Melatonin reverts CGRP expression induced by capsaicin

Melatonina reverte a expressão de CGRP induzida pela capsaicina

Fabiano da Cunha Tanuri[1]
Debora Amado[1]
Eliangela de Lima[1]
Iron Dangoni Filho[2]
Mario Fernando Prieto Peres[2,3]

[1] Departamento de Neurologia/Neurocirurgia, Universidade Federal de São Paulo, Escola Paulista de Medicina, São Paulo, Brazil
[2] Hospital Israelita Albert Einstein, São Paulo, Brazil
[3] Instituto de Psiquiatria, Faculdade de Medicina da Universidade de São Paulo

ABSTRACT

Introduction: CGRP, a neuropeptide synthetized and released in the central nervous system and potent vasodilator, has been implicated in migraine physiopathology. Because of that, there are CGRP targeted therapies that decrease CGRP levels. Melatonin, a pineal gland secretion, has already proved its analgesic effect. We aimed to study CGRP expression in an animal model comparing capsaicin, CGRP and melatonin. **Methods:** We used in our study male animal rats and separated them into groups based in the kind of received solution (control group, capsaicin only and melatonin plus capsaicin). It was prepared brain stem slices and measured the CGRP levels in the trigemino nucleus caudalis (TNC). **Results:** Capsaicin group (N = 5) presented low intensity of GCRP expression and animals that received capsaicin plus melatonin (N = 5) showed high intensity of CGRP expression compared to capsaicin group. **Conclusion:** Melatonin decreases CGRP in an experimental model in rats induced by capsaicin, reducing its inflammatory action in cerebral vessels.

Keywords: Melatonin, CGRP, Animal Model.

RESUMO

Introdução: CGRP, um peptídeo produzido e liberado no sistema nervoso central e potente vasodilatador, tem sido implicado na fisiopatologia da Migrânea. Devido a isso, tem surgido diversas terapias direcionadas ao CGRP que reduzem seus níveis. A melatonina, substância produzida pela glândula pineal, já possui seu efeito analgésico comprovado. Nós objetivamos estudar a expressão do CGRP em um modelo animal comparando capsaicina, CGRP e melatonina. **Métodos:** Foi utilizado em nosso estudo ratos machos adultos e estes foram separados em grupos baseados na solução que recebiam (grupo controle, apenas capsaicina e melatonina mais capsaicina). Foram preparadas fatias dos cérebros dos animais e então medidos os níveis de CGRP no núcleo caudal trigeminal. **Resultados:** Grupo da Capsaicina (N = 5) apresentou baixa intensidade da expressão de CGRP, enquanto aqueles animais que receberam capsaicina mais melatonina (N = 5) mostraram altos níveis de expressão de CGRP quando comparados ao grupo do CGRP. **Conclusão:** No nosso estudo experimental com ratos induzidos por capsaicina notou-se que a melatonina reduz os níveis de CGRP, diminuindo a ação inflamatória nos vasos cerebrais.

Descritores: Melatonina, CGRP, Modelo Animal.

***Correspondence**
Mario Fernando Prieto Peres
E-mail: marioperes@usp.br

Received: January 20, 2019.
Accepted: January 31, 2019.

INTRODUCTION

CGRP (Calcitonin Gene-Related Protein) is a 37-amino acid neuropeptide that belongs to a family of structurally related peptides (e.g. calcitonin, amylin, adrenomodulin). This neuropeptide is synthesized and released from sensory nerves in the central nervous system and gastrointestinal system, where it acts as a potent vasodilator[1,2].

It has been implicated in the mechanisms of migraine, acting along trigeminovascular pathways as a vasodilator and nociceptive initiator[3].

CGRP targeted therapies have been studied for both acute (gepants) and preventive (anti-CGRP monoclonal antibodies) treatment[4].

Decrease CGRP levels contribute to migraine treatment. Other strategies may also improve migraine control by reducing CGRP levels, such as the use triptans, coenzyme Q10, serotonin reuptake inhibitors, exercise, acupuncture and some kinds of food, such as grape pomace, cocoa and ginger extracts[4-9].

Melatonin is the primary secretory product of the pineal gland, an indoleamine derivate of the essential amino acid tryptophan[9]. It has been extensively linked to migraine pathophysiology, due to its capacity of membrane stabilization, anti-inflammatory properties, modulation of serotonin, inhibition of dopamine release, gamma amino butyric acid (GAMA) and glutamate neurotransmission, scavenging toxic free radicals and cerebrovascular regulation[10,11]. Besides, melatonin plays important roles in antinociceptive mechanisms. It has been reported that patients suffer less pain and prolonged latencies thresholds during nighttime. These observations were attributed to high melatonin levels through night and its analgesic effect[12].

Melatonin has been studied as a prophylaxis headache treatment in cluster headache and migraine, but its underlying mechanisms has yet to be determined[11]. We aimed to study the pattern of CGRP expression in an experimental model of headache, comparing capsaicin, melatonin and CGRP levels.

