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Peripheral purinergic receptor modulation influences the trigeminal ganglia nitroxidergic system in an experimental murine model of inflammatory orofacial pain.

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Running title: PPADS local application decreases pain sensitivity

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Abstract

ATP plays a role as an endogenous pain mediator generating and/or modulating pain signalling from the periphery to the central nervous system. The aim of this study was to analyze the role of peripheral purinergic receptors on the modulation of nitroxidergic system at trigeminal ganglia (TG) level monitoring the alteration of nitric oxide synthase isoforms. The Fos positive neurons at brainstem level (spinal trigeminal nucleus) and the pain related behaviour have been also evaluated.

The local administration of P2 purinergic receptor antagonist, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), decreased the face-rubbing activity, nitric oxide synthase isoform expression in TG and Fos expression in spinal trigeminal nucleus after subcutaneous injection of formalin.

These results suggest a role of peripheral P2 purinergic receptors in orofacial pain transmission through the modulation of nitroxidergic system.

Key words: purinergic receptors; trigeminal system; nitroxidergic system; inflammatory pain; mice

Introduction

ATP exerts its effects via P2 receptors, which can be subdivided into two major groups, the P2X and P2Y receptors. They are purinoreceptors classified into G-protein coupled receptors P2Y and ATP-gated cation channels, so-called P2X receptors. In sensory ganglia, all seven cloned mammalian P2X receptors are present (Staikopoulos et al., 2007; Zhang et al., 2005).

During acute inflammation, high concentrations of extracellular ATP have been measured on the sites of tissue injury in experimental animal models and in arthritic patients (Gordon, 1986; Park et al., 1996; Verghese et al., 1996).

ATP released from the damaged peripheral nerve tissue (Koizumi et al., 2004; Zhao et al., 2008). might contribute to initiate neuron and glia activation, inducing the synthesis and release of pro-inflammatory cytokines and nitric oxide (NO) (Inoue, 2006; Martucci et al., 2008). NO is produced

within the nervous cells mainly by two isoforms of nitroxidergic synthase (NOS), a constitutive (neuronal) isoform (nNOS) and an inducible isoform (iNOS), which participate in different way in pain transmission (Jenkins et al., 1994; Martucci et al., 2008; Watkins and Maier, 2003).

Several data support the evidence of pathological related ATP/NO interactions. ATP can act at various purinergic receptors on neurons and glia to increase intracellular calcium (James and Butt, 2001) which can transiently activate NOS (Baader and Schilling, 1996; Baltrons and García, 1997) leading to an increase of NO.

The spinal trigeminal nucleus is subdivided into three parts, i.e., subnucleus caudalis (Sp5C), interpolaris (Sp5I), and oralis (Sp5O), and it receives somatosensory inputs from orofacial areas. The role of the three subnuclei in the trigeminal nociceptive mechanisms is not yet well defined. Nevertheless several studies showed that Sp5C is involved in nociceptive orofacial inputs (Sessle, 1987). Pain induced activation at this level can be associated with Fos expression. This is a nuclear phosphoprotein product of the mammalian c-fos protooncogene generally used to identify the activated neurons involved in pain transmission in the supraspinal areas (Rodella et al., 1998).

Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid tetrasodium salt (PPADS) is a wide range P2 receptor antagonist (Lambrecht, 2000; Lambrecht et al., 1992, Mayer et al., 1998; Sluyter et al., 2001; Stucky et al., 2004; Zhao et al., 2008). The receptors that are more sensitive to PPADS are $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_5$. Although all of them are present in nervous system, $P2X_1$, $P2X_3$ $P2X_5$ receptors have been specifically described in trigeminal ganglia neurons (Chen et al., 1995; Valera et al., 1994).

In order to obtain more information about the relationship among inflammation, nitric oxide and purinergic receptors, the aim of this work was to investigate, in a mouse model of orofacial inflammatory pain, the NOS isoform expression in TG, monitoring also the activated neurons at central level (spinal trigeminal nucleus), through Fos expression, and pain related behavior, after local purinergic receptor modulation.

To better understand the role of P2X receptors present in skin during inflammation, the behavioural test was also performed using A-317491 (5-[[[(3-Phenoxyphenyl)methyl][(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino] carbonyl]-1,2,4-benzenetricarboxylic acid sodium salt hydrate), a novel $P2X_3$ and $P2X_{2/3}$ receptor antagonist, which is able to exclude these receptors highly expressed in ganglia neurons (Jarvis et al., 2002). Finally, the general role of nitric oxide in pain modulation was assessed using TRIM (1-[2-(trifluoromethyl)phenyl] imidazolo), a selective iNOS/nNOS inhibitor (Haga et al., 2003; Handy et al., 1996).

Materials and methods

Animals

Experiments were carried out on 81 C57BL/6J male mice (20-25 gr. Harlan, Italy).

To minimize the circadian variations, the animals were housed in individual cages with food and water *ad libitum* and kept in an animal house at a constant temperature of 22°C with 12 h alternating light-dark cycle. The experiments were performed between 08:00 h and 12:00 h. All effort was made to minimize animal suffering and the number of animals used. The experimental procedures were approved by the Italian Ministry of Health and in accordance with the ethical standards of the Helsinki Declaration and the principles presented in the "Guidelines for the Use of Animals in Neuroscience Research" of the Society for Neuroscience.

The animals were subdivided in 4 experimental groups of 3 animals each: the first group was injected with saline (control, CTR); the second group was injected with the drug (control of the drug); the third group was injected with formalin (FORM); the forth group was treated with the drug at different doses and after 30 minutes injected with formalin (drug+FORM).

Injection site

The formalin, PPADS and A-317491 injections was performed subcutaneously into the right upper lip, just lateral to the nose through a 27-gauge needle into the right vibrissa pad as quickly as possible, with only minimal animal restraint. The TRIM injection was performed intraperitoneally.

Drug treatments

Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid tetrasodium salt (PPADS; Sigma-Aldrich, Milan, Italy) was dissolved in saline and used at doses of 6.25, 12.5 and 25 mg/kg (0.01 ml/10 g), according to Gourine et al. (2005) and Martucci et al. (2008).

