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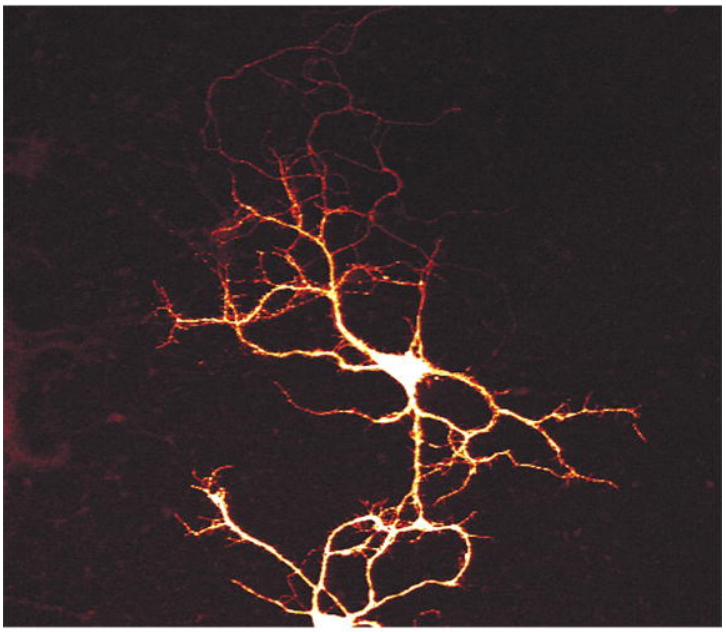

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Research Report

PPADS, a purinergic antagonist reduces Fos expression at spinal cord level in a mouse model of mononeuropathy

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ABSTRACT

Recent evidence suggest that ATP plays a role as an endogenous pain mediator generating and/or modulating pain signaling from the periphery to the spinal cord. In this study we evaluated the effects of intraperitoneal administration of P2 receptor antagonist, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), evaluating pain related behaviours and monitoring the expression of Fos, a marker of activated neurons, in an experimental mouse model of neuropathic pain (sciatic nerve tying). The PPADS administration decreased both tactile allodynia and thermal hyperalgesia in a time and dose dependent manner. The dose of 25 mg/kg PPADS completely reversed nociceptive hypersensitivity. Moreover, non-noxious stimulation induced an increase of Fos positive neurons in the spinal cord of animals with tying of sciatic nerve. PPADS administration partially reversed this increase. These results suggest that PPADS reduces neuronal activation at spinal cord level and that P2 receptors are involved in the retrograde signalling progress exciting sensory spinal neurons.

1. Introduction

The physiological role of ATP-dependent signalling pathway in painful states remains unclear although the ability of ATP to evoke pain has been known for nearly 40 years (Collier et al., 1966). ATP produces its physiological and pharmacological effects via P2X and P2Y receptors. They are purinoreceptors classified into G-protein coupled receptors P2Y and ATP-gated cation channels, so-called P2X receptors. P2X and P2Y receptors, which are localized also in the central and peripheral nervous system, play an important role in excitatory nociceptive processing (Burnstock, 2006; Liu and Salter, 2005) and they can be blocked by specific antagonists and molecules.

ATP is well known to be noxious in humans and animals and its release from the damaged peripheral nerve tissue could contribute to initiate a cascade of neuropathological events that starts at the site of nerve injury and proceeds involving glia, DRG neurons and the spinal cord dorsal horn neurons (Watkins and Maier, 2003). The potential sources of ATP release during sensory transmission in the spinal cord are the central terminals of primary afferent neurons.

Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) is a wide range P2 receptor antagonist (Lambrecht et al., 1992; Lambrecht, 2000). The receptors more sensitive to PPADS are $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_5$. $P2X_1$ receptor is present in dorsal root ganglia, trigeminal ganglia, coeliac ganglia, spinal

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cord, rat brain (Valera et al., 2004; Webb et al., 1996; Collo et al., 1996), urinary bladder, smooth muscle layers of small arteries and arterioles and vas deferens, with lower levels in lung and spleen (Valera et al., 2004; Collo et al., 1996); $P2X_2$ receptor is distributed in bladder, brain, spinal cord, superior cervical ganglia, adrenal medulla, intestine, and vas deferens, with highest levels found in the pituitary gland and vas deferens (Brake et al., 1994), adrenal medulla (Collo et al., 1996); $P2X_3$ receptor is expressed only by a subset of sensory neurons (trigeminal, nodose, and dorsal root ganglia), (Chen et al., 1995; Lewis et al., 1995; Collo et al., 1996); $P2X_5$ receptor is expressed in motoneurons of the ventral horn of the cervical spinal cord, and in neurons of the trigeminal and dorsal root ganglia (Collo et al., 1996).

$P2X_4$ and $P2X_7$ receptors are less sensitive to PPADS with respect to the above cited receptors. Mouse and human $P2X_4$ receptors were found to be sensitivity to PPADS, while rat receptor showed low sensitivity (Jones et al., 2000); $P2X_4$ receptor is expressed in brain, spinal cord, sensory ganglia, superior cervical ganglion, lung, bronchial epithelium, thymus, bladder, acinar cells of the salivary gland, adrenal gland, testis, and vas deferens (Bo et al., 1995; Buell et al., 1996; Collo et al., 1996; Seguela et al., 1996). Rat and human $P2X_7$ receptor was found to have high sensitivity to PPADS, while sensitivity was low in mouse; $P2X_7$ receptor is distributed in brain microglia and astrocytes, presynaptic terminals, bone marrow cells, including granulocytes, monocytes/macrophages and B lymphocytes (Collo et al., 1997; Sim et al., 2004).

Also $P2Y_1$ receptor is sensitive to PPADS (Ralevic and Burnstock, 1998). $P2Y_1$ receptor is expressed in many tissues including sensory neurons, Schwann cells, brain, heart, spleen, lung, liver, skeletal muscle, and kidney (Tokuyama et al., 1995; Mayer et al., 1998; Xiao et al., 2002; Stucky et al., 2004).

Neuropathic pain following peripheral nerve injury; long-term pain associated with inflammation and cancer pain are common and distressing conditions. There is growing recognition of the involvement of purinergic mechanisms in these diseases (Di Virgilio, 2003; Fukuoka and Noguchi, 2002; Kennedy, 2005) acting to develop and maintenance of chronic pain at peripheral level (Fukuoka and Noguchi, 2002). Intraperitoneal ATP induces writhing in mice (Collier et al., 1966; Gyires and Torma, 1984) and when injected into the rat paw causes paw lifting (Bland-Ward and Humphrey, 1997; Tsuda et al., 2000; Tsuda et al., 1999).

Peripheral nerve injury has been shown to regulate the expression of peripheral $P2X_3$ receptor, although the direction of this alteration appears to be dependent by the kind of injury. An increase of the number of $P2X_3$ receptor-immunoreactive DRG neurons was demonstrated after a chronic constriction injury of the sciatic nerve (Novakovic et al., 1999). Accumulation of $P2X_3$ receptor immunoreactivity was observed in nerve endings at the site of injury. This local up-regulation of P2X receptor may be responsible for the development of ectopic purinergic sensitivity at the sites of injury. Indeed, intravenous injection of ATP has been reported to excite afferents in the nerve with chronic constriction injury without affecting nerve fibers on the contralateral side (Chen et al., 1999). These data are supported by studies in knock out mice for $P2X_3$ (Cockayne et al., 2000; Souslova et al., 2000). $P2X_3$ receptor was found to be selectively expressed at high levels in nociceptive sensory neurons (Chen et al., 1995; Lewis et al., 1995) indicating its involvement in chronic conditions, particularly in chronic inflammatory and neuropathic pain (Jarvis et al., 2002). $P2X_4$ receptor on microglia has also been recognised to be involved in neuropathic pain (Inoue, 2006). In addition, disruption of $P2X_7$ receptor gene has been shown to abolish chronic

