

# Effect of periapical inflammation on calcium binding proteins and ERK in the trigeminal nucleus

Mariela C. Canzobre<sup>1,2</sup>, Alejandra R. Paganelli<sup>2,3</sup>, Hugo Ríos<sup>2,3</sup>

<sup>1</sup> Universidad de Buenos Aires, Facultad de Odontología, Cátedra de Histología y Embriología, Buenos Aires, Argentina.

<sup>2</sup> Universidad de Buenos Aires, Facultad de Medicina, I<sup>o</sup> Unidad Académica de Histología, Embriología, Biología Celular y Genética, Buenos Aires, Argentina.

<sup>3</sup> CONICET- Universidad de Buenos Aires, Instituto de Biología Celular y Neurociencias "Prof. E. De Robertis" (IBCN), Buenos Aires, Argentina.

## ABSTRACT

Peripheral inflammation induces plastic changes in neurons and glia which are regulated by free calcium and calcium binding proteins (CaBP). One of the mechanisms associated with the regulation of intracellular calcium is linked to ERK (Extracellular Signal-Regulated Kinase) and its phosphorylated condition (pERK). ERK phosphorylation is important for intracellular signal transduction and participates in regulating neuroplasticity and inflammatory responses. The aim of this study is to analyse the expression of two CaBPs and pERK in astrocytes and neurons in rat trigeminal subnucleus caudalis (Vc) after experimental periapical inflammation on the left mandibular first molar. At seven days post-treatment, the

periapical inflammatory stimulus induces an increase in pERK expression both in S100b positive astrocytes and Calbindin D28k positive neurons, in the ipsilateral Vc with respect to the contralateral side and control group. pERK was observed co-expressing with S100b in astrocytes and in fusiform Calbindin D28k neurons in lamina I. These results could indicate that neural plasticity and pain sensitization could be maintained by ERK activation in projection neurons at 7 days after the periapical inflammation.

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**Key words:** neuroplasticity; pERK kinase; S100b protein; Calbindin D28k; apical periodontitis; trigeminal nucleus.

## Efecto de la inflamación periapical sobre las proteínas fijadoras de calcio y ERK en el núcleo trigeminal

### RESUMEN

La inflamación periférica induce cambios plásticos en las neuronas y en la glía, los cuales están regulados por el calcio libre y las proteínas fijadoras calcio (CaBP). Uno de los mecanismos asociados con la regulación del calcio intracelular está vinculado con la fosforilación de la proteína quinasa ERK. Asimismo, ERK fosforilado es importante para la transducción de señales intracelulares y participa en la regulación de la neuroplasticidad y las respuestas inflamatorias. El objetivo de este estudio es analizar la expresión de dos CaBPs y pERK en astrocitos y neuronas del subnúcleo caudal del trigémino (Vc) después de una inflamación periapical experimental en el primer molar inferior izquierdo en ratas. A los siete días posteriores al tratamiento, el estímulo inflamatorio

periapical induce un aumento en la expresión de pERK, en el número de astrocitos positivos para la proteína marcadora astroglial S100b y en neuronas positivas para Calbindina D28k, en el Vc ipsilateral respecto del lado contralateral y el grupo de control. Además, se observó coexpresión de pERK tanto en astrocitos S100b positivos, como en neuronas fusiformes Calbindin D28k positivas, de la lámina I. Estas observaciones podrían indicar que la neuroplasticidad y la sensibilización al dolor podrían mantenerse mediante la activación de ERK en las neuronas de proyección a los 7 días de la inflamación periapical.

**Palabras clave:** neuroplasticidad; quinasa pERK; proteína S100b; Calbindina D28k; periodontitis apical; núcleo trigeminal.

## INTRODUCTION

Periodontal sensory information is transmitted by the maxillary and mandibular branches of the trigeminal nerve. The principal relay for orofacial

sensory information (A delta and C fibers) are the laminae I, II and V of the trigeminal subnucleus caudalis (Vc), which is the first center involved in orofacial pain modulation<sup>1-4</sup>. After peripheral

inflammatory reaction, the primary afferent neuron at the dorsal horn of the Vc releases glutamate onto the ipsilateral fusiform second-order neurons, which in turn increases the influx of calcium<sup>2</sup>. These fusiform neurons localized in lamina I project the nociceptive information to other higher centers of the central nervous system<sup>4</sup>. At the same time, glia and interneurons of Vc participate in the local modulation of the nociceptive information<sup>1,5,6</sup> and may induce morphological modifications as well as biochemical, molecular, and functional synaptic changes. Some of these plastic changes are regulated by free calcium and Calcium Binding Proteins (CaBP)<sup>7-9</sup>.

