Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 508 (2005) 139–146

www.elsevier.com/locate/ejphar

AM404, an inhibitor of anandamide reuptake decreases Fos-immunoreactivity in the spinal cord of neuropathic rats after non-noxious stimulation

Luigi F. Rodella*, Elisa Borsani, Rita Rezzani, Francesca Ricci, Barbara Buffoli, Rossella Bianchi

Department of Biomedical Sciences and Biotechnology, Division of Human Anatomy, University of Brescia, viale Europa 11, 25123 Brescia, Italy

Received 21 September 2004; received in revised form 14 December 2004; accepted 17 December 2004
Available online 12 January 2005

Abstract

Cannabinoids like anandamide are involved in pain transmission. In this study we evaluated the effects of administrating *N*-(4-hydroxyphenyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide (AM404), an inhibitor of anandamide reuptake and monitoring the expression of c-*fos*, a marker of activated neurons in an experimental model of neuropathic pain (sciatic nerve tying). Fos expression was monitored 14 days after tying of sciatic nerve and 2 h after non-noxious stimulation. We showed that non-noxious stimulation increased Fos-positivity in the dorsal superficial laminae of the lumbar spinal cord of tied animals but not in the control animals. AM404 significantly reduced Fos induction in tied animals. Co-administration of cannabinoid CB_1 receptor, cannabinoid CB_2 receptor and transient receptor potential vanilloid type 1 (TRPV-1) antagonists reduced the effect of AM404 and this reduction was higher using cannabinoid CB_1 receptor antagonist. These results suggest that AM404 could be a useful drug to reduce neuropathic pain and that cannabinoid CB_1 receptor, cannabinoid CB_2 receptor and vanilloid TRPV-1 receptor are involved.

Keywords: Neuropathic pain; Cannabinoid; Cannabinoid CB_1 receptor; Cannabinoid CB_2 receptor; Vanilloid TRPV-1 receptor; Spinal cord; Fos

1. Introduction

Chronic neuropathic pain syndrome is a disabling condition which can be a much greater challenge for the clinician than acute pain. The limitations of treatment for neuropathic pain and the inability to provide relief for many patients have stimulated ongoing studies examining different approaches of treating neuropathic pain (Dworkin, 2002). The neural basis for the neuropathic pain syndromes are poorly known as it is only in the last 15 years that the availability of animal models for these conditions has permitted us to understand some of the physiopathological changes in chronic pain better (Bennett and Xie, 1988; Kim and Chung, 1992).

Over the last 30 years considerable advances have been made in understanding cannabinoid functions in the nervous system. The cannabinoid receptor system and its ligands have both been actively investigated. Two cannabinoid receptors (cannabinoid CB_1 receptor and cannabinoid CB_2 receptor) have so far been described (Rice, 2001) and cloned (Piomelli et al., 1998), but new data suggest there may be a third cannabinoid receptor (cannabinoid CB_3 receptor) (Wilson and Nicoll, 2002) and other new cannabinoid receptor subtypes. Expression of the cannabinoid CB_1 receptor is found mainly on central and peripheral neurons and it has been identified in the brain (Herkenham et al., 1991; Tsou et al., 1998; Ergetova and Elphic, 2000), spinal cord (Hohmann et al., 1999; Sanudo-Pena et al., 1999; Farquhar-Smith et al., 2000) and on dorsal root ganglia

* Corresponding author. Tel.: +39 030 3717485; fax: +39 030 3717408.
E-mail address: rodella@med.unibs.it (L.F. Rodella).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.12.031

neurons (Sanudo-Pena et al., 1999), while the cannabinoid CB_2 receptor was found mainly at a peripheral level (Pertwee, 1997), even if the cannabinoid CB_2 receptor was also found in the brain microglia (Kearn and Hillard, 1999) and cerebellum (Skaper et al., 1996). Two eicosanoids have been identified as endocannabinoids with an agonist effect for cannabinoid CB_1 receptor: anandamide and 2-arachidonylglycerol. Anandamide is widely distributed (Felder et al., 1996) and causes classical cannabinoids effects, including analgesia (Fride and Mechoulam, 1993; Jaggar et al., 1998). Anandamide binds to the cannabinoid CB_1 receptor and causes agonist activity (Di Marzo and Deutsch, 1998), nevertheless some recent data suggest that anandamide also activates cannabinoid CB_2 receptor (Malan et al., 2001; Pertwee and Ross, 2002; Sarker and Maruyama, 2003) and transient receptor potential vanilloid type 1 (TRPV-1), co-expressed with cannabinoid CB_1 receptor (Ross, 2003; Zygmunt et al., 2000). Anandamide is rapidly synthesized on demand and its uptake by neurons is rapid, temperature dependent, saturable and selective, suggesting the presence of a specific membrane transporter (Beltramo et al., 1997; Di Marzo et al., 1994; Di Marzo and Deutsch, 1998). Recently an anandamide transport inhibitor has been developed, i.e. the drug *N*-(4-hydroxyphenyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide (AM404). AM404 is an anandamide re-uptake inhibitor which enhances the antinociceptive and the hypotensive effects of anandamide in vivo (Beltramo et al., 1997; Calignano et al., 1997). Some authors reported that the cannabinoids modulate the nociceptive processes in the neuropathic pain (Calignano et al., 1998; Herzberg et al., 1997) both in the peripheral and central nervous system (Hohmann and Herkenham, 1999; Richardson et al., 1998). Cannabinoid CB_1 receptor appeared to be involved in neuropathic pain transmission even if some studies also showed that cannabinoid CB_2 receptor and vanilloid TRPV-1 receptor are involved in neuropathic pain transmission (Lopez-Rodriguez et al., 2003; Scott et al., 2004). The mechanism and the target of specific metabolic pathway which modulate anandamide in this hyperalgesia model are not clearly understood as indeed the involvement of endogenous cannabinoids has not been fully evaluated. Fos is a nuclear phosphoprotein product of the mammalian c-*fos* protooncogene widely used as morphological marker of pain activated neurons and its expression can be induced in nerve cells by various factors including somato-sensory stimuli. Hunt et al. (1987) showed that the expression of Fos protein in the post-synaptic neurons of the spinal cord dorsal horn of rat was induced by several types of stimuli including noxious stimulation. Subsequently, the expression of Fos was generally used to identify the activated neurons involved in pain transmission in the spinal cord (Harris, 1998) and in the supraspinal areas (Bullit, 1990; Lanteri-Minet et al., 1994; Rodella et al., 1998) and to correlate its expression with the effects of some antinociceptive drugs (Bianchi et al., 2003; Kosai et al., 2001; Rodella et al., 2001).