A-317491 (5-[[[(3-Phenoxyphenyl)methyl][(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino] carbonyl]-1,2,4-benzenetricarboxylic acid sodium salt hydrate; Sigma-Aldrich, Milan, Italy), a novel $P2X_3$ and $P2X_{2/3}$ receptor antagonist, was dissolved in saline at 30, 100, 300 nmol according with McGaraughty et al. (2003).

TRIM (1-[2-(trifluoromethyl)phenyl] imidazolo), a selective iNOS/nNOS inhibitor (Handy et al., 1995; Handy et al., 1996) (Tocris Cookson Ltd, Bristol, UK), was dissolved in 20% DMSO (dimethylsulphoxide; Sigma-Aldrich, Milan, Italy) in saline at concentration of 10, 20, 50 mg/Kg according with Handy et al (1996).

Nociceptive behavioral response

The formalin test was made injecting 2,5% formalin (FORM) according to Luccarini et al. (2006). Following formalin injection all animals were immediately placed in the test box for a 60 min observation period. A nociceptive score was determined measuring the number of seconds that the animals spent rubbing the injected area with the ipsilateral fore- or hindpaw. The recording time was divided into 20 blocks of 3 min. A video camera was used to record the grooming response.

PPADS evaluation at the best dose

The dose-response behavioural analysis showed that PPADS 25 mg/kg was the most efficient dose, which has been used for the immunohistochemical and western blotting evaluation.

Immunohistochemical analysis

Fos expressions were evaluated at 3h after formalin injection and at the same time also iNOS and nNOS. The subsequent time course analysis of iNOS and nNOS was evaluated at 6h, 12h and 24h after formalin injection.

All mice were anaesthetized with Zoletil (60 mg/Kg i.p., Verbatic, France) and transcardically perfused with saline followed by 40 ml of 4% paraformaldehyde in phosphate buffer 0.1 M pH 7.4. After fixation, the brainstem and the trigeminal ganglia of each animal were removed, post-fixed in 4% paraformaldehyde in phosphate buffer for 2 h and cryoprotected overnight in 30% sucrose at 4°C. Frozen serial transverse sections (40 µm thick) of all the brainstem and of the trigeminal ganglia were placed in TBS (Tris-Buffer-Saline) solution. Alternate sections were processed for single immunohistochemistry or toluidine blue-stained for morphological control.

Briefly, the first series of sections was incubated in normal goat serum (10% in TBS plus 0.1% Triton X-100) for 60 min and then incubated in rabbit polyclonal primary antiserum directed against Fos (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) or nNOS (1:500, Chemicon, USA) or iNOS (1:500, Chemicon, USA) diluted in TBS containing 3% normal goat serum and 0.1% Triton X-100, for 24 h at 4°C. After incubation in the primary antiserum, the sections were sequentially incubated in biotinylated goat anti-rabbit immunoglobulins and avidin-biotin peroxidase complex (Vector Labs., Burlingame, CA, USA). The reaction product was visualized using hydrogen peroxide and diaminobenzidine (Sigma, St. Louis, MO, USA) as chromogen.

The immunohistochemistry control was performed by omitting the primary antibody and incubating the sections with non-immune rabbit serum. The distribution of the labelled cells of all animals was charted with the aid of an image analyzer (Immagini e Computer, Milano, Italy).

Western Blotting analysis

nNOS and iNOS expressions were evaluated at 3 hours after formalin injection. All mice were anaesthetised with Zoletil (60 mg/Kg i.p.) and sacrificed by cervical dislocation. The trigeminal ganglia of each animal were removed and immediately frozen in liquid nitrogen and stored at -80°C until the NOSs content expression assay. On the day of NOS determination tissues were defrosted at room temperature, weighed, diluted in lysis buffer (Tris HCl pH 8 50 mM, NaCl 150 mM, Triton 1% 100µl/ml, PMSF 0.6 mM e aprotinina 1µg/ml), homogenized and centrifuged at 13000g at 4°C for 2 min. After protein assay, supernatant was diluted in Laemmli buffer (0.3 M Tris–HCl, pH 6.8, containing 10% SDS, 50% glycerol, 5% dithiothreitol and 0.05% bromophenol blue) to obtain 40 µg of proteins. The proteins were loaded onto a 8% SDS–polyacrylamide gel and then transferred onto a nitrocellulose membrane (Biosciences, Uppsala, Svezia) for 1 h at 4°C. The membrane was blocked with 5% BSA in TBST (20 mM Tris–base, pH 7.6, 137 mM NaCl and 0.1% Tween 20) at 4° C overnight. The next day it was incubated with primary polyclonal antibody directed against mouse nNOS (Cayman Chemical, Ann Arbor, MI, USA) diluted 1:200 or iNOS (Santa Cruz, Biotechnology, Santa Cruz, CA) diluted 1:500 in blocking solution (1% serum albumin bovine) for 2 h at room temperature. After two washing in TBST buffer, the blot was incubated with biotinylated goat anti-rabbit immunoglobulins (Vector Labs., Burlingame, CA, USA) for 1 h at room temperature. Subsequently the blot was detected with addition of avidin-biotin peroxidase complex (Vector Labs., Burlingame, CA, USA). The reaction product was visualized using hydrogen peroxide and diaminobenzidine (Sigma, St. Louis, MO, USA) as chromogen.

Data analysis

Analysis of the behavior was made for 1 h after the injection of formalin by three investigators who were blinded to the animal's group assignment. The data of all animals were analyzed and compared by repeated measures ANOVA (analysis of variance) with Tukey's post test.

The intensity of nNOS and iNOS positive cells in the trigeminal ganglia was estimated at a final X200 magnification by means of integrated optical density by researchers unaware of the animal

group assignment. On the basis of their size, the ganglion cells have been divided into two groups (Andoh and Kuraishi, 2004; Peier et al., 2002; Puri et al., 2006): the small-sized neurons (diameter <20 um) and the medium- and large-sized neurons (diameter >20 um). Grey levels were evaluated as integrated optical density (IOD) with an image analysis program (Image-Pro Plus™ 4.5.1, Milan, Italy).