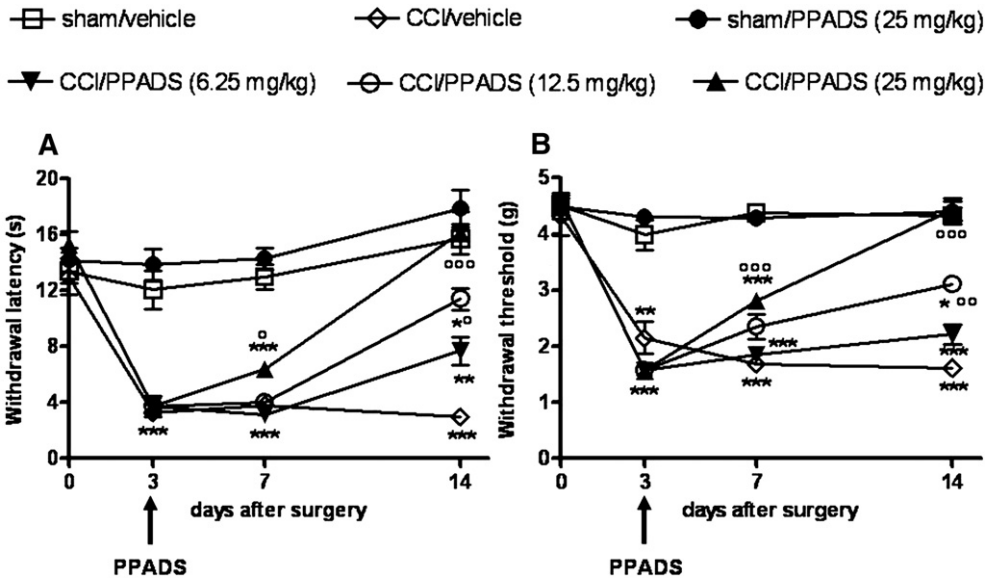

Fig. 1 – Time course of the effect of different doses of PPADS administered daily to neuropathic mice (CCI) beginning from third day after injury, on thermal hyperalgesia (A) and mechanical allodynia (B). A: Withdrawal latency to heat, measured by Plantar test, and B: mechanical threshold of the injured paws, measured by Dynamic Plantar Aesthesiometer, are expressed as s (seconds) and g (grams), respectively. Data represent mean ± SEM of 15–18 mice. *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus sham/vehicle mice; °$p<0.05$, °°$p<0.01$, °°°$p<0.001$ versus CCI/vehicle mice.

inflammatory and neuropathic pain (Chessell et al., 2005). Other works underlined that purinergic sensitivity develops in sensory neurons, after chronic peripheral nerve injury (Zhou et al., 2001). Moreover, other studies (Liu and Tracey, 2000; Stanfa et al., 2000) using intrathecal administration of PPADS showed that peripheral sites are unlikely to have been accessed by the antagonist given by this route. The results showed a lack of antiallodynic effect of the antagonist intrathecally administered after peripheral nerve injury. It implies that spinal P2 receptors play little role in the maintenance of this form of neuropathic pain.

Most of these papers reported the data about $P2X_3$ or $P2X_{2/3}$ receptor, since only recently a role of P2Y receptors in nociception was considered. It is noteworthy that $P2Y_1$ and $P2Y_2$ receptors were found in sensory neurons including nociceptors (Molliver et al., 2002; Xiao et al., 2002; Stucky et al., 2004) and in Schwann cells (Mayer et al., 1998). In addition, in cell culture, PPADS inhibited P2Y activation that is involved in ATP-induced pain (Lakshmi and Joshi, 2005).

The spinal cord is a complex structure with a great number of interactions between neuron–neuron and neuron–glia. In this context the identification of specific neurons involved in pain modulation is an important tool to better define and clarify the spinal cord areas activated after different kind of stimulation. For this purpose, the use of Fos as a morphological marker was useful to identify activated neurons (Bullitt, 1990; Harris, 1998; Rodella et al., 1998) and permitted us to localize the specific spinal cord areas involved in pain modulation. Since ATP released from the damaged peripheral nerve tissue might contribute to initiate neuron and glia activation at peripheral level and trigger of a series of neurochemical events affecting spinal cord pain transmission, we investigated the potential role of purinoceptors as a target of pharmacological treatment of neuropathic pain using both behavioural approach and monitoring Fos expression.

