A2A adenosine receptor regulates glia proliferation and pain after peripheral nerve injury

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Abstract

Peripheral nerve injury produces a persistent neuropathic pain state characterized by spontaneous pain, allodynia and hyperalgesia. In this study, we evaluated the possible involvement of A2ARs in the development of neuropathic pain and the expression of microglia and astrocytes in the spinal cord after sciatic nerve injury. For this purpose, partial ligation of the sciatic nerve was performed in A2A knockout mice and wild-type littermates. The development of mechanical and thermal allodynia, as well as thermal hyperalgesia was evaluated by using the von Frey filament model, the cold-plate test and the plantar test, respectively. In wild-type animals, sciatic nerve injury led to a neuropathic pain syndrome that was revealed in these three nociceptive behavioural tests. However, a significant decrease of the mechanical allodynia and a suppression of thermal hyperalgesia and allodynia were observed in A2AR deficient mice. The expression of microglia and astrocytes was enhanced in wild-type mice exposed to sciatic nerve injury and this response was attenuated in knockout animals. Taken together, our results demonstrate the involvement of A2ARs in the control of neuropathic pain and propose this receptor as an interesting target for the development of new drugs for the management of this clinical syndrome.

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1. Introduction

Neuropathic pain can be caused by primary lesion or dysfunction in the nervous system and is associated with the appearance of abnormal sensory signs, such as alldynia (pain as a result of a stimulus which does not normally produce pain), hyperalgesia (an increased response to a stimulus which is normally painful) or spontaneous pain [3,8]. The clinical manifestations of neuropathic pain are difficult to treat even with potent analgesic compounds [2].

The purinergic system plays a relevant physiological role in the control of pain at the central and peripheral level [39,40]. The purine nucleoside adenosine is an ubiquitous endogenous neurotransmitter [18] which acts on four G-protein coupled receptors A1R, A2AR, A2BR and A3R [21], and the endogenous ligand adenosine can enhance or decrease nociception depending on the receptor subtype activated [41]. Genetic and pharmacological studies have shown that the stimulation of A2AR is pronociceptive while the stimulation of A1R is
thought to be antinociceptive. Indeed, mice lacking A2AR exhibit an increased nociceptive threshold after direct stimulation of peripheral sensory nerves or an inflammatory reaction [23,33]. Pharmacological studies, using A2AR ligands also demonstrated their role in the control of nociception. The local administration, into the hindpaw, of the adenosine A2AR agonist, CGS 21680 produced mechanical hyperalgesia in rats [29] and the systemic administration of the A2AR antagonist SCH 58261 attenuated the nociceptive responses in both acute and inflammatory tests in mice [23,27], suggesting a relevant role of adenosine A2ARs in peripheral nociceptive pathways. However, few data are available on the implication of the A2ARs in neuropathic pain.

Microglia, the resident macrophages of the central nervous system, modulates neuronal changes occurring during the development and maintenance of several chronic pain states, including neuropathic pain [47,48]. Microglia respond quickly to peripheral nerve injury and releases several cytokines, which are known to produce neuron sensitization in the spinal cord [17]. Microglia express A1Rs, A2ARs, and A3Rs, but not A2BRs [25]. Although the proliferation and/or apoptosis of microglia are regulated by several adenosine receptors, the secretory activity of these cells appears to be stimulated by A2AR [17,20]. Moreover, A2AR stimulation enhances the proliferation and activation of astrocytes secondary to a nervous injury [7,26], while the A2AR antagonists 1,3-dipropyl-7-methylxanthine (DPMX) and SCH58261 have opposite effects [7].

In order to clarify the role of A2ARs in the modulation of neuropathic pain, we have used knockout mice deficient in A2AR [33] to evaluate the consequences of this deletion, in the development and expression of pain after sciatic nerve injury. We have also investigated the expression of microglia and astrocytes after sciatic nerve ligation in these A2AR mutant mice.