On the basis of these findings, the aim of our study was to evaluate the effects of the chronic administration of AM404 and the involvement of cannabinoid CB_1 receptor, cannabinoid CB_2 receptor and vanilloid TRPV-1 receptor monitoring the expression of c-*fos* in the dorsal horn of lumbar spinal cord in chronic neuropathic pain model (Bennett and Xie, 1988) after non-noxious stimulation.

2. Material and methods

Experiments were carried out on 80 male Sprague–Dawley rats (200 g b.wt.). 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. The animals were subdivided into three surgical groups: in the first group (60 animals) the left sciatic nerve was tied producing a chronic constriction injury (CCI); the second group (10 animals) was the sham operated animals and the third group (10 animals) was the control non-operated animals (NAIVE).

2.1. Surgical procedure

The rats were anesthetized by intraperitoneal injection of sodium pentobarbitone (40 mg/kg), and the left 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. Then four ligatures were loosely tied around the nerve at 1–2 mm distance using 4-0 chromic gut suture material according to the method described by Bennett and Xie (1988). The sham operated animals however had the left sciatic nerve exposed at the same level, without ligature.

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 (Hunt et al., 1987; Catheline et al., 1999), where cannabinoid receptors and particularly cannabinoid CB_1 receptors were well expressed (Farquhar-Smith et al., 2000). After the surgical procedure, the animals had the pat of the ligatured side lifted up.

2.2. Experimental protocols

We used three different experimental protocols. The first to evaluate the Fos expression comparing chronic constriction injured, sham and NAIVE groups; the second to evaluate the effects of different doses of AM404 and the third to evaluate the effect of co-administration of AM404 with other drugs that modulate cannabinoid and vanilloid receptors.

For this purpose, AM404 was also administrated in association with cannabinoid CB_1 receptor, cannabinoid CB_2 receptor and vanilloid TRPV-1 receptor antagonists. In particular we used AM251 (*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide), a selective cannabinoid CB_1 receptor antagonist (Akerman et al., 2004; Dogrul et al., 2003); AM630 (6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl](4-methoxyphenyl), a cannabinoid CB_2 receptor antagonist (Hosohata et al., 1997) and capsazepine (*N*-[2-(4-Chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide), a vanilloid TRPV-1 receptor antagonist (Harrison et al., 2003). All the drugs were purchased by Tocris Cookson, Bristol, UK.

2.2.1. First experimental protocol: evaluation of Fos expression in chronic constriction injured, sham and NAIVE rats

In the first experiment we evaluated the induction of Fos positive neurons by non-noxious stimulation in chronic constriction injured, sham and NAIVE rats. The chronic constriction injured (n=10), sham (n=10), and NAIVE (n=10) animals were subdivided in two sub-groups: 1) the first (n=5) consisted of stimulated animals, 2) and the second (n=5) of non-stimulated animals. 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 (40 mg/kg i.p.). All rats of the stimulated subgroups received the non-noxious stimulus three times a minute every 30 s on the 14th post-operative day.

2.2.2. Second experimental protocol: dose–response analysis for AM404

A group of 25 chronic constriction injured and stimulated rats were studied at 14th day after the surgery. The animals were treated with different concentration of AM404 and subdivided into five groups: 1) vehicle only (n=5) (physiological saline containing 10% DMSO (dimethylsulphoxide; Sigma, St. Louis, USA)), 2) AM404 1 mg/kg (n=5), the animals received AM404 (1 mg/kg/day), 3) AM404 5 mg/kg (n=5), the animals received AM404 (5 mg/kg/day), 4) AM404 10 mg/kg (n=5), the animals received AM404 (10 mg/kg/day), 5) AM404 20 mg/kg (n=5), the animals received AM404 (20 mg/kg/day). The subcutaneous AM404 administration was performed, daily for 14 days after the surgery with the last injection 1 h before the stimulation. The non-noxious stimulation protocol was the same as previous described.