2. Results

2.1. *PPADS effect on thermal hyperalgesia and mechanical allodynia*

The evaluation of nociceptive hypersensitivity was performed at 0, 3, 7, 14 days after surgery and 24 h after last PPADS/saline administration in order to avoid acute interference.

CCI mice showed a significant decrease of withdrawal latency and withdrawal threshold with respect to the sham-operated animals. This effect was present only in the paw ipsilateral to the injury and starting at 3 days after the surgery up to the end of the experiment. Seven days after injury, only the animals treated with 25 mg/kg PPADS (administration starting from 3 days from the lesion), significantly attenuated both thermal hyperalgesia and mechanical hypersensitivity. Fourteen days after injury the values became not statistically different from the sham-operated animals presenting a physiological threshold. The other PPADS doses were effective after prolonged administration and the effect was dose-related on both thermal (r^2: 0.6221, $p<0.01$) and mechanical (r^2: 0.8812, $p<0.001$) nociceptive hypersensitivity. There was no effect of 25 mg/kg PPADS repeated administration on nociceptive response of sham animals. The data are summarized in Fig. 1.

In the experiment in which we treated the animals only with a single dose of PPADS (25 mg/kg) on the 14th day, the PPADS administration reversed mechanical allodynia (Fig. 2B), measured after 1 h but not after 24 h from the treatment, without affecting thermal hyperalgesia at any time (Fig. 2A).

2.2. *Evaluation of Fos expression*

Fos immunoreactivity appeared as a brown staining in the nucleus of the neurons. In the NAIVE non-stimulated/stimulated groups and treated with vehicle or PPADS (25 mg/kg) the number of Fos positive neurons in laminae I–II and III–VI was very low without any significant difference between the different groups of laminae.

The Fos distribution pattern shown in the sham groups both at 7th and 14th day was similar to the NAIVE.

In chronic constriction injured/non-stimulated and vehicle treated animals we observed a slight but significant increase of Fos positive neurons in laminae I–II, while in laminae III–VI

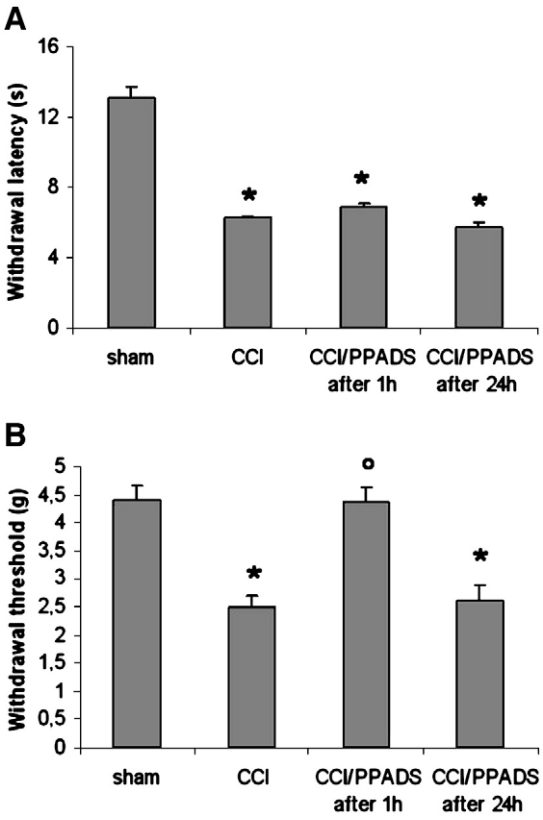

Fig. 2 – Effect of acute administration of PPADS 25 mg/kg to neuropathic mice (CCI) at 14th day on thermal hyperalgesia (A) and mechanical allodynia (B) after 1 h or 24 h. CCI and sham mice were acutely injected with saline. A: Withdrawal latency to heat, measured by Plantar test, and B: mechanical threshold of the injured paws, measured by Dynamic Plantar Aesthesiometer, are expressed as s (seconds) and g (grams), respectively. Data represent mean ± SEM. *$p<0.001$ versus sham mice; °$p<0.001$ versus CCI mice.