2. Materials and methods

2.1. Animals

Mice lacking A2ARs were generated as previously reported [33]. In order to homogenize the genetic background of the mice, the first generation heterozygotes were backcrossed for 30 generations on a CD1 background (Charles River, France) with selection for the mutant A2AR gene at each generation. Heterozygote/heterozygote mating produced wild-type and knockout littermates for subsequent experiments. Breeding couples were periodically renovated by crossing heterozygote mice with wild-type CD1 females (Charles River, France) in order to maintain a genetically diverse outbred background. A2AR knockout mice and wild-type littermates (30–35 g) were housed five per cage in temperature (21 ± 1 °C) and humidity (55 ± 10%) controlled rooms, with a 12-h light/12-h dark cycle (light between 8:00 AM and 8:00 PM). Food and water were available ad libitum. Mice were handled for one week before starting the experiments. Animal procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the local Ethical Committee (CEEA-PRBB). All efforts were made to minimize the number of mice used and the suffering. All experiments were performed under blind conditions.

2.2. Surgery

A partial ligation of the sciatic nerve at mid-thigh level was used to induce neuropathic pain, as previously described [36,10]. Briefly, mice were anaesthetized with isoflurane (induction, 5%; surgery, 2%) and the common sciatic nerve was exposed at the level of the mid-thigh of the right hind paw. At ~1 cm proximally to the nerve trifurcation, a tight ligature was created around 33–50% of the sciatic nerve using 9–0 18-inch non-absorbable virgin silk suture (Alcon® surgical, TX, USA), leaving the rest of the nerve ‘undamaged’. The muscle was then stitched, and the incision was closed with wound clips. Control animals (sham-operated mice) underwent the same surgical procedure except that the sciatic nerve was not ligated.

2.3. Nociceptive behavioural tests

Hyperalgesia to noxious thermal stimulus and allodynia to cold and mechanical stimuli were used as outcome measures of neuropathic pain by using the following behavioural models.

Thermal hyperalgesia was assessed, in the plantar test (Ugo Basile, Varese, Italy) as previously reported [10,24,32] by measuring paw withdrawal latency in response to radiant heat. A cut-off time of 20 s was used to prevent tissue damage in the absence of a response. The mean paw withdrawal latencies for the ipsilateral and contralateral hind paws, respectively, were determined from the average of three separate trials, taken at 5-min intervals to prevent thermal sensitization and behavioural disturbances.

Mechanical allodynia was quantified by measuring the hind paw withdrawal response to von Frey filament stimulation, as previously reported [10,11]. The filament of 0.4 g was used at first. Then, the strength of the next filament was decreased when the animal responded or increased when the animal did not respond. This up-down procedure was stopped four measures after the first change in animal responding. The threshold of response was calculated by using the up-down Excel program generously provided by Basbaum’s laboratory (UCSF, San Francisco, USA). Clear paw withdrawal, shaking or licking was considered as a nociceptive-like
response. Both ipsilateral and contralateral hind paws were tested.

Thermal alldynia to cold stimulus was assessed by using the hot/cold-plate analgesia meter (Columbus, OH, USA), as previously described [5,10]. The number of elevations of each hind paw was recorded in the mice exposed to the cold plate (5 ± 0.5 °C) for 5 min. A score was calculated for each animal as the difference of number of elevations between ipsilateral and contralateral paw.

2.4. Experimental protocol

Animals were first habituated for one hour to each different experimental test once daily for 4 days. After the habituation period, baseline responses were established during 2 consecutive days for each paradigm in the following sequence: von Frey model, plantar test (30 min later) and cold-plate test (15 min later). All the behavioural tests were performed on the same group of animals. One day after baseline measurements, sciatic nerve injury was induced. A2A knockout mice and their wild-type littermates were tested in each paradigm on the same group (30 min later) and cold-plate test (15 min later). All the different experimental test once daily for 4 days. After the surgical procedure using the same experimental sequence as for baseline responses. In the time course study, the days chosen for the behavioural measurements were 3, 7 and 17. In each of these days, different animals of each group (3–4 per group) were sacrificed and the spinal cords were removed, frozen and preserved for the immunohistological studies without previous perfusion.