2.2.3. Third experimental protocol: evaluation of AM404 alone and co-administered with AM251, AM630 and capsazepine on Fos expression

A group of 25 chronic constriction injured and stimulated rats were studied at 14th day after the surgery and subdivided into five groups: 1) AM404 treated (n=5), the animals received AM404 (10 mg/kg/day), 2) AM404 and AM251 co-administered (n=5), the animals received AM404 (10 mg/kg/day) AM251 (1 mg/kg/day), 3) AM404 and AM630 co-administered (n=5), the animals received AM404 (10 mg/kg/day) and AM630 (1 mg/kg/day), 4) AM404 and capsazepine co-administered (n=5), the animals received AM404 (10 mg/kg/day) and capsazepine (1 mg/kg/day), 5) vehicle only (n=5). The subcutaneous administration of the drugs was performed daily for 14 days after the surgery with the last injection 1 h before the stimulation. The non-noxious stimulation protocol was the same as previous described. The drug doses corresponded to those used in previous studies (Del Arco et al., 2002; Giuffrida et al., 2000) and showing a significant reduction of Fos positive neurons in our dose response experiments. Therefore, the chronic treatment (14 days) was chosen on the basis of our preliminary observations showing the better response to AM404 in a daily administration (data not shown).

2.2.3.1. Fos immunohistochemistry. Two hours after the stimulation the animals were anaesthetised with sodium pentobarbital (40 mg/kg i.p.) and transcardically perfused with saline followed by 1 L 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 from each rat was determined in each case the spinal cord in situ using a dissecting microscope and measuring the distance between the points of entry of the most rostral and most caudal rootlets of the L4, L5 and L6 dorsal root. Frozen serial transverse sections (25 μm thick) of all segments were collected in phosphate-buffered saline. We collected all the sections of the L4–L6 segments. For Fos immunoreactivity, we stained one section every 10 for a total of 25 sections per animal. Some sections were Nissl-stained for morphological control. 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 of all animals was charted with the aid of

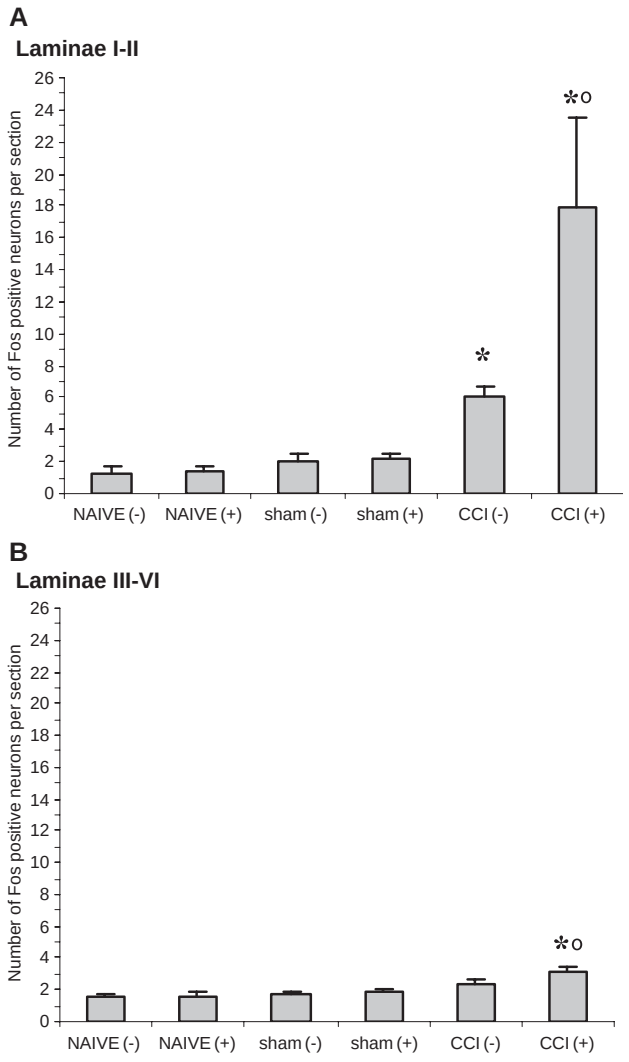

Fig. 1. Statistical evaluation of Fos immunoreactivity in L4–L6 spinal cord laminae I–II (A) and III–VI (B) in naive (NAÏVE), sham and rats with chronic constriction injury (CCI) on 14th postoperative day. NAIVE (−) ($n=5$): non-operated animals without stimulation; NAIVE (+) ($n=5$): non-operated animals with stimulation; sham (−) ($n=5$): sham operated animals without stimulation; sham (+) ($n=5$): sham operated animals with stimulation; CCI (−) ($n=5$): animals with tied nerve without stimulation; CCI (+) ($n=5$): tied nerve with stimulation. Values are mean±S.D. and represent the number of neurons per section; *$P<0.05$ compared to NAIVE, °$P<0.05$ with respect to CCI (−).

an image analyzer (Immagini e Computer, Milano, Italy). The density 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. Cell counts were made in all the processed sections at a final ×40 magnification. The number of Fos-positive cells of each processed section of each spinal cord was divided by the number of counted tissue sections, in order to evaluate the average number of labelled cells for each animal. The data of all animals were analyzed and compared by analysis of variance (ANOVA) and by a Bonferroni multiple comparison test.