METHODS

Animals

The ethical committee of the Universidade Federal de São Paulo (UNIFESP) approved all experimental protocols. All efforts were made to minimize animal suffering following the proposal of International Ethical Guideline for Biomedical Research[13]. Wistar adult male rats (250–300 g) housed under environmentally controlled conditions in a 12 hours light/dark cicle 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 = 5): animals that received capsaicin solution (200 nM) only; 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

Capsaicin stimulation

For this procedure, all rats were anesthetized with pentobarbital (40 mg/kg i.p.) and a surgical opening was made in the region between the scalp and C1 (first cervical vertebra). An amount of 10 ml 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[14]. To avoid capsaicin outflow, the needle was only removed 10 min after injection.

Perfusion and immuno-histochemistry

The rats were anesthetized with pentobarbital overdose (120 mg/kg) after two hours infusion, 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 ml) were prepared on a cryostat microtome at -20ºC and collected in PBS with sodium azide (0.1%) to Nissl staining and immuno-histochemistry. Sections were rinsed three times 5 min in PBS, pre-treated with 0.3% H2O2 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 hours at room temperature. Sections were incubated for 48 hours at 4ºC in PBS solution containing 2% BSA, 2% normal serum 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% 3,30-diaminobenzidine tetrahydrochloride (Sigma) and with 0.002% H2O2. Sections were then mounted in slides and dehydrated through alcohol to xylene and coverslipped with Entellan (Merck).

Nissl staining

Brain stem slices (40 ml) 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

The CGRP expression sections in TNC layer I/II were counted at 0 to - 1 mm caudal to obex. Representative images of the brainstem slices were digitalized using the Image 1.61 system. The images were transformed into black and White. The image analysis were performed in the anterior region of the TNC, which presented the same area analyzed in all the cuts. It was quantified the optic density from the negative obteined of the images, through the grayscale analysis of the Image Tool program in "pixels" unit. The white color "pixels" were quantified and the results were expressed as mean 3 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

The studied groups presented difference in the CGRP expression analyzed through the densitometry. Control group that received vehicle into the cisterna magna showed high intensity of CGRP expression (VEI: 733,95 3 144,08) in TNC (layer I/II). In contrast, we observed that animals submitted to trigeminal stimulation of intracisternal capsaicin presented low intensity of CGRP expression (CAP: 295,1 3 49,93). This number is significantly different when we compare CAP x VEI ($p < 0.001$). On the other hand, animals that received intraperitoneal melatonin 20 min before the capsaicin stimulation presented high intensity of CGRP expression (CAP + MEL: 584,02 3 133,59) when compared to animals that received capsaicin only ($p < 0.05$) and similar to VEI group.

The results of immunohistochemical and quantification through CGRP expression optic density were observable in figures 1, 2 and 3.

DISCUSSION

We found in this experimental study direct relation of the levels of Melatonin and Capsaicin in rats model, which were exposed to capsaicin and melatonin injection. After that, it was measured the CGRP density and it showed decrease of its density when associated to capsaicin. However, when we measure melatonin and capsaicin both together, its levels increase and almost normalize. A similar study, has already evidenced data about the relation of melatonin and pineal gland in neurovascular headaches' pathophysiology[15].

Melatonin reverts CGRP alteration induced by capsaicin due to the inhibition of CGRP-induced increase

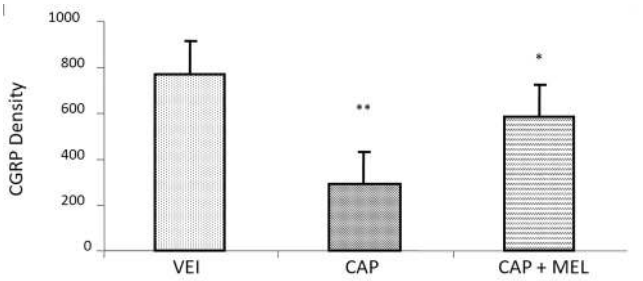

Figure 1. Photomicrographs of CGRP expression in TNC (layer I/II). A: Tissue from animal that received vehicle. B: Tissue from capsaicin-injected animal. C: Tissue from animal that received capsaicin and melatonin. Detail shows the area used to quantify by optic density. x200, scale bar 55 Qc.