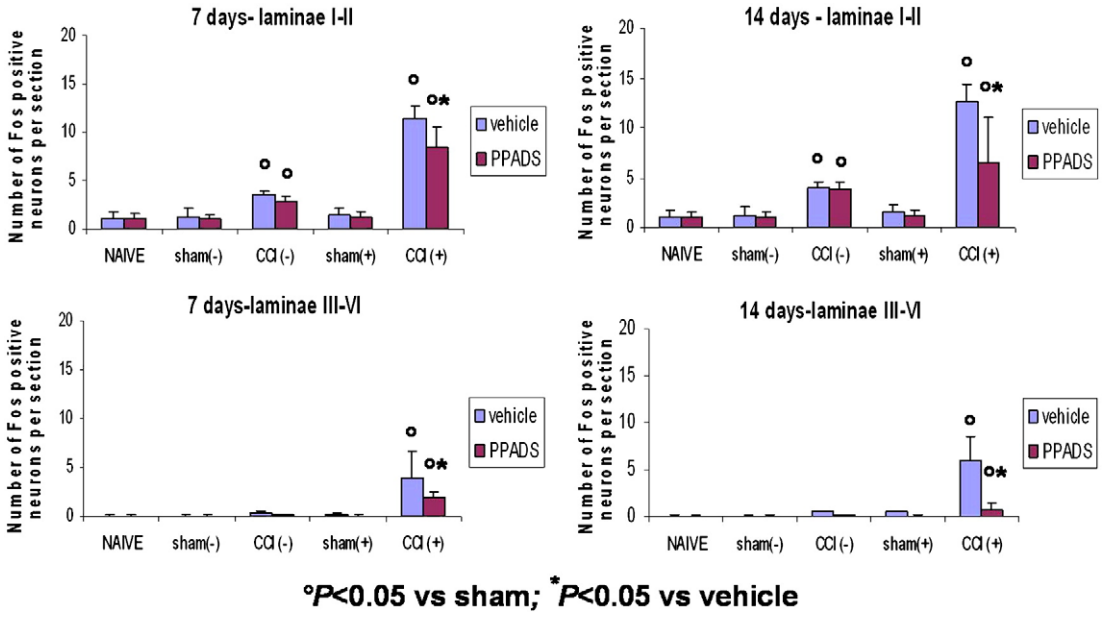

Fig. 3 – Statistical evaluation of Fos immunoreactivity in L4–L6 spinal cord laminae I–II and III–VI in NAIVE, sham and rats with chronic constriction injury (CCI) on 7th and 14th postoperative day. NAIVE ($n=3$): non-operated animals without stimulation; sham (–) ($n=3$): sham operated animals without stimulation; sham (+) ($n=3$): sham operated animals with stimulation; CCI (–) ($n=5$): animals with tied nerve without stimulation; CCI (+) ($n=3$): animals with tied nerve and stimulation. Values are mean and represent the number of neurons per section ± S.D.; °$p<0.05$ versus NAIVE, *$p<0.05$ with versus CCI (–).