The calcium binding protein S100b is expressed only in mature astrocytes<sup>10,11</sup>, and Calbindin D28k, calretinin, parvalbumin are present in neurons<sup>12,13</sup>. One of the mechanisms associated with the regulation of intracellular calcium is linked to the mitogen-activated protein kinase (MAPK) pathways. Some of these pathways are important for intracellular signal transduction and participate in regulating neural plasticity and inflammatory responses<sup>9,14,15</sup>. There are four well-characterized subfamilies of MAPKs: Extracellular signal-Regulated Kinase (ERK) 1/2, ERK5, c-Jun N-terminal Kinase (JNKs) and p38<sup>16,17</sup>. Some authors have shown a sequential activation of ERK immediately after a peripheral injury. Initially, ERK is transiently phosphorylated (pERK) in dorsal horn neurons, but only for a period of few hours after injury, then in the microglia and some weeks later in astrocytes<sup>14,18,19</sup>. It is assumed that activation of ERK in glial cells is important for the maintenance of pain states<sup>15,20</sup>.

However, there are no studies linking astroglia and neuron activation of trigeminal nociceptive pathway after apical periodontitis. The aim of this study is to analyze the expression of calcium binding proteins and ERK activation in astrocytes and neurons in the trigeminal subnucleus caudalis (Vc) at seven days after experimental periapical inflammation in rats.

## MATERIALS AND METHODS

This study was carried out on 16 female Wistar rats, 50 days old (weight about 140-170 gr). The animals in these experiments were used according to the Guide for the Care and Use of Laboratory Animals, National Academy of Science, USA and the experimental protocol was authorized by the Ethics

Committee of the School of Dentistry (8/1 1/2012-40) and Medicine (CD n° 2057/14) of Buenos Aires, Argentina. They were housed in a room at about 24 °C, 12 h light/dark cycle and free access to water and standard laboratory rat food. The animals were divided into an experimental and a control group, (n= 8 rats per group), in order to perform immunohistochemistry, immunofluorescence and Western blot analysis. All rats were deeply anaesthetized by intraperitoneal injection of ketamine (100 mg/kg) plus xylazine (14 mg/kg). The rats in the experimental group were operated on the left mandibular first molar to remove the occlusal enamel and dentin, in order to locate the pulp chambers and root canal orifices. The working length of root canal (occlusal-apical) was previously standardized by our laboratory at 4 mm. Later, apical preparation of mesial and distal root canals was done by instrumentation up to a 25 K-file (Dentsply-Maillefer, Ballaigues, Switzerland). After drying with sterile paper points (Sure Endo - Excel Dental Supplies Ltd, mesial and distal root canals were filled with gutta-percha cones (Sure Endo- Excel Dental Supplies Ltd.) and ZOE-based sealers (Grossman's sealer- Farmadental) by the lateral compaction technique. Grossman's sealer was manipulated following the manufacturer's recommendations. Each cavity was then sealed with glass ionomer restorative cement (Fuji II LC). After recovery from anesthesia, animals were monitored for signs of discomfort or pain, and body weight was monitored until they were euthanized. After the first day post-treatment, all operated animals returned to normal behavior, including feeding. Animals in the control group were not exposed to the experimental treatment and received only the anesthetic protocol. All animals in both groups were euthanized at 7 days. Experimental (n=5) and control (n=5) animals were anesthetized with ketamine/xylazine and fixed transcardially using a modification<sup>6</sup> of the original perfusion method described by Gonzalez Aguilar and De Robertis<sup>21</sup>. After euthanasia, brains were removed and post-fixed for 4 hours at 4-5° C. Subsequently, the brains were washed 3 times in phosphate buffer saline (PBS) (1 hour) and then stored at 4 °C in PBS containing 0.01% sodium azide. Cryoprotection was performed by immersion in 10% and 30% buffered sucrose (120 min. and overnight respectively). After that, brainstems were frozen at -20°C. Cryostat brainstem sections (30 µm) were sectioned using a slide microtome and collected in phosphate

buffer saline (PBS) to obtain floating sections for immunohistochemistry. In this study, we selected coronal sections from spinal cord cervical levels C4 to -5.6 interaural levels (6 – 1.5 mm caudal to obex)<sup>22</sup>.