3. Results

3.1. First experimental protocol: evaluation of Fos expression in chronic constriction injured, sham and NAIVE rats

In the NAIVE non-stimulated and stimulated groups the number of Fos positive neurons in laminae I–II and III–VI was very low without any significant difference between two the different groups of laminae. The Fos distribution pattern shown in the sham non-stimulated and stimulated animals was similar to the NAIVE. In the chronic constriction injured/non-stimulated animals we observed a slight but significant increase of Fos positive neurons in laminae I–II, while in laminae III–VI there was a slight non-significant increase of the number of Fos positive neurons compared with the NAIVE and sham groups. In the chronic constriction injured/stimulated group 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 the chronic constriction injured/non-stimulated and chronic constriction injured/stimulated, the positive neurons were found ipsilaterally from L4 to L6 spinal segments. The different intensity of immunostaining was observed not only in the different laminae but also within the single lamina. Neurons with intensely stained nucleus appeared interspersed with others displaying lightly stained nuclei. The data on the distribution of the labelled cells in the spinal cord in the different laminae are summarized in Fig. 1.

3.2. Second experimental protocol: dose–response analysis for AM404

The dose–response curve for AM404 in chronic constriction injured and stimulated animals showed that

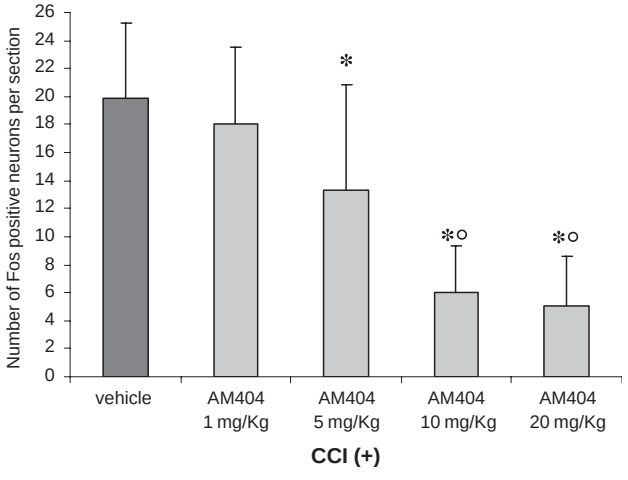

Fig. 2. Dose–response evaluation of AM404 effect, monitoring Fos immunoreactivity in L4–L6 spinal cord laminae I–VI in CCI (+) animals (tied nerve with stimulation) on 14th postoperative day. Values are mean±S.D. and represent the number of neurons per section; *$P<0.05$ compared to vehicle, °$P<0.05$ compared to AM404 at the dose of 5 mg/kg.

the more efficient dose in a chronic subcutaneous treatment of 14 days was of 10 mg/kg/day. We showed that the administration of AM404 at the dose of 20 mg/kg/day did not change significantly the number of Fos positive neurons in dorsal laminae with respect to the dose of 10 mg/kg. On the other hand the dose of 10 mg/kg/day is more efficient with respect to 1 and 5 mg/kg/day (Fig. 2).