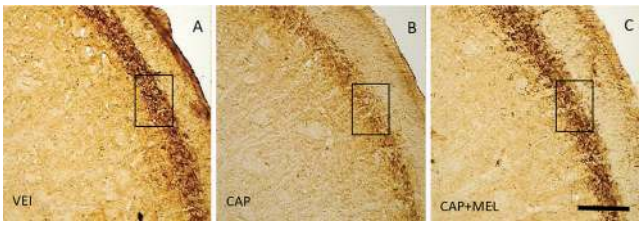

Figure 2. Photomicrographs of CGRP expression in TNC (layer I/II). A: Tissue from animal that received vehicle. B: Tissue from capsaicin-injected animal. C: Tissue from animal that received capsaicin and melatonin. Detail shows the area used to quantify by optic density. x200, scale bar 55 Qc. A', B' and C' represents the negative of the cut images used for quantification by density optic.

in adenylate cyclase. It has already been proposed that CGRP and melatonin may share an active role in the maintenance of arterial tone in cerebral vasculature. Several studies have demonstrated that melatonin causes constriction of rat cerebral arteries[16-18].

We hypothesized melatonin could revert capsaicin effect owing to its ability of avoiding capsaicin. These data are in accordance with previous studies showing melatonin is able to produce a significant inhibition against the neurogenic pain caused by capsaicin. It has been reported the melatonin antinociceptive effect by central administration of it, showing its high lipid solubility, capacity to penetrate the blood-brain-barrier and produce a significant inhibition against the neurogenic pain by activating supraspinal sites. In another study, it was presented that melatonin has the ability of avoid capsaicin effect of initiation and limit the development of "central sensibilization"[19].

Clinical implications

It is an exciting moment to migraine specialists and patients. There is one approved CGRP receptor antagonist and some others being studied. Thus, some doubts about it and its interaction to other established drugs will need to be answered in the next years. Melatonin has been showed as a potential candidate for migraine treatment, including a Brazilian study that

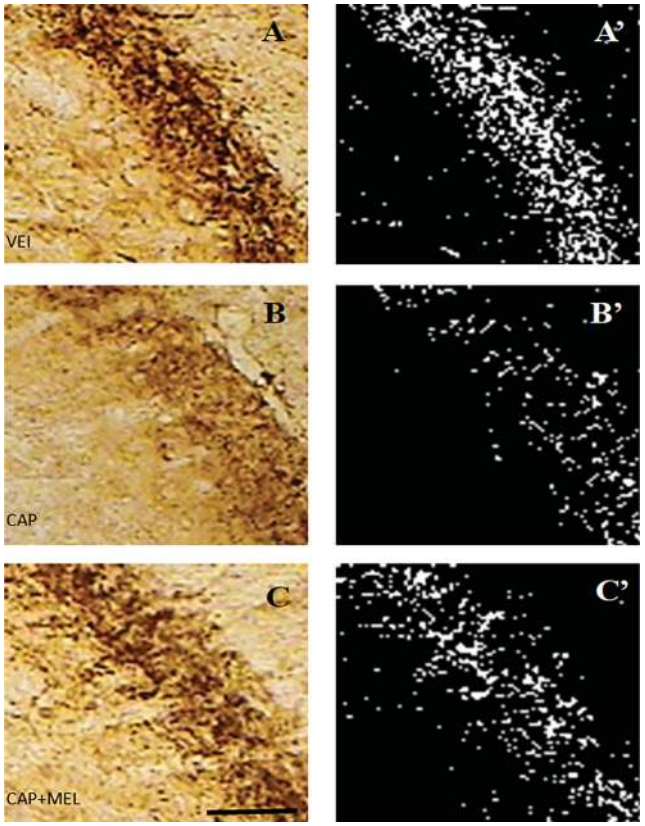

Figure 3. Quantification of CGRP expression through optic density in rats that received: VEI – vehicle (n = 5); CAP – rats that received capsaicin (n = 5); CAP + MEL – rats that received capsaicin and melatonin (n = 5). Rats were killed 60 min after injection. Cells were counted in 40 Qm sections sampled in the TNC layers I and II at 0 to – 1 mm caudal to the obex (3 secctions). *$p < 0.05$ compared with capsaicin-treated animals; **$p < 0.001$ compared with vehicle only.

found significant headache response with melatonin as a migraine prevention[20].

Our study brings new questions and challenges to headache societies: may migraine patients taking melatonin still respond to CGRP antagonists? Is the decrease of CGRP the real explanation for melatonin improvement in headache disorders? Or do these drugs have synergistic effect?

Melatonin has been associated to CGRP decreased in patients with pure menstrual migraine[21]. It was investigated the melatonin capability of reduce inflammation through decreasing CGRP and inducible nitric oxide synthase. At the beginning of the 2000s, it was proposed that melatonin could inhibit CGRP vasodilatation effect and increases cAMP in rats' arteries[22].

LIMITATIONS

CGRP should be measured in other brain structures besides trigeminal nucleus caudalis, such as trigeminal ganglion, cerebral ventricles, meningeal afferents and even medullary components of the TNC, as well as structures outside the central nervous system (CNS), like skin[23], gastrointestinal tract, lymphocytes[24] and thymus[25].

CONCLUSION

Melatonin decreases CGRP in an experimental model in rats induced by capsaicin, reducing its inflammatory action in cerebral vessels.

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