The right (unstimulated, contralateral) side of each brainstem was marked on the ventral face with a blade for identification of the paired control side (Fig 1A). Immunostaining was done by our immunohistochemical technique protocol in free floating sections with a procedure using an enzyme method with chromogen; avidin- biotin complex (ABC) or immunofluorescence<sup>6</sup>. The primary antibodies used were: anti astroglial calcium binding protein S100b (rabbit polyclonal, Sigma-Aldrich Cat# HPA015768, RRID:AB\_1856538, 1:800), Calbindin D28-K (rabbit polyclonal, CB38, Swant Immunochemicals, Cat# CB38, RRID:AB\_2721225, 1:5000) and phosphorylated ERK (Santa Cruz Biotechnology Cat# sc-7383, RRID:AB\_627545, 1:1000). Negative controls were performed by replacing primary antibody with goat serum. Briefly, for the ABC method, sections were washed in TS for 40 minutes and then incubated with appropriate biotin conjugated secondary antibody 1:2000 (Jackson Immuno Research). When immunofluorescence labeling was performed, anti S100b or Calbindin D28-K was combined with pERK. After washing for 40 minutes with TS, rhodamine red or fluorescein isothiocyanate (FITC) conjugated secondary antibodies were used. Western blot analyses were performed on brainstem extracts from control (n=3) and operated rats (n=3). Brainstems were cut along in the sagittal plane to obtain the ipsilateral and contralateral side of Vc of each group. Tissues were homogenized in a Potter-Elvehjem homogenizer, in 5 vol of TS containing 1 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, and protease inhibition cocktail (Sigma-Aldrich). The homogenate was centrifuged at 12000 g, 4°C for 20 min and the supernatant was assayed for protein concentration with the BCA Protein Assay Kit (Thermo Scientific Pierce), according to the manufacturer's instructions. Aliquots of supernatant corresponding to 30 µg of total protein were loaded onto SDS-12% polyacrylamide gels. The proteins were electrotransferred to a PVDF membrane in Tris-glycine-methanol buffer and examined with different antibodies.

Membranes were blocked for 1 h at room temperature in a blocking solution containing 5%

normal goat serum, 0.1 %, Tween-20 in PBS (pH 7.4). The membrane was then incubated overnight at 4°C with primary antibodies in the blocking solution. The following primary antibodies were used in the western blot experiments: rabbit polyclonal S100b (1:1000), rabbit polyclonal Calbindin D28-K (1:1000), mouse monoclonal pERK (1:1000) and mouse monoclonal GAPDH (Glyceraldehyde 3-phosphate dehydrogenase, Santa Cruz Biotechnology Cat# sc-166574, RRID:AB\_2107296; 1:5000) as loading control.

Membranes were rinsed three times with 0.1% Tween-20 in PBS for 10 min each, followed by incubation for 1 h at room temperature with HRP donkey anti-mouse IgG or anti-rabbit IgG, 1:2000 (Millipore). The blots were washed three times for 20 min each and then processed for DAB/Ni immunohistochemistry. Development of peroxidase activity was carried out with 0.04% w/v 3,3' diaminobenzidine (Sigma-Aldrich) plus 2.5% w/v nickel ammonium sulphate (Baker) and 0.01% H<sub>2</sub>O<sub>2</sub>. The reaction was stopped by adding tap water. The hemimandibles were dissected, post-fixed overnight and radiographed. Then they were decalcified in 10% EDTA/PBS for about 5 weeks, dehydrated, clarified in toluene and embedded in Paraplast. They were cut at 10 µm longitudinal sections, collected on glass slides and stained with hematoxylin-eosin to analyze the degree of periapical injury and extent of inflammatory tissue after canal instrumentation and filled up to the apex. All stained sections were photographed in a Zeiss Axiophot light microscope equipped with epifluorescence by switching between FITC and rhodamine filter sets. Photomicrographs were captured at 24 bits per pixel resolution (8 bpp x 3 colors) using an Olympus Q-color 5 camera, and identical light exposure parameters. The morphometric analysis was performed using Image Pro 6 (Media Cybernetics, Silver Springs, MD, USA). Based on normal morphological criteria, on each side and two levels of Vc per animal, the immunoreactive cells were quantified per dorsal area of 0.01 mm<sup>2</sup>.