The density of the Fos in the brainstem was evaluated in Sp5C, Sp5I and Sp5O using a quantitative method by researchers unaware of the animal group assignment. Fos-positive cell counts were made in all the processed sections at a final X200 magnification. Total counts were taken from each section and assigned to specific components of the brainstem trigeminal complex. Cytoarchitecturally identified regions of the spinal trigeminal nucleus including Sp5C, Sp5I and Sp5O were examined for Fos-positive cells. Rostrocaudal levels of these subnuclei were referenced to bregma according to coordinates provided by Franklin and Paxinos (1997). Moreover, a set of serial transverse 40 µm sections stained with toluidine blue (Sigma, St. Louis, MO, USA) was prepared to better identify the area of Sp5 subnuclei. We analyzed the following according to the bregma coordinates: Sp5O sections were collected from -5.68 mm to -6.48 mm, Sp5I from -6.48 mm to -7.48 mm and Sp5C from -7.48 mmm to -8.48 mm.

Immunoreactive bands of Western Blot analysis were analyzed using a computer-based densitometry image program. Grey levels were evaluated as integrated optical density (IOD) with an image analysis program (Image-Pro Plus™ 4.5.1, Milan, Italy).

The data of all animals from immunohistochemistry and western blotting were analyzed and compared by repeated measures ANOVA and by a Bonferroni multiple comparison test.

Results

Nociceptive behavioral response

Formalin injected animals showed sustained face-rubbing episodes with vigorous face-wash strokes directed to the perinasal area (whisker pad, upper lip and nostril) with the ipsilateral forepaw, sometimes with the hindpaw. The ipsilateral forepaw was often accompanied in its movements by the contralateral forepaw. Occasionally, animals strongly pulled on their vibrissa with their ipsilateral forepaw. This nociceptive response presented a typical biphasic time course with an early and short-lasting (3–5 min) first period of activity followed, after a 10–15 min quiescent period, by a second prolonged (20–45 min) phase.

Behaviour analysis for PPADS

Animals pre-treated with PPADS at different doses (6.25, 12.5, 25 mg/Kg) and injected with formalin showed a decrease in face rubbing activity directly correlated with the drug dose. The dose of 25 mg/kg was the most efficient.

So, the effect of the acute pre-treatment of PPADS at the highest dose (25 mg/kg) has been evaluated.

Control animals showed only face-grooming episodes for during all analysis time.

PPADS treated animals displayed a nociceptive score not significantly different from control animals.

Formalin injected animals showed sustained face-rubbing episodes as described previously.

Animals pre-treated with PPADS and injected with formalin showed a significant decrease of nociceptive score with respect to the formalin animals. Particularly, its presented a less pronounced early phase and a less marked and lasting second rubbing period.

The data are summarized in Fig. 1.

Behaviour analysis for A-317491 and TRIM

Animals pre-treated with A-317491 and injected with formalin presented a statistical less pronounced early phase and a delayed second rubbing period respect to the formalin group in all the three doses used. Respect to 25 mg/Kg PPADS group, the animals present a delayed and statistically higher second phase.

Animals pre-treated with different doses of TRIM and injected with formalin showed a significant decrease of nociceptive score with respect to the formalin animals. Particularly, it presented a statistical less pronounced early phase and a second rubbing period shorted between 21' and 38' with the highest dose. Respect to 25 mg/Kg PPADS group, the animals treated with the highest dose presented a statistical increase in rubbing only between 24' and 27', the other time points were statistically lower.

The behavioral response after administration of each single drug at the highest dose was not statistically different respect to the control group (data not shown).

The data are summarized in Fig. 2.

Immunohistochemical evaluation after 25mg/Kg PPADS treatment: time course of nNOS and iNOS expression in TG

The time course of nNOS showed a rapid increase of the protein at 3h in FORM animals, especially in small neurons. During 24 h nNOS gradually decrease towards control values both for small and medium- and large-sized neurons. The PPADS treatment partially limited the increase of nNOS. The data are summarized in Fig. 3a.

The time course of iNOS showed a rapid increase of the protein at 3h in FORM animals. During 24 h iNOS gradually decrease reaching control values both for small and medium- and large-sized neurons. The PPADS treatment partially limited the increase of iNOS reaching control value at 6h. The data are summarized in Fig. 3b.

Immunohistochemical evaluation at 3 hours from formalin injection

The nNOS and iNOS immunoreactivity was localized in the cytoplasm and the nuclei were unstained.

In control animals, the nNOS staining was low in small, medium and large neurons and fibers; iNOS staining was not found.

In PPADS treated animals, the nNOS staining was similar to the control animals, without any significative difference between the ipsilateral or contralateral TG. The iNOS expression was not found.

In formalin injected animals, a significant increase in nNOS staining was observed particularly in small neurons appearing well evident and dark brown stained respect to the control group. In addition several fibers, axons and surrounding Schwann cells were immunoreactive. The increase of nNOS expression was more evident ipsilaterally respect to injection site. iNOS staining was very faint in all neurons and a moderate staining was found in the fibers particularly in ipsilateral TG.

In animals pre-treated with PPADS and injected with formalin, only a moderate nNOS staining was observed in small neurons and in the fibers tending to the control conditions; a recovery to normal iNOS staining was reached.

The data are summarized in Fig. 4.

Immunohistochemical evaluation after 25mg/Kg PPADS treatment at 3h: Fos expression in brainstem

In control animals, the number of Fos positive neurons in Sp5C, Sp5I and Sp5O was very low without any significant difference between the different areas of the trigeminal nucleus.

In PPADS treated animals, the number of Fos positive neurons in Sp5C, Sp5I and Sp5O was very low without significant difference between the different areas of the trigeminal nucleus.

In formalin injected animals, the number of Fos positive cells increased in Sp5O and in the Sp5C areas mainly ipsilaterally in comparison with control animals. The formalin-related increase of immunopositivity were found mainly in subnucleus oralis and in the ipsilateral side. In animals pre-treated with PPADS and injected with formalin, a significant decrease of Fos positive neurons was observed bilaterally in the spinal trigeminal nucleus compared to the formalin group.

The data about the distribution of the labeled cells in Sp5O and Sp5C in the different groups are summarized in Fig. 5.

Western Blotting analysis after 25mg/Kg PPADS treatment at 3h: nNOS

In the TGs control animals, the nNOS expression was moderate.

