cervical cord was stored overnight in

the same fixative and then placed in a cryoprotectant (30% sucrose in 0.1 M PB, pH 7.4). Coronal serial sections (40 μm) were prepared on a cryostat microtome at −20°C and collected in PBS with sodium azide (0.1%) to Nissl staining and immuno-histochemistry. c-fos protein expression was characterized in free-floating sections according to the following protocol. Sections were rinsed three times 5 min in PBS, pre-treated with 0.3% H_2O_2 in PBS for 15 min, rinsed three times 5 min in PBS and pre-incubated in 10% bovine serum albumin (Calbiochem) and 2% normal serum (Vector) in PBS for 2 h at room temperature. Sections were incubated for 48 h at 4°C in PBS solution containing 2% BSA, 2% normal serum, anti-c-fos antibody (1:1,000; Calbiochem) and 0.3% Triton X-100 in PBS. Following three washes in PBS, the sections were incubated in a PBS solution contained biotinylated rabbit IgG (1:200) (Vector) for 2 h at room temperature. Sections were rinsed three times 5 min in PBS and incubated with the avidin–biotin–peroxidase complex (Vector) in PBS for 1 h and 30 min at room temperature. Sections were rinsed twice 5 min in PBS and 5 min in Tris–HCl (pH 7.6) and revealed with 0.06% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and with 0.002% H_2O_2. Sections were then mounted in slides and dehydrated through alcohol to xylene and coverslipped with Entellan (Merck).

Nissl staining

Brain stem slices (40 μm) were hydrated in alcohol solutions of decreased concentration followed by staining in 0.5% cresyl Violet acetate (Sigma) diluted in 0.1 M acetate buffer pH 4.0. Slices were dehydrated, coverslipped and analyzed by light microscopy optic Zeiss Axiolab.

Quantifications

c-fos immunoreactive cells in the TNC layer I/II were counted at 0 to −1 mm caudal to obex. Three sections per animal were analyzed. The count of c-fos immunoreactive cells was performed using magnification of 200× under a microscope optical system Zeiss Axiolab. Representative images of the brain stem slices were digitalized using NIH Image 1.61 system. Cell counting was performed blinded by three observers and the results are expressed as the mean ± standard deviation.

Statistical analysis

Data were analyzed using one-way analyses of variance (ANOVA) followed by Tukey's Q test. A value of $P < 0.05$ was accepted as significant.

Results

Nissl-stained coronal sections of brain stem in control rats showed a preserved cytoarchitecture (Fig. 1a) and the TNC (layer I/II) was observable in Fig. 1b. Sections of the other groups showed similar or normal Nissl staining (data not shown).

The pattern of c-fos expression was different in the studied groups. Controls rats receiving vehicle in the cisterna magna showed a small number of c-fos-positive cells (VEI: 9.8 ± 2.42; $P < 0.01$) in the TNC (layer I/II) as well as the sham-operated rats (SH: 3.26 ± 0.15; $P < 0.001$), pinealectomized rats (PX: 2.3 ± 0.78; $P < 0.001$) and rats that received capsaicin and melatonin (CAP + MEL: 10.53 ± 2.78; $P < 0.01$), when compared to capsaicin-treated animals (CAP: 25.7 ± 8.78). On the other hand, pinealectomized rats that received capsaicin presented the highest number of c-fos-positive cells (PX + CAP: 41.5 ± 6.68; $P < 0.01$). A diffuse staining in regions that were not considered in the present work was observed. The results of c-fos expression in the immuno-histochemistry and the quantification of these c-fos-positive cells are observed in Figs. 2 and 3, respectively.