Results are shown as mean and SEM. Data were statistically compared by two-way analysis of variance (ANOVA) followed by the post-hoc Bonferroni test. Statistical difference was considered significant for p-values less than 0.05. All statistical analyses were performed using "Graph Pad Prism" (version 5.03; Graph Pad Software).

## RESULTS

### Histological analysis of periapical tissues

Conventional histological stain was used to evaluate the periapical inflammatory reaction. The histological analysis in the experimental group showed the extent of damage and the inflammatory reaction on the left mandibular first molar at 7 days post treatment. The connective tissue of the periodontal ligament showed an acute inflammatory infiltrate (arrow in Fig. 1B) after preparation of root canal and was filled up to the apex. This reaction was usually accompanied by widening of the periodontal ligament at the expense of resorption of the cortical alveolar bone. At 7 days, the histopathological reaction would be compatible

with a diagnosis of apical periodontitis and probably sensory receptor stimulation (Fig. 1B). In the control group and the right hemimandible of the experimental group, the molars showed normal histological features (Fig. 1C). None of them showed any histological signs of an inflammatory process in the periodontal ligament.

### Astrocytes and neurons activation in the subnucleus caudalis

To determine whether the periapical inflammation can induce activation of astrocytes and neurons on the subnucleus caudalis, we analyzed the expression of S100b in mature astrocytes, Calbindin D28k in neurons and pERK in astrocytes and neurons present in the subnucleus caudalis (Vc). In the

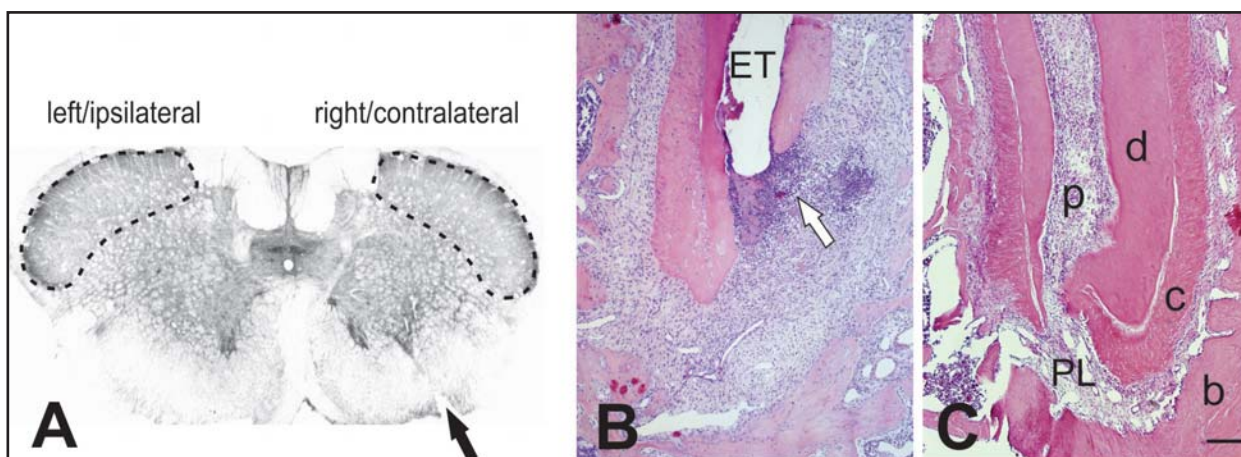


Fig. 1: A: coronal sections of paired ipsilateral and contralateral subnucleus caudalis (Vc). The right (contralateral) side of each brainstem was marked on the ventral face with a blade (arrow) for identification of the paired control side. B-C: Sections of apical zone on the left (B) and right (C) mandibular first molar of experimental group and stained with hematoxylin and eosin. B: Image showing an inflammatory reaction in the periodontal ligament (white arrow) seven days after root canals instrumentation and filled with gutta-percha cones and ZOE-based sealers (Grossman's sealer- Farmadental). p= pulp; d= dentin; c= cement; b= alveolar bone; PL= periodontal ligament; ET= endodontic treatment. Scale bar= 100um.