In PPADS treated animals, nNOS expression in TG was similar to the control animals.

In formalin injected animals, nNOS was overexpressed mainly in ipsilateral TG.

Instead, in animals pre-treated with PPADS and injected with formalin, we observed a significant decrease of nNOS mainly in the ipsilateral TG.

The data are summarized in Fig. 6.

Western Blotting analysis after 25mg/Kg PPADS treatment at 3h: iNOS

In the TG control animals, iNOS expression was not observed.

In PPADS treated animals, iNOS expression in the TG was similar to the control animals..

In formalin injected animals, iNOS was overexpressed in ipsilateral TG.

Instead, in animals pre-treated with PPADS and injected with formalin, we observed a significant decrease of iNOS in the ipsilateral TG.

The data are summarized in Fig. 6.

Discussion

These results suggest the involvement of peripheral P2 receptors in orofacial inflammatory pain transmission at peripheral level and that purinergic receptor activation modulates the nitroxidergic system in TG. The data are supported by the analysis of Fos expression at central level (spinal trigeminal nucleus) and pain related behaviour. Pain sensation as a result of P2 receptors activation is greatly increased in inflamed tissue. During acute inflammation, high concentrations of extracellular ATP have been measured on the sites of tissue injury in experimental animals and in arthritic patients (Gordon, 1986; Park et al., 1996; Verghese et al., 1996). The source of ATP is not only from damaged cells, but also from non-damaged endothelial cells (Boettger et al., 2007) and

from keratinocytes, where the increase in sodium channel expression may contribute to pain by inducing epidermal ATP release and resulting in excessive activation of P2X receptors on primary sensory axons (Zhao et al., 2008). Nanomolar levels of ATP can act at various purinergic receptors on neurons, glia, and endothelial cells to increase intracellular calcium (James and Butt, 2001) and increases in intracellular calcium can cause NO release from neurons and glia (Queiroz et al., 1997). An elevated P2X receptor activity can also result from the enhanced expression of this receptor in inflamed tissue and can contribute to abnormal pain responses associated with inflammatory injuries (Wirkner et al., 2007).

According with this observations, our results showed that the local application of PPADS in the inflamed site produces a reduction in pain-related behaviour. The animals pre-treated with PPADS and injected with formalin showed a significant decrease of nociceptive score with respect to the formalin injected animals, with an early phase less pronounced and second rubbing period less marked and lasting. Other data in literature suggest the role of PPADS in antinociception. The intraperitoneal administration of PPADS after chronic constriction injury of the sciatic nerve was able to inhibit both thermal hyperalgesia and mechanical allodynia (Borsani et al., 2008; Martucci et al., 2008) and its intrathecal pre-treatment significant suppresses both the first and the second phase of formalin related behaviour (Tsuda et al., 1999). Moreover, because both phases of formalin-evoked nociceptive behaviors were potentiated by a selective allosteric enhancer of $P2X_3$ receptor function (Jarvis et al., 2002), the involvement of $P2X_3$ receptors in acute and inflammatory pain is of strong likelihood. Our results, using A-317491, showed a general decrease in pain behaviour, that is traduced in a delayed second phase with values statistically higher respect to the animals treated with PPADS. These results suggest also an important role of the other types of P2 receptor that could be also localized in other cells.

About the modulation of nitroxidergic system the behavioral test performed with TRIM underlined the important role of NO in pain modulation, decreasing drastically both the first and the second phase respect to the formalin group. These data support other observations in which have

been established the involvement of the three isoforms of NOS in inflammatory pain, using also knock-out mice (Boettger et al., 2007; Kolesnikov et al., 2009).

Using the drug PPADS, we showed an alteration of the nitroxidergic system at TG level. In particular we observed an increase of nNOS and iNOS in formalin injected animals especially at 3h with a decreasing trend in 24h. It is noteworthy to underline that the heavy nNOS-immunostained small neurons are primarily involved in nociception. Moreover, we observed, for the first time, a decrease of nNOS and iNOS expression in animals pre-treated with PPADS and injected with formalin. These data support the hypothesis that the inhibition of purinergic transduction signal negatively modulates the nitroxidergic system in TG, especially in small neurons. Some reports suggest that nNOS and iNOS in dorsal root ganglia may participate in the development and/or maintenance of mechanical hypersensitivity after nerve injury (Guan et al., 2007; Martucci et al., 2008) or in inflammatory pain (Boettger et al., 2007). Jansen-Olesen et al. (2005) observed an increase in iNOS expression in trigeminal ganglia cells to the serum free stressful stimulus as a molecular mechanism mediating the adaptive response.

So, ATP released from the damaged peripheral nerve tissue might contribute to initiate neuronal and glial activation, inducing the synthesis and release of NO and also pro-inflammatory cytokines (Inoue, 2006). Consequently, the ATP induced Ca2+ influx via purinergic receptors influencing NOS activity (Shen et al., 2005).

Going up in the central nervous system, we monitored the influence of PPADS peripheral treatment on central neuronal activation (spinal trigeminal nucleus). Many studies have shown that the most caudal part of the trigeminal sensory complex, i.e., Sp5C, is the essential projection site for the nociceptive orofacial inputs (Sessle, 1987). In this specific subnucleus, our data showed an increase in the number of Fos positive neurons, that is a neuronal activation marker, after formalin injection in the ipsilateral part and its decrease with PPADS pre-treatment. In addition, our data showed an increase of Fos positive neurons both in ipsilateral and in contralateral Sp5O. These results suggest a role in nociceptive transmission of perioral area for both the subnuclei and they are