Discussion

Our results show experimental evidence for the putative role of melatonin and pineal gland in the pathophysiology of neurovascular headaches. A high number of c-fos

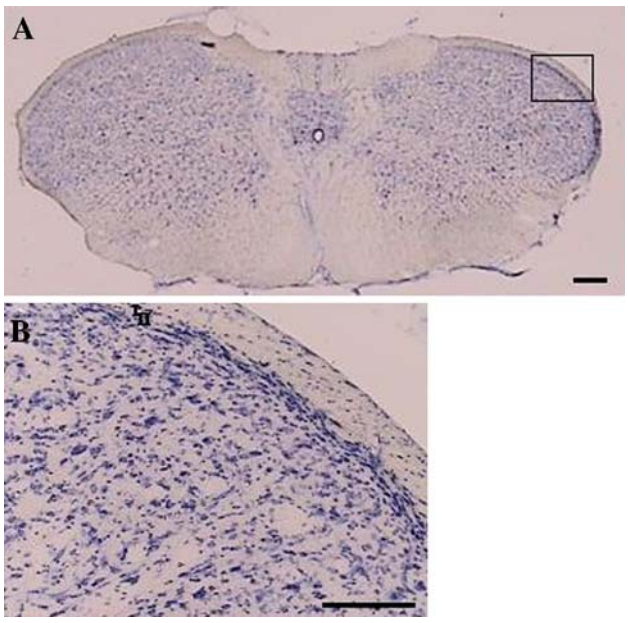

Fig. 1 Photomicrographs of Nissl-stained coronal sections of brain stem. **a** Brain stem, ×20, *scale bar* 300 μc. **b** Trigeminal nucleus caudalis (TNC), ×200, *scale bar* 150 μc

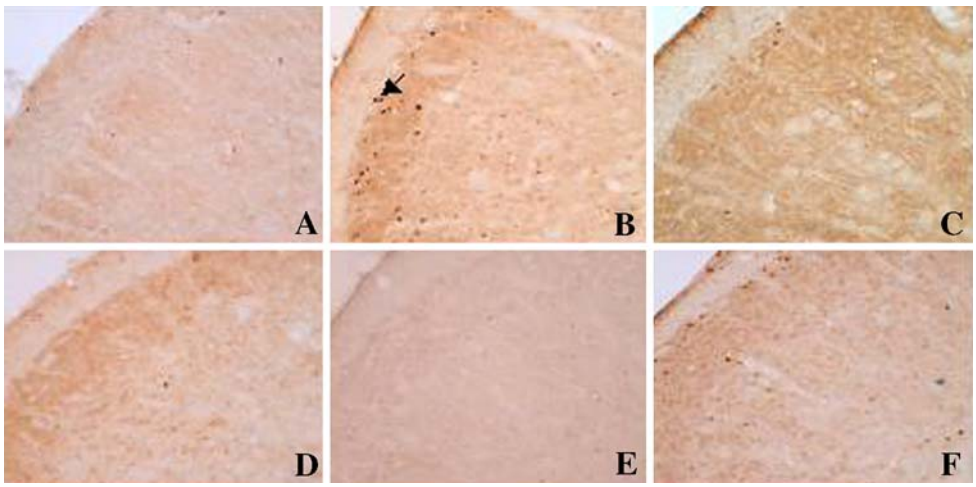

Fig. 2 Photomicrographs of the Fos expression in the TNC/II layers. **a** Tissue from animal that received vehicle; **b** tissue from capsaicin-injected animal; **c** tissue from animal that received capsaicin and melatonin; **d** tissue from pinealectomized animal; **e** tissue from sham-operated animal; and **f** tissue from pinealectomized rat that received capsaicin injection. ×200, *scale bar* 150 μc. *Arrow* shows positive-like staining that was counted as c-fos-positive cells