**Table 1: Astrocytes and neurons activation in the subnucleus caudalis after periapical inflammation.**

	S100b astrocytes	Calbindin D28K neurons	Calbindin D28K fusiform neurons	pERK Glia /neurons
<b>CONTROL GROUP</b>				
Ipsilateral side	10.57 ± 0.67	11.94 ± 1.57	0.63 ± 0.09	28.43 ± 3.52
Contralateral side	10.66 ± 1.00	13.94 ± 0.72	0.68 ± 0.08	31.19 ± 2.48
<b>EXPERIMENTAL GROUP</b>				
Ipsilateral side	16.23 ± 1.76	16.34 ± 0.88	1.18 ± 0.14	35.35 ± 4.09
Contralateral side	13.76 ± 2.32	14.46 ± 1.01	0.83 ± 0.14	31.71 ± 4.42

The results in the table are expressed as mean and SEM. Data (control Vs experimental group) were statistically compared by two-way analyses of variance (ANOVA) followed by the post-hoc Bonferroni test. Note the significant increase in the ipsilateral Vc in the experimental group with respect to their paired contralateral Vc (\*) and to control group (#).  $p < 0.05$ .

experimental group, the number of S100b positive astrocytes per area was significantly increased on the ipsilateral side ( $16.23 \pm 1.76$ ) as compared to the paired contralateral side ( $13.76 \pm 2.32$ ) and to the control group ( $10.57 \pm 0.67$ ) (Table 1 and Fig. 2 A-B).

The expression pattern of Calbindin D28K, at 7 days after periapical inflammation, demonstrates that the number of the CB positive neurons in the superficial lamina of the ipsilateral Vc ( $16.34 \pm 0.88$ ) was significantly greater than in the contralateral side ( $14.46 \pm 1.01$ ) and the control group ( $11.94 \pm 1.57$ ). Additionally, in the lamina I, we analyzed the number of fusiform neurons showing morphological features with an elongated soma and two long primary extensions emerging from each end of the soma. We quantified ( $0.63 \pm 0.09$  cells/area) in the control group and ( $1.18 \pm 0.14$  cells/area) in the ipsilateral side of the experimental group showed statistically significant differences (Table 1 and Fig. 2 C-D).

Finally, we quantified the pERK expression in different cell populations of Vc at 7 days of peripheral inflammation. The number of positive cells per area was found to increase significantly on the ipsilateral side ( $35.35 \pm 4.09$ ) compared to the paired contralateral side ( $31.71 \pm 4.42$ ) (Table 1 and Fig. 2 E-F).

Western blot analysis also revealed an increase in the amount of S100b, CB D28K and pERK proteins on the ipsilateral side of the Vc in the experimental group (Fig. 3).

Moreover, we analyzed ERK activation linked calcium binding proteins in different cell populations related to plasticity. To identify pERK in astrocytes or neurons, double immunofluorescence labeling with S100b or Calbindin D28K was performed, respectively. We observed that pERK and S100b colocalize in the cell body and processes of astrocytes. In addition, pERK expression was observed in some cells with different morphology from astrocytes, and which did not