corroborated by other experimental observations reported in literature. First of all, electrophysiological studies performed in rat and monkey (Dallel et al., 1988) indicate that one or several of the three rostral divisions of the trigeminal sensory complex, i.e., the nucleus principalis (Pr5), Sp5O, and Sp5I, may also be involved in the transmission of orofacial pain. The role of Sp5O in intraoral nociception has been demonstrated (Woda et al., 1977; Dallel et al., 1999), and a possible involvement of Sp5C was reported by Watanabe et al. (2002). On the other hand, some works suggest the role of Sp5O in perioral nociceptive mechanisms (Dallel et al., 1999; Luccarini et al., 1998). In fact, several data suggest that the rostral relay for some oral/perioral nociceptive messages is in Sp5O. Sp5O lesions observed in humans or performed in animals induced a significant decrease in the nociceptive sensations or behaviors triggered by intraoral (He et al., 2000) but also perioral noxious stimuli (Pickoff-Matuk et al., 1986). These data suggest a difference in specialization between Sp5O and Sp5C in the processing of the nociceptive message. Sp5O being preferentially involved in transient oral/perioral nociception, while Sp5C may be the only trigeminal structure specialized in sustained nociception (Duale et al., 1996). Other authors reported only a neuronal activation in Sp5C after formalin perioral stimulation but not in the other subnuclei (Nagamatsu et al., 2001; Otahara et al., 2003). In addition in the rat, Pajot et al. (2000) underline the primary role of Sp5C respect of Sp5O in nociceptive transmission, suggesting a specie-specific subnuclear involvement.

In addition, we observed also a general contralateral activation in brainstem. It has been observed also in other studies (Mineta et al., 1995), considering that the mechanisms producing contralateral hyperalgesia are unknown but may be explained by the existence of interneurons connecting neurons of both sides (Koganemaru et al., 2000). There are several reports of trigeminal primary afferent projections to the contralateral subnucleus caudalis (Arvidsson and Gobel, 1981; Jacquin et al., 1990)

In conclusion, our results suggest a key role for the endogenous ATP in peripheral pain processing involving the P2 receptors localized in skin tissue. Moreover, we have demonstrated that the events

subsequent to inflammatory induction involve NO at TG level rapidly, which could modulate the nociceptive pathways in the central nervous system. On the basis of our results, the PPADS could represent a new therapeutic tool for the inflammatory pain.

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Figure legends

Figure 1

Time course of the face-rubbing activity observed after subcutaneous injection of saline (CTR), the highest dose of PPADS (25 mg/Kg), formalin (FORM) or PPADS (6,25 mg/Kg or 12,5 mg/Kg or 25 mg/Kg) and formalin (PPADS+FORM) into the right upper lip. The mean number of seconds that each mouse spent rubbing is plotted for each 3-min block over the 60-min post-injection observation period. Data represent mean ± S.D.; *P<0.05 versus CTR animals; °P<0.05 versus FORM; ‘P<0.05 versus PPADS (6,25 mg/Kg) + FORM animals; ˜P<0.05 versus PPADS (12,5 mg/Kg) + FORM animals.

Figure 2

Time course of the face-rubbing activity observed after subcutaneous injection of saline (CTR), formalin (FORM), the best dose of PPADS (25mg/Kg) and formalin (PPADS+FORM) and (a) A-317491 (30 nmol or 100 nmol or 300 nmol) and formalin (A-317491+FORM) or (b) TRIM (6,25 mg/Kg or 12,5 mg/Kg or 25 mg/Kg) and formalin (TRIM+FORM) into the right upper lip. The mean number of seconds that each mouse spent rubbing is plotted for each 3-min block over the 60-min post-injection observation period. Data represent mean ± S.D.; *P<0.05 versus CTR animals; °P<0.05 versus FORM animals; ‘P<0.05 versus A-317491 (30 nmol) or TRIM (6,25 mg/Kg) + FORM animals; ˜P<0.05 versus A-317491 (100 nmol) + FORM animals.

Figure 3

Time course of (a) nNOS and (b) iNOS immunoreactivity in ipsilateral trigeminal ganglia in saline treated animals (CTR), formalin treated animals (FORM) and PPADS (25 mg/Kg) and formalin treated animals (PPADS+FORM) after 3, 6, 12 and 24h from formalin injection.

Values are mean ± S.D. and represent the IOD; *P<0.05 versus the same neuron group of CTR animals; °P<0.05 versus the same neuron group of FORM animals.

Figure 4

Microphotographs of nNOS (a–c) and iNOS (a'-c') immunoreactivity in TG neurons in control animals (CTR) (a and a'); formalin treated animals (FORM) (b and b') and PPADS and formalin treated animals (PPADS + FORM) (c and c'). Arrows indicate nNOS and iNOS-positive neurons. Bar 30 µm.

Statistical evaluation of nNOS ipsilateral (d) and contralateral (e) and iNOS ipsilateral (d') and contralateral (e') trigeminal ganglia immunoreactivity in CTR, PPADS, FORM and PPADS+FORM animals. Values are mean ± S.D. and represent the IOD; *P<0.05 versus CTR animals; °P<0.05 versus FORM animals.

Figure 5

Microphotographs of Fos-positive neurons in the ipsilateral spinal trigeminal nucleus: (a) subnucleus caudalis (Sp5C) of saline treated animals (CTR), (b) subnucleus caudalis (Sp5C) of formalin treated animals (FORM), (c) subnucleus caudalis (Sp5C) of PPADS and formalin treated animals (PPADS+FORM), (a') subnucleus oralis (Sp5O) of saline treated animals (CTR), (b') subnucleus oralis (Sp5O) of formalin treated animals (FORM), (c') subnucleus oralis (Sp5O) of PPADS and formalin treated animals (PPADS+FORM). Arrows indicate Fos-positive neurons. Bar 50 µm.

Statistical evaluation of Fos immunoreactivity in ipsilateral (d) and contralateral (d') spinal trigeminal nucleus in CTR, PPADS, FORM and PPADS+FORM animals. Values are mean ± S.D. and represent the number of neurons per section; *P<0.05 versus CTR animals; °P<0.05 versus FORM animals.

Figure 6

Statistical evaluation of nNOS (a', b') and iNOS (a'', b'') expression in ipsilateral (a', a'') and contralateral (b', b'') trigeminal ganglia in saline treated animals (CTR), PPADS treated animals (PPADS), formalin treated animals (FORM) and PPADS and formalin treated animals

(PPADS+FORM). Values are mean ± S.D. and represent the IOD; *P<0.05 versus CTR animals; °P<0.05 versus FORM animals.