2.5. Histochemistry and immunohistochemistry

Microglia and astrocyes expression were evaluated by using rabbit polyclonal antibodies Iba1 (Wako, Pure Chemical Industries, Ltd., Japan) and anti-glial fibrillary acid protein (GFAP, DakoCytomation, Denmark) staining, respectively. After removal, the spinal cords were embedded in optimal cutting temperature compound (O.C.T.) and frozen at −80 °C. Spinal cords were cut in 25 μm sections in cryostat and mounted in Star frost slides. The tissue was fixed with PFA 4% for 20 min. After three washes in PB 0.1 M, the slides were incubated for 2 h in blocking solution containing 3% normal goat serum and 0.3% Triton X-100 in 0.15 M phosphate buffer (NGS-T-PB). Primary antibodies recognizing Iba1 (1:125) and GFAP (1:750) were diluted in the blocking solution and incubated overnight at 4 °C. Sections were washed three times in 0.1 M PB and incubated for 1 h in a goat anti-rabbit biotinylated antibody (Vector Laboratories, Burlingame, CA) with a 1/500 dilution in NGS-T-PB. After being washed three times in PB 0.1 and two times in Tris buffer (0.1 M, pH 7.6). Finally, sections were incubated for 2 min in a Tris buffer solution containing 0.5% 3,3′-diaminobenzidine (DAB; Sigma, Madrid, Spain), and developed by H2O2 addition.

For the GFAP and Iba1 staining, the results are expressed as mean of a minimum of 9 lumbar sections counts, 3–4 per animal. Sections were examined and recorded with a Leica DMR microscope equipped with digital camera Leica DFC 300 FX and analysed with Leica Qwin V3 software measuring the percentage of stained occupied area over the specific region of interest (within 348 × 260 pixels rectangle on the dorsal horn, lamina I–V).

2.6. Statistical analysis

Data obtained in the plantar test, cold plate test and von Frey filament stimulation were compared each experimental day by using two-way ANOVA (genotype and treatment as between factors of variation), followed by a one-way ANOVA when required. The same sequence was followed to analyse the astrocytes and microglia expression. The differences between means were considered statistically significant when the P value was below 0.05. SPSS statistical package was used.

3. Results

3.1. Development of neuropathic pain in A2AR knockout mice and wild-type littermates

3.1.1. von Frey filament stimulation model (mechanical allodynia)

Sciatic nerve injury led to a profound decrease of the threshold for evoking withdrawal at the hind lateral paw to a mechanical stimulus in both genotypes, but this response was significantly attenuated in A2AR knockout mice (Fig. 1A). Baseline values were similar in both genotypes. Sham operation did not produce any modification of nociceptive responses in wild-type or in knockout mice. Nerve injury in the wild-type mice led to a significant decrease of the threshold for evoking hind paw withdrawal to mechanical stimulation on the injured side. This mechanical allodynia appeared on the first measurement after surgery (day 3) and persisted for the whole duration of the experiment. Indeed, a significant effect of nerve injury was revealed on day 3 (P < 0.001), day 6 (P < 0.001), day 8 (P < 0.001), day 10 (P < 0.001), day 13 (P < 0.001), day 15 (P < 0.001) and day 17 (P < 0.001) after surgery (one-way ANOVA vs. sham-operated). A2AR knockout mice exposed to sciatric nerve injury also showed significant allodynia on day 3 (P < 0.001), day 6 (P < 0.001), day 8 (P < 0.001), day 10 (P < 0.001), day 13 (P < 0.01), day 15 (P < 0.01)
and day 17 ($P < 0.05$) after surgery (one-way ANOVA vs. sham-operated animals). However, a significant decrease of mechanical allodynia was observed in A2AR knockout mice on day 3 ($P < 0.01$), day 6 ($P < 0.01$), day 8 ($P < 0.001$), day 10 ($P < 0.01$), day 13 ($P < 0.001$), day 15 ($P < 0.001$) and day 17 ($P < 0.001$) when compared to the wild-type group (one-way ANOVA). Withdrawal latencies of the contralateral paw were not modified in any experimental group (Fig. 1B).