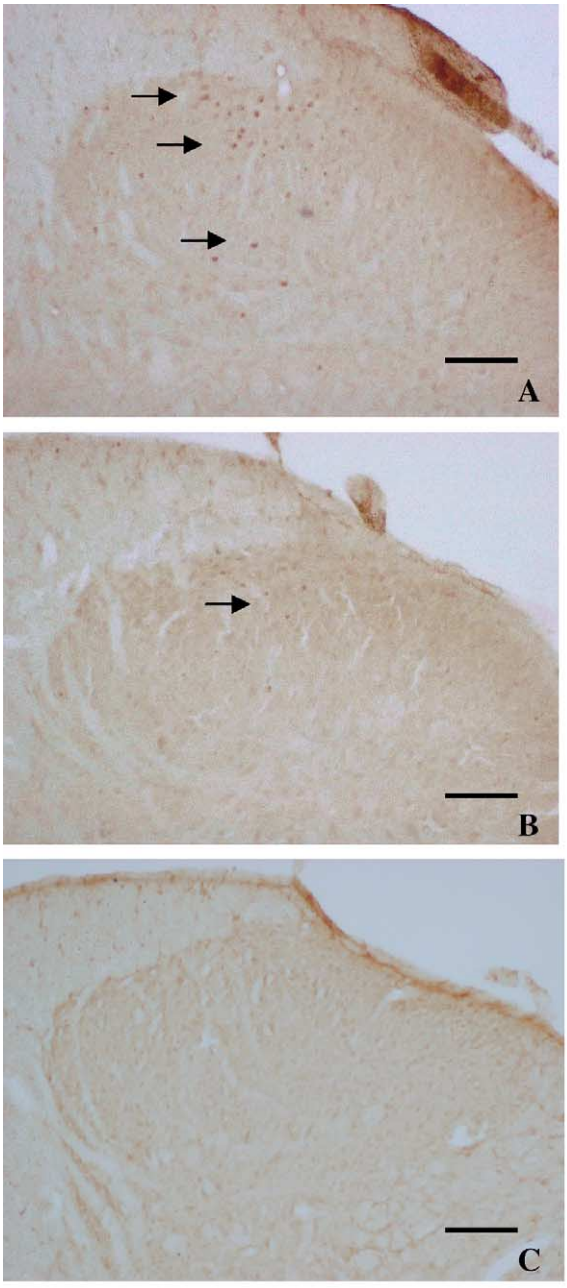

Fig. 3. Fos-positive neurons in the spinal cord (L5 level) of tied and stimulated animals (CCI+): (A) vehicle treated animals, (B) AM404 treated animals, (C) negative control for the specificity of the primary antibody. Arrows indicate Fos-positive neurons. Bar 100 μm.

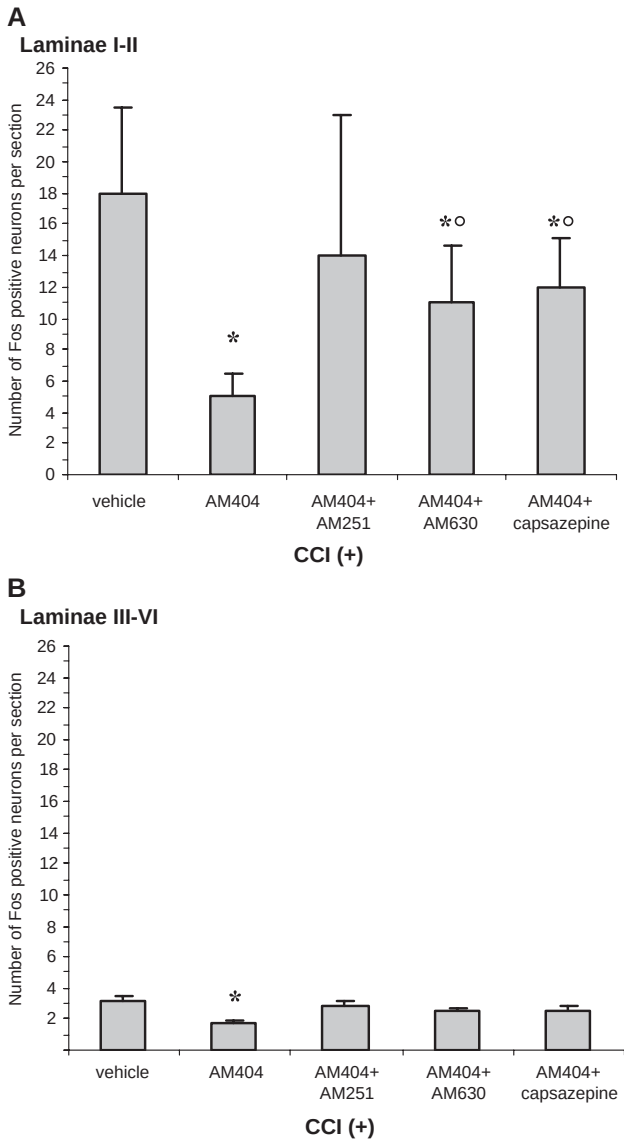

Fig. 4. Statistical evaluation of Fos immunoreactivity in L4–L6 spinal cord laminae I–II (A) and III–VI (B) in CCI (+) animals (tied nerve with stimulation) on 14th postoperative day. Vehicle (n=5): vehicle injected animals; AM404 (n=5): AM404 injected animals; AM404+AM251 (n=5): AM404 plus AM251 injected animals; AM404+AM630 (n=5): AM404 plus AM630 injected animals, AM404+capsazepine (n=5): AM404 plus capsazepine injected animals. Values are mean±S.D. and represent the number of neurons per section; *P<0.05 compared to vehicle, °P<0.05 with respect to AM404.

3.3. Third experimental protocol: evaluation of AM404 alone and co-administered with AM404 with AM251, AM630 and capsazepine on Fos expression

In the chronic constriction injured and stimulated/vehicle injected animals, numerous Fos-positive neurons were detected scattered mainly in the superficial laminae I–II. Some positive neurons were also scattered in laminae III–VI. In the chronic constriction injured/stimulated and AM404 treated animals, we observed a significant decrease of Fos positive neurons in the laminae of the

dorsal horn of the lumbar spinal cord L4–L6 segments (Fig. 3). In the chronic constriction injured/stimulated animals and co-treated with AM404 and AM251 we observed a slight significant decrease of Fos positive neurons in laminae I–II compared to the chronic constriction injured/stimulated/vehicle injected animals. In the chronic constriction injured/stimulated animals co-treated with AM404 and AM630 we observed a significant decrease of Fos positive neurons in laminae I–II compared to the chronic constriction injured/stimulated/vehicle injected. In the chronic constriction injured/stimulated animals co-treated with AM404 and capsazepine we observed a significant decrease of Fos positive neurons in laminae I–II with respect to the chronic constriction injured/stimulated/vehicle injected animals. In laminae III–VI the number of Fos positive neurons did not show any significant change either in the animal co-treated with AM404 and AM251 or AM630 or capsazepine compared to the chronic constriction injured/stimulated/vehicle injected animals. The data on the distribution of the labelled cells in the spinal cord in the different laminae are summarized in Fig. 4.