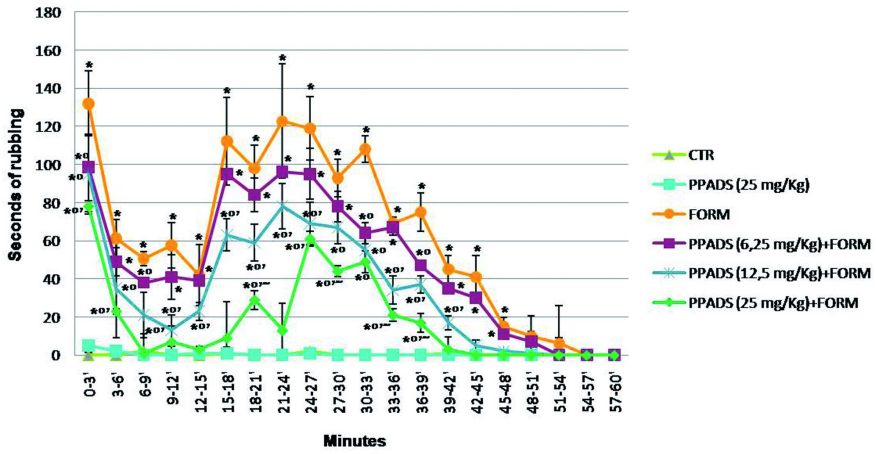

Figure 1
Time course of the face-rubbing activity observed after subcutaneous injection of saline (CTR), best dose of PPADS (25 mg/Kg), formalin (FORM) or PPADS (6,25 mg/Kg or 12,5 mg/Kg or 25 mg/Kg) and formalin (PPADS+FORM) into the right upper lip. The mean number of seconds that each mouse spent rubbing is plotted for each 3-min block over the 60-min post-injection observation period. Data represent mean ± S.D.; *P<0.05 versus CTR animals; °P<0.05 versus FORM; ‘P<0.05 versus PPADS (6,25 mg/Kg) + FORM animals; ˜P<0.05 versus PPADS (12,5 mg/Kg) + FORM animals.
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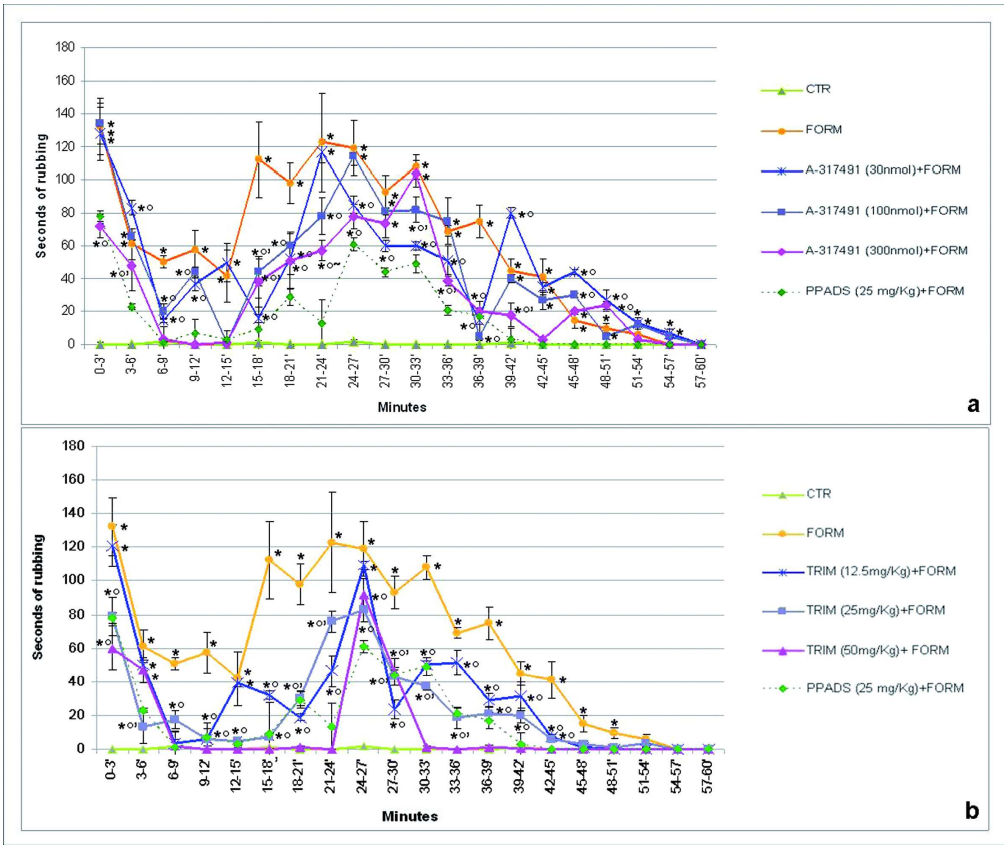