3.1.2. Plantar test (thermal hyperalgesia)

Sciatic nerve ligation decreased paw withdrawal latency to thermal stimulus only in wild-type mice and this response was abolished in mice lacking A2AR (Fig. 2A). Baseline values were similar in both genotypes. Sham operation did not produce any modification of nociceptive responses in wild-type or in knockout mice. A marked and long-lasting decrease of the paw withdrawal latencies was observed in the ipsilateral paw of wild-type mice exposed to sciatic nerve injury from day 3 to day 17 ($P < 0.001$) after surgery (one-way ANOVA vs. sham-operated animals). In contrast, thermal hyperalgesia was observed in A2AR knockout mice only on day 3 ($P < 0.05$), but not in the behavioural measurements performed during the remaining days. Indeed, significant decrease of such a hyperalgesia was observed in A2AR knockout mice on day 3 ($P < 0.001$), day 6 ($P < 0.001$), day 8 ($P < 0.001$), day 10 ($P < 0.001$), day 13 ($P < 0.001$), day 15 ($P < 0.001$) and day 17 ($P < 0.001$) when compared to the wild-type group (one-way ANOVA). Withdrawal latencies of the contralateral paw were not modified in any of the experimental groups (Fig. 2B).

3.1.3. Cold plate test (thermal alldynia)

Sciatic nerve ligation enhanced the score values (see Section 2) during the cold thermal stimulation only in wild-type mice, and this response was abolished in
knockout mice (Fig. 3A). Baseline score values were similar in both genotypes. Sham operation did not produce any modification of nociceptive response neither in wild-type nor in knockout animals. Wild-type mice exposed to sciatic nerve injury significantly increased the score values after surgery, revealing the development of thermal allodynia on day 3 \((P < 0.01)\), day 6 \((P < 0.01)\), day 8 \((P < 0.01)\), day 10 \((P < 0.001)\), day 13 \((P < 0.01)\), day 15 \((P < 0.01)\), day 17 \((P < 0.05)\) after surgery (one-way ANOVA vs. sham-operated). In contrast, thermal allodynia was not developed in A2AR knockout mice. Indeed, a significant decrease of such an allodynia was observed when compared A2AR knockout mice on day 3 \((P < 0.05)\), day 6 \((P < 0.05)\), day 8 \((P < 0.01)\), day 10 \((P < 0.001)\), day 13 \((P < 0.001)\), day 15 \((P < 0.01)\), day 17 \((P < 0.05)\) with wild-type mice (one-way ANOVA) (Fig. 3).

3.2. Microglia cells and astrocytes are involved in the decreased manifestation of neuropathic pain in A2A knockout mice

Glial cells are involved in neuropathic pain and express A2ARs [25]. Therefore, we have evaluated glial response in the dorsal horn of spinal cord after sciatic nerve injury or sham-operation in A2AR knockout and wild-type mice. A time course study was performed to determine the glial response on days 3, 7 and 17 after sciatic nerve injury.

A significant enhancement of the microglia cell staining was observed on day 3 in the dorsal ipsilateral horn of animals with nerve injury compared to sham control in both A2AR knockout and wild-type mice \((P < 0.05)\), (one-way ANOVA) (Fig. 4A). On days 7 and 17 a significant enhancement of microglial activation in the dorsal ipsilateral horn of animals with nerve injury compared to sham control was observed \((P < 0.05)\), only in wild-type mice but not in A2AR knockout (one-way ANOVA) (Figs. 4A and 6A). A significant difference in the microglia expression between genotypes \((P < 0.05)\), was also revealed on days 7 and 17 after nerve injury (one-way ANOVA) (Figs. 4A and 6A).