4. Discussion

Our results showed that non-noxious stimulation increased Fos-positivity in the dorsal superficial laminae of the lumbar spinal cord of tied rats but not in control animals. These results gave us the morphological evidence of neuronal activation in the dorsal horn of the spinal cord, underlying the presence of allodynia in tied rats. The slight induction of Fos expression in chronic constriction injured/non-stimulated animals agreed with the data of Catheline et al. (1999) and Kosai et al. (2001) showing that tying could activate some spinal nerve fibers and dorsal horn neurons of spinal cord.

The distribution of Fos positive neurons in the ipsilateral lumbar dorsal horn of spinal cord of chronic constriction injured/stimulated animals agrees with the findings suggesting that in the chronic constriction injured rats the non-noxious mechanical stimulation enhanced c-Fos expression mainly in laminae I–II, while in laminae III–VI there was only a slight increase (Kosai et al., 2001). The same stimulation did not induce Fos expression in laminae I–II in intact rats (Harris, 1998). 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, inducing the Fos expression as suggested by other authors (Catheline et al., 1999; Nakamura and Myers, 1999; Woolf et al., 1992). In addition we showed for the first time that AM404 delivered chronically after tying of the sciatic nerve, significantly decreased the pain inducing Fos expression of the sensitive neurons of the lumbar spinal cord in a dose-dependent manner showing that the dose of 10 mg/kg is the lowest dose that produces a strong decrease of Fos immunostaining. This finding suggests a significant reduction of neuronal activation in this central area by the pharmacological action of AM404. These results agree with the findings showing that AM404 decreased neuronal activation inhibiting the reuptake of anandamide (Calignano et al., 1997), an endocannabinoid which is able to interact mainly with cannabinoid CB_1 receptor (Di Marzo and Deutsch, 1998) but also with cannabinoid CB_2 receptor and with vanilloid TRPV-1 receptor (Malan et al., 2001; Pertwee and Ross, 2002; Sarker and Maruyama, 2003; Ross, 2003; Zygmunt et al., 2000). The interaction between AM404 and cannabinoid receptors is debated. Glaser et al. (2003) suggested that AM404, acting as cannabinoid indirect agonist, inhibited the fatty acid amide hydrolase and Breivogel et al. (2004) observed that the action of endocannabinoids in the presence of AM404 was due to inhibition of their degradation. However, a role of AM404 as direct agonist was not supported either in vitro or in vivo studies (Beltramo et al., 1997; Calignano et al., 1997). However, at present, the anandamide uptake pathway is still unanswered (Hillard and Jarrahian, 2003). The existence of anandamide transporter is based on an anandamide uptake process that is temperature-dependent, selective and saturable and in addition several studies identified compounds that inhibit anandamide accumulation, including AM404 (Beltramo et al., 1997; Glaser et al., 2003; Hillard et al., 1997; Ligresti et al., 2004). However other authors believe that anandamide uptake was a process of simple diffusion (Day et al., 2001; Deutsch et al., 2001; Glaser et al., 2003). Nevertheless, the results of our study were not addressed to solve this question.

The co-administration of AM404 with AM251, a cannabinoid CB_1 receptor antagonist, caused a slight decrease of Fos positive neurons compared to the chronic constriction injured/stimulated animals and suggest that cannabinoid CB_1 receptor was not the only receptor involved. This idea was supported by the fact that both the co-administration of cannabinoid CB_2 receptor and vanilloid TRPV-1 receptor antagonists induced a decrease of Fos positive neurons compared to the chronic constriction injured/stimulated animals that was significantly less great that AM404 alone. On the whole, these data underline the pivotal role of cannabinoid CB_1 receptor involved in anandamide pathway. Moreover, according to Lopez-Rodriguez et al. (2003) and Scott et al. (2004), we showed an involvement of cannabinoid CB_2 receptor and vanilloid TRPV-1 receptor in the neuropathic pain transmission. These data agree with morphological studies indicating the presence of cannabinoid CB_1 receptor in laminae I and II of the spinal cord (Farquhar-Smith et al., 2000; Salio et al., 2001; Salio et al., 2002a,b; Tsou et al., 1996; Tsou et al., 1998); the co-expression of vanilloid TRPV-1 receptor with cannabinoid CB_1 receptor (Ross, 2003; Zygmunt et al., 2000) and showing the induction of cannabinoid CB_2 receptor in rat lumbar spinal cord in

chronic pain models (Zhang et al., 2003), just where we observed the greatest decrease of Fos-positive neurons after the administration of AM404. On the whole, these results support the pharmacological role of cannabinoids modulation in pain treatment. In particular, since it is well known that the psychotropic effects of cannabinoids are an obstacle to the development of cannabinoid based therapy for the treatment of neuropathic pain (Perez-Reyes, 1999), the availability of drugs that interact with the endocannabinoid system can be useful to by-pass the adverse effect of exogenous cannabinoid administration. Finally, our results, showing that AM404 administration reduced pain-induced Fos activity in the lumbar spinal cord, confirm and give anatomical support to the hypothesis that the inhibition of the reuptake of endocannabinoid can be useful to reduce neuropathic pain. Therefore, AM404 seems to be a promising molecula for the treatment of neuropathic pain. Nevertheless, further histochemical, behavioural and pharmacological studies could be addressed in this way.