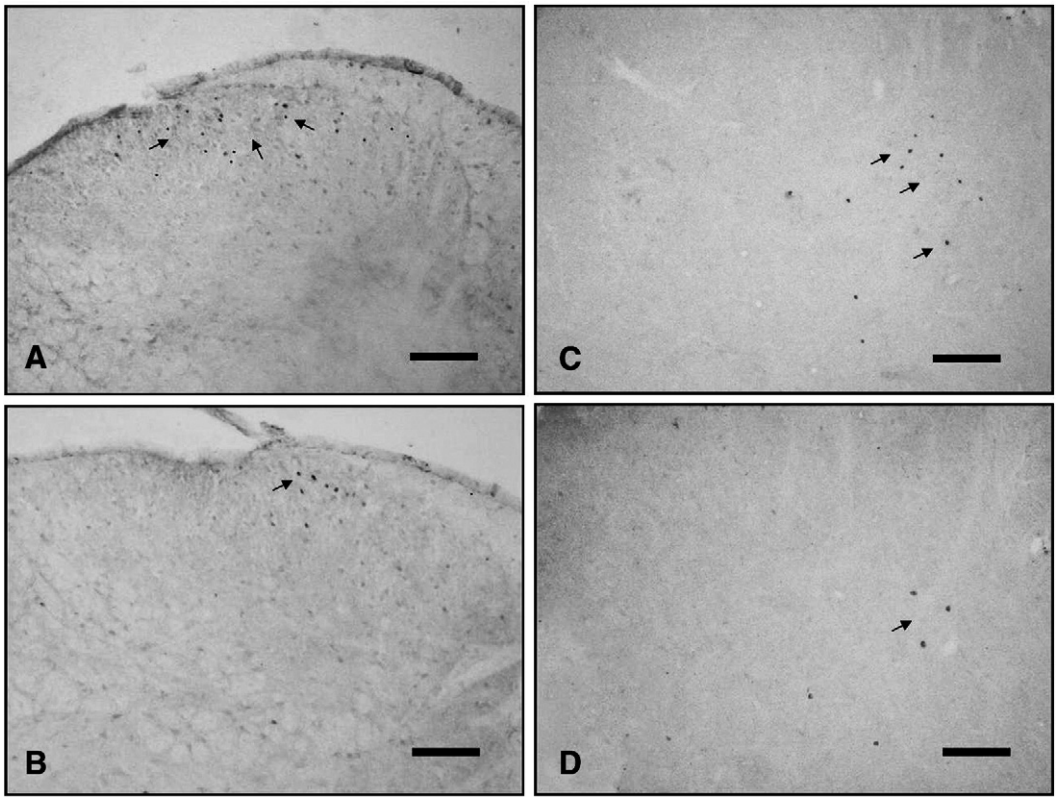

Fig. 4 – Fos-positive neurons in the spinal cord (L5 level) of tied and stimulated animals (CCI+): (A) laminae I–II of vehicle treated animals, (B) laminae I–II of PPADS treated animals, (C) laminae III–VI of vehicle treated animals, (D) laminae III–VI of PPADS treated animals. Arrows indicate Fos-positive neurons. Bar 50 μm.

there was a slight non-significant increase of the number of Fos positive neurons compared to the NAIVE and sham groups. Moreover, we did not observe significant difference between vehicle and PPADS (25 mg/kg) treated animals from the third day after surgery both at 7th and 14th day. In the chronic constriction injured/stimulated and vehicle treated animals the number of Fos positive neurons significantly increased compared to the NAIVE, mainly in the laminae I–II and slightly in laminae III–VI.

In chronic constriction injured/stimulated and vehicle treated animals we observed no significant difference of Fos positive neurons in laminae I–II and III–VI compared to the chronic constriction injured/stimulated and vehicle treated animals both to 7th and 14th day. In chronic constriction injured/stimulated and PPADS (25 mg/kg) treated animals from the third day after surgery, at 7th day we observed a significant decrease of Fos positive neurons in laminae I–II and III–VI compared to the chronic constriction injured/stimulated and vehicle injected animals. After 14 days we observed an additional decrease of number of Fos positive neurons. The data about the distribution of the labelled cells in the spinal cord in the different groups and laminae are summarized in Figs. 3 and 4.

In chronic constriction injured groups, the positive neurons were found mainly ipsilaterally from L4 to L6 spinal segments.

3. Discussion

The results of this study suggest the involvement of P2 receptors in chronic constriction injured mice, underlying the ability of P2 receptor antagonist PPADS to decrease spinal cord neurons activation.

We have analyzed the activation of sensitive spinal dorsal horn neurons after PPADS treatment in a mice model of neuropathy using Fos immunohistochemistry, showing that PPADS treatment reduced the number of Fos positive neurons in this area. These results are corroborated by behavioral data showing the ability of PPADS to inhibit thermal hyperalgesia and mechanical allodynia in a dose dependent manner. In addition we showed that the chronic administration of PPADS produced a long lasting effect with respect to the single dose.

Our results, about the distribution of Fos positive neurons in mice, agree with the data in rats using the same model (Kosai et al., 2001). Moreover, according to Harris (1998), the same non-noxious stimulation did not induce Fos expression in laminae I–II in intact rats.