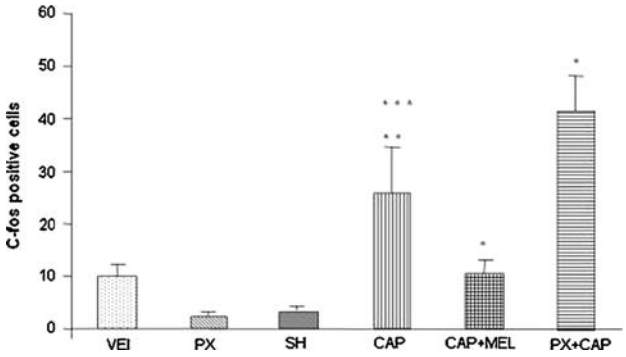

Fig. 3 Quantification of the c-fos protein immunoreactive cells in rats that received: *VEI*, vehicle ($n = 5$); *SH*, sham-operated rats ($n = 5$); *PX*, pinealectomized rats ($n = 5$); *PX + CAP*, pinealectomized rats that received capsaicin injection ($n = 7$); *CAP*, rats that received capsaicin ($n = 6$); *CAP + MEL*, rats that received capsaicin and melatonin ($n = 5$) and were killed 120 min after injection. Cells were counted in 40 μm sections sampled in the TNC layers I and II at 0 to −1 mm caudal to obex (3 sections). $^{*}P < 0.01$ compared with capsaicin-treated animals; $^{**}P < 0.001$ compared with vehicle only, sham-operated and pinealectomized rats

capsaicin-induced immunoreactive cells in TNC I and II layers in pinealectomized rats was found.

In previous studies, intracisternal capsaicin evoked c-fos expression over the rostro-caudal extent of the TNC [30, 31]. Capsaicin selectively actives nociceptive fibers and the principal nociceptive innervation of blood vessels of the subarachnoid space and dura mater originated in the trigeminal ganglion. According to Kemper [32], the number of c-fos immunoreactive cells in TNC I/II is capsaicin dose-dependent and indicates a direct and specific relationship between c-fos expression and the capsaicin concentration used. In the present work, the trigeminovascular system was activated by 200 nM capsaicin. The ventrolateral, mediolateral and dorsomedial part of the TNC are innervated by, respectively, the ophthalmic, maxillary and mandibular branches of the trigeminal nerve. All three branches carry fibers that innervate the meninges and meningeal vasculature [33], which were demonstrated in the present study by the increased c-fos expression in all dorsoventral parts of the TNC I/II after the intracranial capsaicin administration. This increase was intensified by the pinealectomy procedure.

Melatonin reduces lipid peroxidation process, enhances the antioxidative enzymes activities and scavenges toxic-free radical. Furthermore, melatonin inhibits the production of adhesion molecules that promote the sticking of leukocytes to endothelial cells, attenuating transendothelial cell migration and edema [34]. It inhibits the synthesis of prostaglandins acting via anti-inflammatory effect and reduces the upregulation of the pro-inflammatory cytokines, interleukins and TNFα [35]. Melatonin inhibits the activity of nitric oxide synthase and dopamine release, potentiates the inhibitory effect of GABA in the central nervous system, modulates the serotonin receptors and potentiates the opioid analgesic. Its structure is similar to indomethacin that inhibits COX activity [36, 37]. Thus, several mechanisms could be involved in our results.

Experimental studies demonstrated that pinealectomy increases the damage in focal cerebral ischemia and facilitates the epileptogenic process in rats, showing that melatonin has a neuroprotective action [38, 39].

In this study, we have found a great number of c-fos immunoreactive cells capsaicin induced in TNC I/II layers in pinealectomized rats, when compared with the control group, indicating that pineal gland may be potentially involved in the pathophysiology of neurovascular headaches. This finding was not observed when animals received melatonin intraperitoneally.

Several studies have evidenced low levels of melatonin in blood of patients with migraine, chronic migraine and cluster headache chronic [40, 41]. This data suggest an inadequate release of this hormone, which could be responsible for decreasing its protective role against pain,

via several mechanisms described above. The exact mechanism, however, has yet to be determined in future studies. It is less likely that other structures or substances may play a role in these findings, since melatonin is the main secretory product of the pineal gland and no other brain structure were affected during pinealectomy.

Melatonin is a potential candidate for migraine treatment [42]. A significant headache response was found in a study looking the effect of melatonin 3 mg/day for migraine prevention [43].

Melatonin has also been studied in cluster headache and other trigemino-autonomic cephalgias with preliminary good results. Our study is supporting melatonin supplementation as an effective suppressing agent of trigeminovascular activation in an experimental model.

Conclusion

Our data provide experimental evidence to support the role of melatonin and pineal gland in the pathophysiology of neurovascular headaches.

Conflict of interest None.

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