Acknowledgement

This study was supported by the MURST grant.

References

Akerman, S., Kaube, H., Goadsby, P.J., 2004. Anandamide is able to inhibit trigeminal neurons using an in vivo model of trigeminovascular-mediated nociception. J. Pharmacol. Exp. Ther. 309, 56–63.

Beltramo, M., Stella, N., Calignano, A., Lin, S.Y., Makriyannis, A., Piomelli, D., 1997. Functional role of high-affinity anandamide transport, as revealed by selective inhibition. Science 2777, 1094–1097.

Bennett, G.J., Xie, Y.K., 1988. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33, 87–107.

Bianchi, R., Rezzani, R., Borsani, E., Rodella, L., 2003. mGlu5 receptor antagonist decreases Fos expression in spinal neurons after noxious visceral stimulation. Brain Res. 960, 263–266.

Breivogel, C.S., Walker, J.M., Huang, S.M., Roy, M.B., Childers, S.R., 2004. Cannabinoid signalling in rat cerebellar granule cells: G-protein activation, inhibition of glutamate release and endogenous cannabinoids. Neuropharmacology 47, 81–91.

Bullit, E., 1990. Expression of c-*fos*-like protein as a marker for neuronal activity following noxious stimulation in the rat. J. Comp. Neurol. 296, 517–530.

Calignano, A., La Rana, G., Beltramo, M., Makriyannis, A., Piomelli, D., 1997. Potentiation of anandamide hypotension by the transport inhibitor, AM404. Eur. J. Pharmacol. 337, R1–R2.

Calignano, A., La Rana, G., Giuffrida, A., Pomelli, D., 1998. Control of pain initiation by endogenous cannabinoids. Nature 394, 277–281.

Catheline, G., Guen, S.L., Honore, P., Mbesson, J., 1999. Are there long-term changes in the basal or evoked Fos expression in the dorsal horn of the spinal cord of the mononeuropathic rat? Pain 80, 347–357.

Day, T.A., Rakhsham, F., Deutsch, D.G., Barker, E.L., 2001. Role of fatty acid amide hydrolase in the transport of the endogenous cannabinoid anandamide. Mol. Pharmacol. 59, 1369–1375.

Del Arco, I., Navarro, M., Bilbao, A., Ferrer, B., Pomelli, D., Rodriguez De Fonseca, F., 2002. Attenuation of spontaneous opiate withdrawal in mice by the anandamide transport inhibitor AM404. Eur. J. Pharmacol. 454, 103–104.

Deutsch, D.G., Glaser, S.T., Howell, J.M., Kunz, J.S., Puffenbarger, R.A., Hillard, C.J., Abumrad, N., 2001. The cellular uptake of anandamide is coupled to its breakdown by fatty acid amide hydrolase (FAAH). J. Biol. Chem. 276, 6967–6973.

Di Marzo, V., Deutsch, D.G., 1998. Biochemistry of the endogenous ligands of cannabinoid receptor. Neurobiol. Dis. 5, 386–404.

Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J.C., Pomelli, D., 1994. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature 372, 686–691.

Dogrul, A., Gul, H., Akar, A., Yildiz, O., Bilgin, F., Guzeldemir, E., 2003. Topical cannabinoid antinocicception: synergy with spinal sites. Pain 105, 11–16.

Dworkin, R.H., 2002. An overview of neuropathic pain: syndromes, symptoms, signs, and several mechanisms. Clin. J. Pain 18, 343–349.

Ergetova, M., Elphic, M.R., 2000. Localization of cannabinoid receptors in the rat brain using antibodies to the intracellular C-terminal of CB1. J. Comp. Neurol. 422, 159–171.

Farquhar-Smith, W.P., Ergetova, M., Bradbury, E.J., McMahnon, S.B., Rice, A.S.C., Elphick, M.R., 2000. Cannabinoid CB1 receptor expression in rat spinal cord. Mol. Cell. Neurosci. 15, 510–521.

Felder, C.C., Nielsen, A., Briley, E.M., Palkovits, M., Priller, J., Axelrod, J., Nguyen, D.N., Richardson, J.M., Riggin, R.M., Koppel, G.A., Paul, S.M., Becker, G.W., 1996. Isolation and measurement of the endogenous cannabinoid receptor agonist, anandamide, in brain and peripheral tissues of human and rat. FEBS Lett. 393, 231–235.

Fride, E., Mechoulam, R., 1993. Pharmacological activity of the cannabinoid receptor agonist, anandamide, a brain constituent. Eur. J. Pharmacol. 231, 313–314.