A significant increased in astrocytes expression was revealed in the ipsilateral dorsal of animals with nerve injury compared to sham control only in wild-type mice but not in A2AR knockout, 3, 7 and 17 days after nerve injury \((P < 0.01)\) (one-way ANOVA) (Figs. 5B and 6B).
A significant difference in the astrocytes expression between genotypes was also revealed on days 3 (P < 0.05), 7 (P < 0.01) and 17 (P < 0.05) after nerve injury (one-way ANOVA) (Figs. 5B and 6B).

No differences were observed among the different experimental groups in the expression of microglia or astrocytes in the contralateral dorsal horn of the spinal cord (Figs. 4B and 5B).

4. Discussion

In this study, we have investigated the role played by A2ARs in the behavioural and histological manifestations of neuropathic pain in mice after partial sciatic nerve injury. All the behavioural symptoms of neuropathic pain were decreased in A2AR knockout mice during the whole experimental period. A significant decrease in mechanical allodynia was observed, although there was an important difference between the ipsi versus contralateral paw. However, thermal hyperalgesia and thermal allodynia were abolished in A2AR knockout mice. The fact that the animals present mechanical allodynia but not thermal hyperalgesia or thermal allodynia, could be due to the different mechanisms underling thermal versus mechanical allodynia [36]. Indeed, nerve injury-induced thermal allodynia is more closely related to the neurochemical reorganization of dorsal horn and primary afferent neurons than the mechanical allodynia [36]. In agreement, the increased sensitivity to thermal stimulation observed after nerve injury in rats requires activity of a small-diameter, unmyelinated C fibers in contrast to the Aβ-mediated mechanical allodynia [31,36]. A significant attenuation in the microglia and astrocytes expression
was also revealed in the A2AR mutant animals during the time course study. Spinal cord samples were removed on day 3 after sciatic nerve injury which corresponds to the first day of the behavioural measurements, on day 7 when there was described that microglia reaches the maximal expression [13], and on day 17 which corresponds to the last day of the behavioural measurements. A significant increase in microglia expression was observed 3 days after partial nerve injury, in the ipsilateral side in both genotypes. However on this day, the astrocytes activation appears only in wild-type mice. An important activation of both microglia and astrocytes expression was revealed 7 and 17 days after nerve injury in wild-type animals but not in mutant mice. Nerve injury has been reported to promote activation of glial cells in the spinal cord, and activated glia may contribute to the initiation and maintenance of neuropathic pain [41,49].

Hypertrophy of both microglia and astrocytes is responsible for the release of inflammatory and pain modulator factors, including reactive oxygen species, nitric oxide, arachidonic acid, leukotrienes, prostaglandins, excitatory amino acids, nerve growth factors and enkephalins [30,49]. Different studies have demonstrated that spinal microglial activation precedes astrocytes activation [14,45] and that astrocytes proliferation is more closely related to the maintenance of pain behaviour in neuropathic pain models [14,42–44]. In accordance with these studies, the behavioural manifestations of neuropathic pain follow a parallel time course with the activation of astroglia, which was absent along the entire experimental sequence in the A2AR knockout mice in our study. Microglia express A2ARs which appear to be responsible for the stimulation of secretory and proinflammatory activity of these cells [25]. Thus, A2ARs stimulation upregulates cyclooxygenase 2 and releases prostaglandin E2 [20]. Other injury stimuli, such as hypoxia, trauma and inflammation have shown to enhance A2ARs density at the astrocytes level [15], which lead to an increase in proliferation and activation of these cells [7,26]. After nerve injury, there is an increase in extracellular level of adenosine [41], that is released from a variety of cells types such as neurons, neutrophils, mast cells, or fibroblasts and it comes also from the ATP, as a result of its dephosphorylation by ecto-5′-nucleotidase [41]. Although the actions of adenosine are mainly protective, in some circumstances, the ARs stimulation aggravates tissue damage mainly by A2ARs [16]. In our study, the adenosine released as a result of nerve injury could act on the A2ARs expressed by microglia and astrocytes generating the activation of these cells which are responsible for the further neuroinflammation process occurring during neuropathic pain.