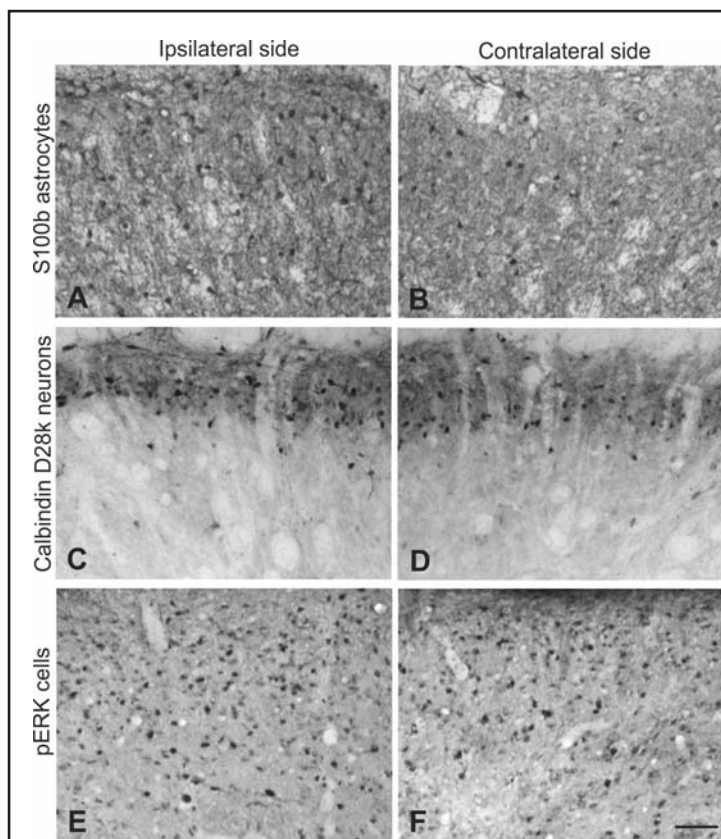


Fig. 2: Coronal sections of brainstems showing the superficial (I and II) and deeper laminae (III) on both sides of Vc of experimental group. (A-B): Images showing S100 b positive astrocytes. (C-D): Experimental group, showing Calbindin D28k positive neurons. (E-F): Experimental group, showing pERK positive cells. The ipsilateral side (A-C-E) showed a significant increase in the number of immunoreactive cells relative to their paired contralateral Vc (B-D-F) for the three study proteins. Moreover, the ipsilateral side of Calbindin D28K (C) showed an increase in the number of fusiform neurons in superficial laminae relative to the contralateral side.

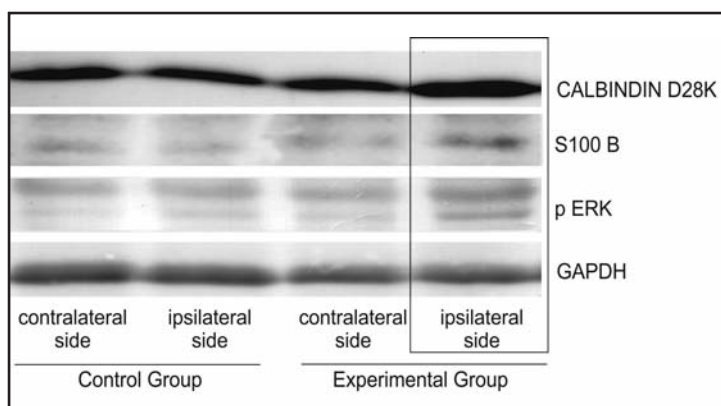


Fig. 3: Western Blot analysis showed an increase in the amount of Calbindin D28K, S100b and pERK proteins on the ipsilateral side of the experimental group 7 days after periapical inflammation. GAPDH was used as a loading control.