The induction of Fos expression in the spinal cord could be explained by the fact that myelinated primary afferents conducting non-noxious stimuli activated the spinal neurons, as suggested by other authors (Catheline et al., 1999; Nakamura and Myers, 1999; Woolf et al., 1992).

In chronic constriction injured/stimulated and PPADS injected animals we observed a significant decrease of Fos expression, especially in laminae I–II, with respect to the vehicle treated group. CCI induces a peripheral and central release of NO and anti/proinflammatory cytokines from Schwann cells, macrophages and activated glia or microglia (Ledeboer et al., 2005). So, ATP released from the damaged peripheral nerve tissue might contribute to initiate neuronal and glial activation, inducing the synthesis and release of proinflammatory cytokines and NO (Créange et al., 1997; Levy et al., 1999). These results agree with the findings showing the ability of PPADS, administered systemically or locally, to reduce evoked Fos expression at spinal cord level in a rat model of postoperative pain (Tsuda et al., 2001). We can suppose that PPADS-sensitive P2 receptors located in the injured area may be the major target site of PPADS in producing the antiallodynic effect. These considerations support recent data demonstrating that suramin and PPADS have a low effect on the primary fibers-evoked responses of the dorsal horn neurons in normal and neuropathic rats (Stanfa et al., 2000). The involvement of extra-cellular ATP and P2 receptors in neuropathic pain has been reported in different models and several cellular systems (Inoue, 2006) and PPADS is known to affect, although with different affinity, both P2Y and P2X receptors and their subtypes (Ralevic and Burnstock, 1998). Neuropathic pain models and sciatic nerve transection cause modification in P2 receptor expression at DRG and/or spinal cord level. In fact, it has been shown with an up-regulation in neuropathic models of P2Y1 and P2X3 both at DRG (Xiao et al., 2002; Novakovic et al., 1999; Tsuzuki et al., 2001) and at spinal cord level (Yang et al., 2004; Novakovic et al., 1999; Tsuzuki et al., 2001). Moreover, the disruption of the $P2X_7$ receptor gene abolishes chronic inflammatory and neuropathic pain that is upregulated in chronic neuropathic pain patients (Chessell et al., 2005). Finally, these observations suggest a role of endogenous ATP in neuropathic pain syndromes, supporting previous proposal that ATP leaked from the damaged cells induces the tissue injury-evoked pain (Burnstock, 2000; Ralevic and Burnstock, 1998; Tsuda et al., 2000).

4. Experimental procedures

4.1. *Animals*

Experiments were carried out on 60 C57BL/6J male mice (20–25 g 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 efforts were made to minimize animal suffering and the number of animals used. The experimental procedures were approved by the Italian Ministry of Health.

The animals were subdivided into 3 surgical groups: in the first group (24 animals) the right sciatic nerve was tied producing a chronic constriction injury (CCI); the second group (24 animals) was the sham operated animals and the third group (12 animals) was the control non-operated animals (NAIVE).

4.2. *Surgical procedure*

The chronic constriction injury (CCI) model was originally described by Bennett and Xie (1988) for rats and was adapted for mice in this study. Animals were anaesthetized with sodium pentobarbitone (60 mg/kg, intraperitoneal (i.p.), 0.1 ml/10 g) and the right sciatic nerve was exposed at the level of the mid-thigh by blunt dissection and separated from the adhering tissue immediately proximal to its trifurcation. Three ligatures were loosely tied around the nerve at about 1 mm distance using 4-0 chromic gut suture material until they elicited a brief twitch in the respective hind, taking care to preserve epineural circulation. In this animal model of chronic pain the ligatures were

placed around the sciatic nerve causing a constriction without damaging nerve fibres and so transporting their inputs into the laminae I–IV of the dorsal horn of the spinal cord (Catheline et al., 1999; Hunt et al., 1987). Sham-operated animals (sciatic exposure without ligation) were used as controls.

4.3. *Drug treatment*

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 (0.05 ml/10 g). Doses were chosen according to those employed by Gourine et al. (2005). PPADS or saline were administered i.p. to neuropathic and sham-operated mice once a day for 11 days, starting from the third day after surgery.