Time course of the face-rubbing activity observed after subcutaneous injection of saline (CTR), formalin (FORM), the best dose of PPADS (25mg/Kg) and formalin (PPADS+FORM) and (a) A-317491 (30 nmol or 100 nmol or 300 nmol) and formalin (A-317491+FORM) or (b) TRIM (6,25 mg/Kg or 12,5 mg/Kg or 25 mg/Kg) and formalin (TRIM+FORM) into the right upper lip. The mean number of seconds that each mouse spent rubbing is plotted for each 3-min block over the 60-min post-injection observation period. Data represent mean ± S.D.; *P<0.05 versus CTR animals; °P<0.05 versus FORM animals; ‘P<0.05 versus A-317491 (30 nmol) or TRIM (6,25 mg/Kg) + FORM animals; ˜P<0.05 versus A-317491 (100 nmol) + FORM animals.
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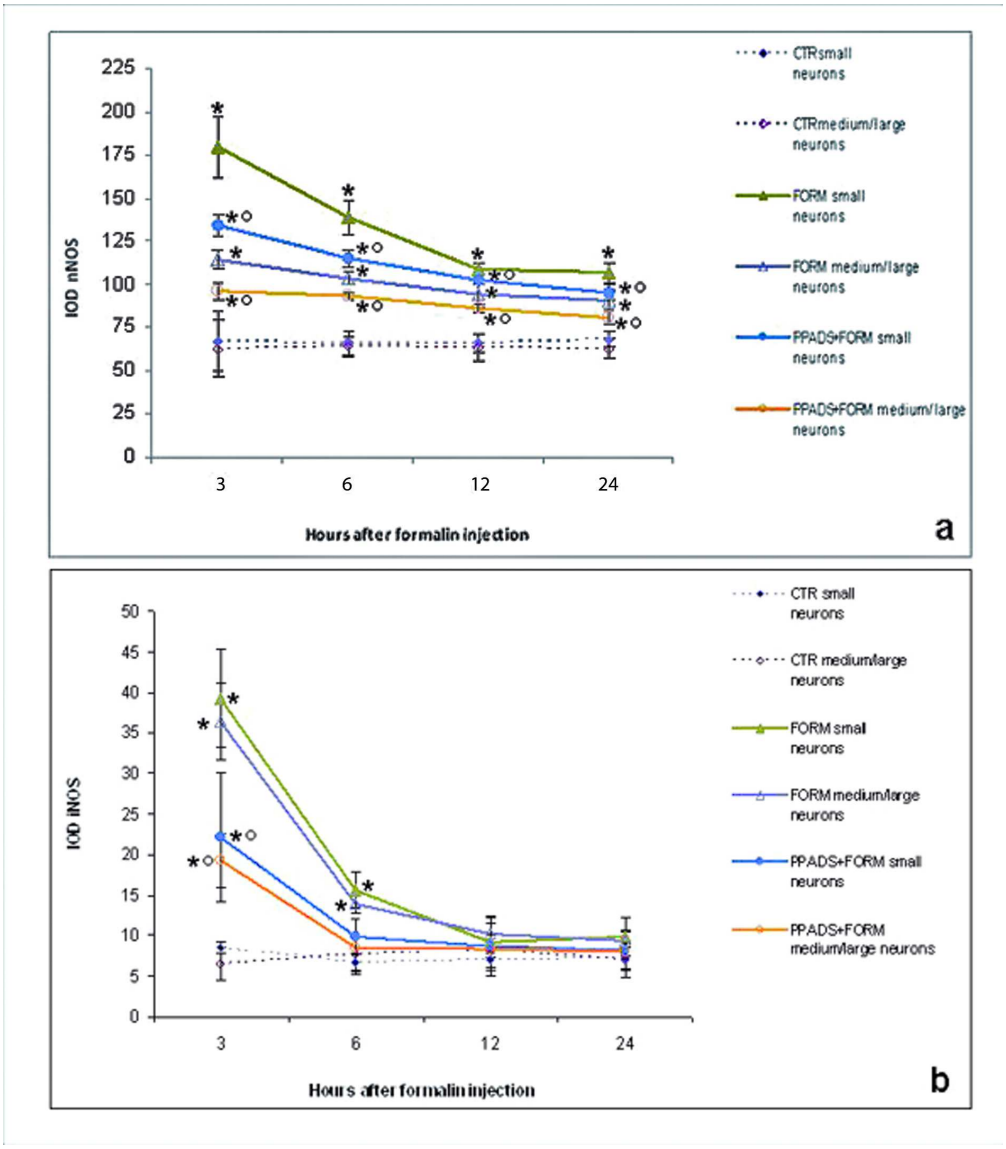

Time course of (a) nNOS and (b) iNOS immunoreactivity in ipsilateral trigeminal ganglia in saline treated animals (CTR), formalin treated animals (FORM) and PPADS (25 mg/Kg) and formalin treated animals (PPADS+FORM) after 3, 6, 12 and 24h from formalin injection.
Values are mean ± S.D. and represent the IOD; *P<0.05 versus the same neuron group of CTR animals; °P<0.05 versus the same neuron group of FORM animals.

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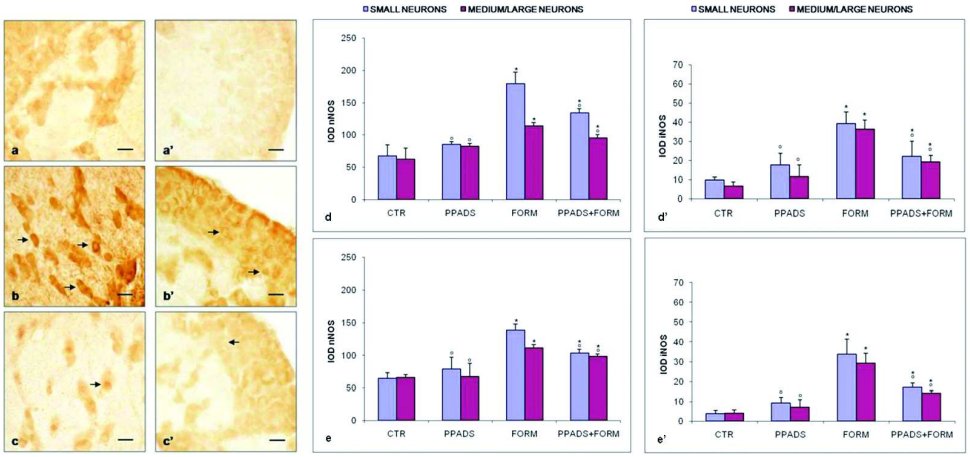

Microphotographs of nNOS (a–c) and iNOS (a'-c') immunoreactivity in TG neurons in control animals (CTR) (a and a'); formalin treated animals (FORM) (b and b') and PPADS and formalin treated animals (PPADS + FORM) (c and c'). Arrows indicate nNOS and iNOS-positive neurons. Bar 30 μm. Statistical evaluation of nNOS ipsilateral (d) and contralateral (e) and iNOS ipsilateral (d') and contralateral (e') trigeminal ganglia immunoreactivity in CTR, PPADS, FORM and PPADS+FORM animals. Values are mean ± S.D. and represent the IOD; *P<0.05 versus CTR animals; °P<0.05 versus FORM animals.