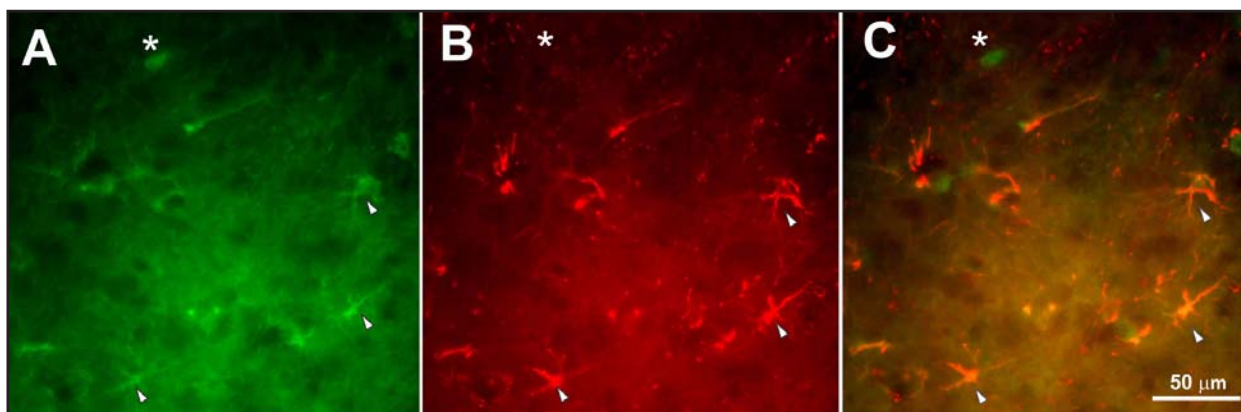


Fig. 4: Double immunofluorescence of S100b and pERK. A: pERK expression in cells of the superficial laminae (arrowheads). B: S100b expression in the cell body and processes of astrocytes (arrowheads). C: double labeled for pERK (green) and S100b (red). Note the presence of cells with different astrocyte morphology in laminae I of ipsilateral Vc. It presents pERK expression but it is not S100b IR cell (asterisk). Scale bar= 50μm.

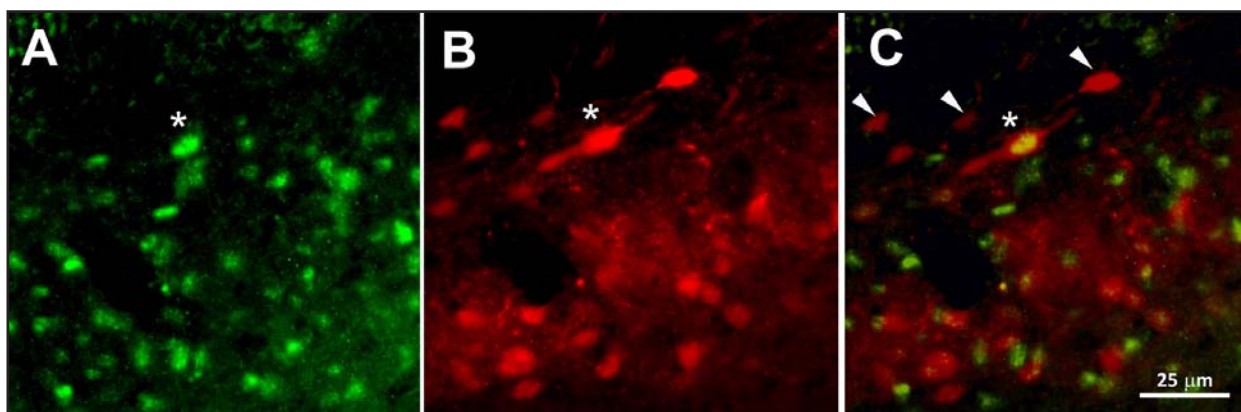


Fig. 5: Double immunofluorescence of pERK and Calbindin D28K in the ipsilateral side of the Vc in the experimental group. A: pERK expression in cells of the lamina I B: Calbindin D28K expression in neurons of superficial laminae. C: Double labeling with pERK (green) and Calbindin D28k (red). Note that CB D28k and pERK colocalize in fusiform neurons in laminae I of ipsilateral Vc at 7 days. Other neurons only express Calbindin D28k (arrowheads) Scale bar= 25μm.

express S100b (Fig. 4 C asterisk). Double immunofluorescence labeling with pERK and Calbindin D28K showed that these proteins colocalize in fusiform neurons presents in lamina I of the ipsilateral Vc at seven days after the peripheral injury. (Fig. 5 C asterisk).

## DISCUSSION

Periapical inflammation is a common problem in dental practice, especially related to endodontic treatment, because canal instrumentation or root canal sealers frequently induce an inflammatory reaction in the periodontal ligament (apical periodontitis)<sup>23,24</sup>. It has been demonstrated that ZOE-based sealers are direct activators of periodontal sensory neurons<sup>23,25</sup>. There is usually greater

incidence of post-endodontic pain following treatment of teeth with vital pulp<sup>23</sup>. One possibility is that periapical vital tissue promotes abnormal accumulation of fluid in the interstitium (edema) with consequent compression and activation of mechanoreceptors or free nerve endings<sup>24</sup>. Another possibility is that peripheral inflammatory injuries induce central sensitization, keeping the circuit in a pathological state related to the development of hyperalgesia/allodynia<sup>26,27</sup>. Previous studies observed that rats have responses that are similar to human periapical inflammatory reaction<sup>28,29</sup>. In this study, we considered that the apical inflammation induced by root canal treatment is a good experimental model for studying plasticity in the trigeminal subnucleus caudalis (Vc). However,

there are few studies linking the activation of nociceptive pathway after apical periodontitis.