Several pharmacological and genetic studies have previously evaluated the involvement of A2ARs in other nociceptive manifestations such as acute and inflammatory pain. Thus, mice lacking A2ARs and wild-type animals receiving the A2AR antagonist SCH 582610 showed an increased nociceptive threshold in the hot plate, tail immersion and a decreased response to formalin test [23,27,33]. The same A2AR antagonist, SCH 58261, also exhibited antinociceptive effects in the writhing test [4]. Moreover, caffeine, a non-specific adenosine receptor antagonist causes antinociception in rats and mice in acute and inflammatory pain models [22,33,41]. The antinociceptive effects of caffeine might be mediated by both the adenosine A3ARs and A2BRs [23]. These previous studies are in agreement with our findings proposing a pronociceptive and proinflammatory action of A2ARs. In contrast to these data, other studies reported an opposite effect of A2ARs on pain. Thereby, intrathecal administration of the A2AR agonist DPMA, attenuated formalin-induced pain behaviour in the rats [50].

Pain transmission and analgesia include complex circuits involving sites in the periphery, the spinal cord, ascending and descending central pathways [3]. In the periphery, the A2ARs are present in sensory nerves terminals [27] and in the dorsal root ganglia [28]. Therefore, A2ARs seem also to play a pronociceptive role at the peripheral level [1,19,40]. Previous studies have reported controversial data on the presence of A2ARs at the spinal cord level. Thus, A2AR was identified in the rat [9,12] but not in the mouse spinal cord [1]. Husey et al. [27] attributed the hypoalgesia found in the A2AR knockout mice to the peripheral mechanism. They have previously shown [1] the absence of A2ARs in the spinal cord of wild-type mice using the quantitative autoradiography. This technique is not sensible enough to detect low receptors expression. Recently, we have found by RTPCR the presence of A2AR mRNA in spinal cord of wild-type mice (unpublished data), supporting that a role for spinal A2ARs in nociception cannot be ruled out. However, at present there are no data regarding the expression of A2ARs in mice spinal cord of neuropathic pain animal models. In the spinal cord, nerve injury induced microglial activation comprises the activation of pre-existing resident microglia, as well as the recruitment of bone marrow-derived peripheral macrophages [51] which express A2ARs [6,41]. This fact could explain the presence of A2ARs in the spinal cord and support our results, showing that the deletion of A2ARs induces a decrease in glial activation.

In the brain, A2ARs are found predominantly in the dorsal and ventral striatum, brain structures which do not play a major role in pain regulation [37]. However, A2ARs are also expressed with lower density in other brain regions such as amygdala, thalamus and hypothalamus [37], which have a relevant role in pain modulation. Moreover, A2ARs activation in the brain enhances the release of glutamate from glial cells [34,38]. Excessive accumulation of glutamate can lead
to synaptic deregulation and, may be in part responsible for the neuropathic-pain related central sensitization. Thus, a potential role of A2ARs in the [46] central integration of pain cannot be ignored.

The A2ARs stimulation produces an increase in the intracellular levels of the cyclic adenosine monophosphate (cAMP). In this line, a recent study shows that the inhibition of cAMP pathway attenuates the behavioural symptoms of neuropathic pain and reduces phosphorylation of cyclic adenosine monophosphate response element-binding in the spinal cord after partial sciatic nerve ligation in rats [35].

This study contributes to a better understanding of the function of A2ARs in pain modulation and demonstrates the crucial role of these receptors in the control of neuropathic pain at the spinal cord level. The genetic deletion of A2ARs reduced all the behavioural and histological manifestations of pain after nerve injury and this response could be due to the inhibition of the inflammatory responses that are linked to the microglia and astrocytes activation in the spinal cord. Thus, our findings support the notion that A2ARs could be an interesting target for the development of new drugs for the management of the clinical manifestation of neuropathic pain.

Conflict of interest

Authors have no conflicting interests.

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References