In addition, in a separate experiment, we tested the behavioural effect in neuropathic mice of a single dose of PPADS (25 mg/kg) after 1 h and 24 h from the administration.

4.4. *Thermal hyperalgesia and mechanical allodynia*

Responses to thermal and mechanical stimuli were measured before and 3, 7 and 14 days after the surgical procedure for the PPADS chronic treatment. Measurements were performed on both the ipsilateral and contralateral hind paws of all mice.

For the acute treatment, 6 mice were sciatic nerve tied as previously described and 3 mice were sham operated. At 14th day, a single dose of PPADS were administered and after 1 h and 24 h the behavioural tests were performed.

Thermal hyperalgesia was assessed using the Hargreave's test (Ugo Basile, Comerio, Italy) (Hargreaves et al., 1988). Animals were placed in an elevated transparent box and the plantar surface of the hindpaw exposed to a beam of radiant heat (beam diameter 0.5 cm and intensity 20 I.R.). As the mouse withdraws its paw, the sudden drop in reflected energy stops the timer. Withdrawal latency is recorded in seconds.

Mechanical allodynia was assessed using a dynamic plantar aesthesiometer (Ugo Basile, Comerio, Italy), an automated version of the von Frey hair assessment. Animals were placed on an elevated wire mesh bottomed cage and the rigid tip of a von Frey-filament (punctate stimulus) was applied to the skin of the midplantar area of the hind paw. It begins to exert an upwards force, ranging up to 5 g in 20 s, until the paw is withdrawn. The force required to elicit a withdrawal responses is measured in grams. Withdrawal threshold of ipsilateral and contralateral paws was measured four times and the value was the mean of the four evaluations.

The data of all animals were analyzed and compared by repeated measures ANOVA (analysis of variance) with Tukey's post test.

4.5. *Fos immunohistochemistry*

Fos expression was evaluated at 7th and 14th days after surgery. The PPADS dose used was of 25 mg/kg/day which gave the best performance in behavioural tests. The CCI and sham animals were subdivided in two subgroups: stimulated and non-stimulated animals. The induction of Fos positive neurons by non-noxious stimulation in chronic constriction injured, sham and NAIVE mice was evaluated according to Kosai et al. (2001). The stimulus was elicited by gently rubbing the plantar surface using a piece of leather glove while the animals were under anaesthesia with sodium pentobarbitone (60 mg/kg i.p.). All mice of the stimulated groups received the non-noxious stimulus three times a minute every 30 s on the 14th post-operative day. 1.5 h after the stimulation the animals were anaesthetised with sodium pentobarbitone (60 mg/kg i.p.) and transcardically perfused with saline followed by 40 ml of 4% paraformaldehyde in phosphate buffer 0.1 M pH 7.4. After fixation, the lumbar spinal cord (L4–L6) of each animal was removed, post-fixed in 4% paraformaldehyde in phosphate buffer for 2 h and cryoprotected overnight in 30% sucrose at 4 °C. We identified the lumbar spinal cord segments by the presence of the lumbar enlargement. The lumbar L4–L6 segments was determined in each case with the spinal cord in situ using a dissecting microscope and measuring the distance between the points of entry of the most rostral and the most caudal rootlets of the L4, L5 and L6 dorsal root. Frozen serial transverse sections (15 μm thick) of all the L4–L6 segments were placed on glass slides, dried and processed for Fos immunohistochemistry. Briefly, the first series of sections was incubated in normal goat serum (10% in phosphate-buffered saline containing 0.1% Triton X-100) for 30 min and then incubated in rabbit polyclonal primary antiserum directed against Fos (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 in phosphate-buffered saline 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 in the ipsilateral dorsal horn of all animals was charted using a light microscope equipped an image analyzer (Immagini e Computer, Milano, Italy). The number of the labelled cells in the lumbar spinal laminae, conventionally grouped into two groups (I–II and III–VI), was evaluated using a quantitative method by researchers unaware of the animal group assignment. The data of all animals were analyzed and compared by repeated measures ANOVA and by a Bonferroni multiple comparison test.

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