After peripheral inflammatory reaction, the glia and interneurons of Vc participate in the local modulation of the nociceptive information<sup>1,5,6</sup>. According to this idea, in a previous study, we demonstrated that a pulpal inflammatory process in adult rats generated plastic changes in S100b positive astrocytes, in the ipsilateral side of Vc. On this side, the expression of protein was significantly greater than in the control animals, which displayed low expression of S100b, suggesting its role in pulpal nociceptive pathway<sup>26</sup>. The calcium binding protein S100b is synthesized and released by activated astrocytes and is involved in the regulation of intracellular free calcium or calcium homeostasis and in the activation of some kinases<sup>10,12</sup>.

Moreover, Calbindin D28K (CB), another member of the calcium-binding protein family, is expressed in neurons of medulla and Vc<sup>13</sup>. Most CB positive neurons in the dorsal horn are involved in transmission and modulation of the nociceptive pathway<sup>30</sup>. We have reported different subtypes of Calbindin D28k immunohistochemistry positive neurons in the trigeminal subnucleus caudalis, and the morphology and distribution on the dorsal laminae I of the Vc<sup>31</sup>.

In addition, our results show that the experimental model of periapical inflammation induces plastic changes in calcium-binding proteins S100b and calbindin D28K expressed in astrocytes and neurons of ipsilateral subnucleus caudalis, respectively. Moreover, we observed an increase in the number of fusiform cells present in ipsilateral lamina I, considered excitatory projection neurons that would transmit nociceptive information to the thalamus.

On the other hand, pERK is actually involved in the response to potentially harmful, abiotic stress stimuli and considered a fast molecular marker in injury or nociception. pERK has been shown to localize to cytoplasm, nucleus and projections<sup>32</sup>.

There is currently evidence that MAPKs expression in neurons or glial cells plays an essential role in the development and maintenance of persistent pain<sup>33,20</sup>. According to these concepts, here we demonstrate that pERK-IR cells were observed mostly in the ipsilateral dorsal horn after experimental apical periodontitis.

It is noteworthy that S100b and Calbindin D28K colocalize with pERK in astrocytes or neuron of Vc, respectively. We did not quantify co-expression of CaBPs and pERK in cells of Vc. Studies reported in the literature found that ERK is phosphorylated in dorsal horn neurons transiently for a period of few hours after stimulus and some weeks later in astrocytes<sup>18,19,14</sup>. However, we demonstrated that Calbindin D28K and pERK colocalize in some fusiform neurons at lamina I of ipsilateral Vc, at a later time-point (7 days) than was described previously. This indicates that some Vc projection neurons presents in lamina I, show long-term pERK activity at 7 days, and could maintain the nociceptive information to thalamus and cortex. This result could be interpreted through the increase of S100b and pERK proteins in astrocytes at the ipsilateral Vc, indicative of glial activation. Astrocytes that respond to alterations in neuronal environments and nanomolar levels of extracellular S100b could be released by activated astrocytes<sup>10</sup> and act on neurons, possibly inducing ERK pathway activation. However, more results are required to confirm this hypothesis.

## CONCLUSION

Prevention and management of acute periapical pain is an integral part of endodontic treatment in patients. We find that apical periodontitis stimulus increases S100b, Calbindin D28k and pERK protein expression in astrocytes and neurons of the ipsilateral Vc and suggest that ERK activation in fusiform calbindin neurons in lamina I (nociceptive projection neurons), could prolong neural plasticity and pain sensitization for 7 days.

## FUNDING

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## CORRESPONDENCE

Dra. Mariela C. Canzobre  
Cátedra de Histología, Facultad de Odontología,  
MT de Alvear 2142 1er piso sector A.  
C1121ABG CABA, ARGENTINA.  
marielacanzobre@yahoo.com.ar

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