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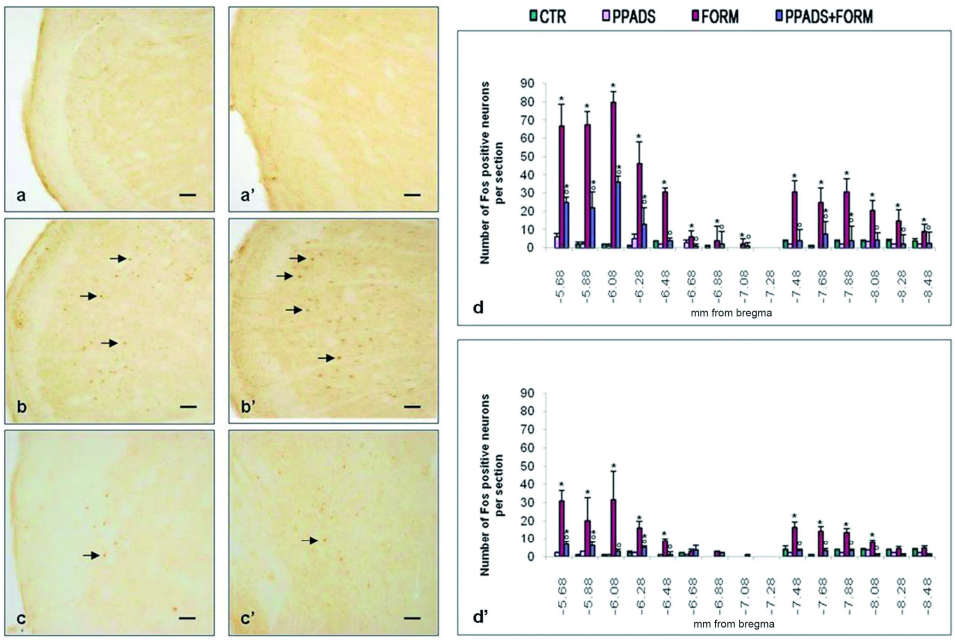

Microphotographs of Fos-positive neurons in the ipsilateral spinal trigeminal nucleus: (a) subnucleus caudalis (Sp5C) of saline treated animals (CTR), (b) subnucleus caudalis (Sp5C) of formalin treated animals (FORM), (c) subnucleus caudalis (Sp5C) of PPADS and formalin treated animals (PPADS+FORM), (a') subnucleus oralis (Sp5O) of saline treated animals (CTR), (b') subnucleus oralis (Sp5O) of formalin treated animals (FORM), (c') subnucleus oralis (Sp5O) of PPADS and formalin treated animals (PPADS+FORM). Arrows indicate Fos-positive neurons. Bar 50 µm. Statistical evaluation of Fos immunoreactivity in ipsilateral (d) and contralateral (d') spinal trigeminal nucleus in CTR, PPADS, FORM and PPADS+FORM animals. Values are mean ± S.D. and represent the number of neurons per section; *P<0.05 versus CTR animals; °P<0.05 versus FORM animals.

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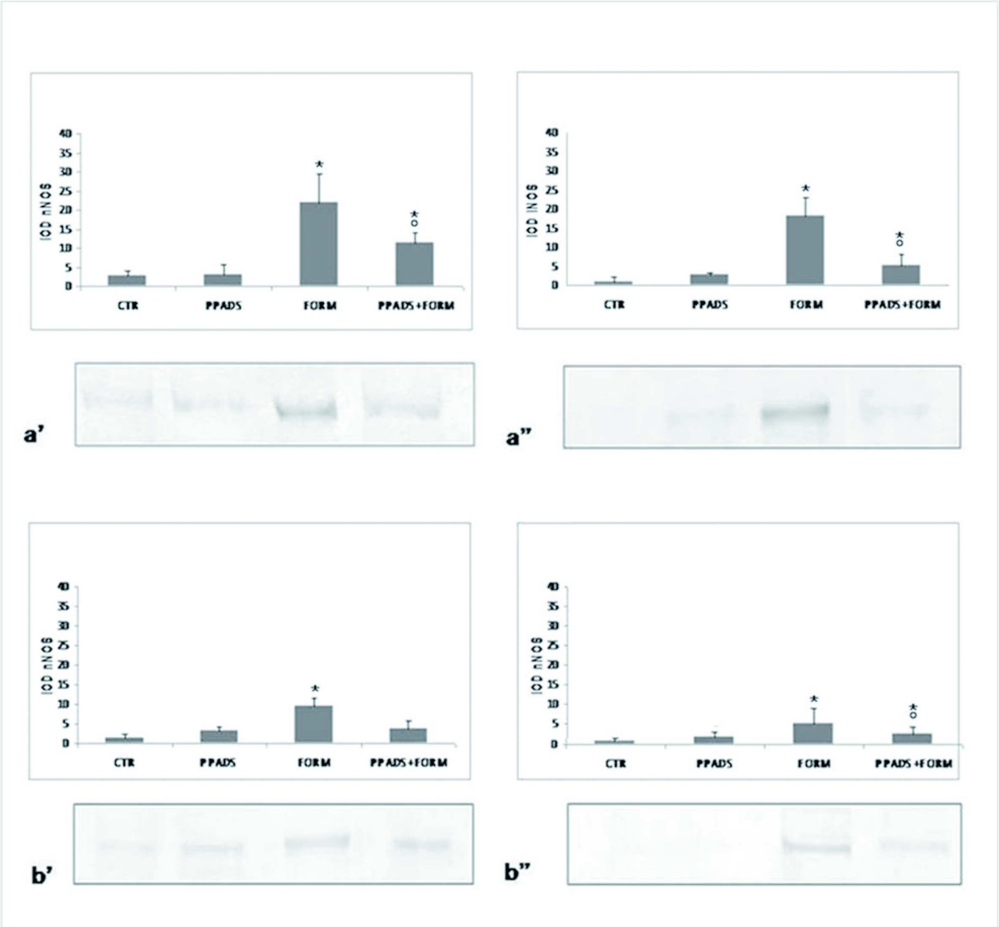

Statistical evaluation of nNOS (a', b') and iNOS (a'', b'') expression in ipsilateral (a', a'') and contralateral (b', b'') trigeminal ganglia in saline treated animals (CTR), PPADS treated animals (PPADS), formalin treated animals (FORM) and PPADS and formalin treated animals (PPADS+FORM). Values are mean ± S.D. and represent the IOD; *P<0.05 versus CTR animals; °P<0.05 versus FORM animals.
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