Giuffrida, A., Rodriguez de Fonseca, F., Nava, F., Loubet-lescoulie, P., Piomelli, D., 2000. Elevated circulating levels of anandamide after administration of the transport inhibitor, AM404. Eur. J. Pharmacol. 408, 161–168.

Glaser, S., Abumrad, N., Fatade, F., Kaczocha, M., Stuholme, K., Deutsch, D., 2003. Evidence against the presence of an anandamide transporter. Proc. Natl. Acad. Sci. U. S. A. 100, 4269–4274.

Harris, J.A., 1998. Using c-*fos* as a neuronal marker of pain. Brain Res. Bull. 45, 1–8.

Harrison, S., De Petrocellis, L., Trevisani, M., Benvenuti, F., Bifulco, M., Geppetti, P., Di Marzo, V., 2003. Capsaicin-like effects of *N*-arachidonoyl-dopamine in the isolated guinea pig bronchi and urinary bladder. Eur. J. Pharmacol. 487, 175–182.

Herkenham, M., Lynn, A.B., Johnson, M.R., Melvin, L.S., De Costa, B.R., Rice, K.C., 1991. Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. J. Neurosci. 11, 563–583.

Herzberg, U., Eliav, E., Bennett, G.J., Kopin, I.J., 1997. The analgesic effect of *R*(+)-WIN55212-2 mesylate, a high affinity cannabinoid agonist, in a rat model of neuropathic pain. Neurosci. Lett. 221, 157–160.

Hillard, C.J., Jarrahian, A., 2003. Cellular accumulation of anandamide: consensus and controversy. Br. J. Pharmacol. 140, 802–808.

Hillard, C.J., Edgemond, W.S., Jarrahian, A., Campbell, W.B., 1997. Accumulation of *N*-arachidonoylethanolamine (anandamide) into cerebellar granule cells occurs via facilitated diffusion. J. Neurochem. 69, 631–638.

Hohmann, A.G., Herkenham, M., 1999. Cannabinoid receptors undergo axonal flow in sensory nerves. Neuroscience 92, 1171–1175.

Hohmann, A.G., Briley, E.M., Herkenham, M., 1999. Pre- and postsynaptic distribution of cannabinoid and mu-opioid receptors in rat spinal cord. Brain Res. 822, 17–25.

Hosohata, Y., Ouock, R.M., Hosohata, K., Makrivannis, A., Consroe, P., Roeske, W.R., Yamamura, H.I., 1997. AM630 antagonism of cannabinoid-stimulated [35S] GTP gamma S binding in the mouse brain. Eur. J. Pharmacol. 19, R1–R3.

Hunt, S.P., Pint, A., Evan, G., 1987. Induction of c-*fos*-like protein in spinal cord neurons following sensory stimulation. Nature 328, 632–634.

Jaggar, S.I., Hasnie, F.S., Sellaturay, S., Rice, A.S.C., 1998. The anti-hyperalgesic actions of the cannabinoid anandamide and the putative CB, agonist palmitoylethanolamide investigated in models of visceral and somatic inflammatory pain. Pain 76, 189–199.

Kearn, C.S., Hillard, C.J., 1999. A model for the cannabinoid action in microglia. Int. Cannab. Res. Soc., Symposium on the Cannabinoids, Burlington, Vermont, p. 44.

Kim, S.H., Chung, J.M., 1992. An experimental model for peripheral neuropathy produced by segmental nerve ligation in the rat. Pain 50, 355–363.

Kosai, K., Tateyama, S., Ikeda, T., Uno, T., Nishimori, T., Takasaki, M., 2001. MK-801 reduces non-noxious stimulus-evoked Fos-like immunoreactivity in the spinal cord of rats with chronic constriction nerve injury. Brain Res. 910, 12–18.

Lanteri-Minet, M., Weil-Fugazza, J., De Pommery, J., Menetrey, D., 1994. Hindbrain structures involved in pain processing as revealed by the expression of c-Fos and other immediate early gene proteins. Neuroscience 58, 287–298.

Ligresti, A., Morera, E., Van Der Stelt, M., Monory, K., Lutz, B., Ortar, G., Di Marzo, V., 2004. Further evidence for the existence of a specific process for the membrane transport of anandamide. Biochem. J. 380, 265–272.

Lopez-Rodriguez, M.L., Viso, A., Ortega-Gutierrez, S., 2003. VR1 receptor modulators as potential drugs for neuropathic pain. Mini Rev. Med. Chem. 3, 729–748.

Malan Jr., T.P., Ibrahim, M.M., Deng, H., Liu, Q., Mata, H.P., Vanderah, T., Porreca, F., Makriyannis, A., 2001. CB2 cannabinoid receptor-mediated peripheral antinociception. Pain 93, 239–245.

Nakamura, S., Myers, R.R., 1999. Myelinated afferents sprout into lamina II of L3–5 dorsal horn following chronic constriction nerve injury in rats. Brain Res. 818, 285–290.

Perez-Reyes, M., 1999. The psychologic and physiologic effects of active cannabinoids. In: Nahas, G.G., Sutin, K.M., Harvey, D., Arguell, S. (Eds.), Marihuana and Medicine. Humana Press, New Jersey, pp. 245–252.

Pertwee, R.G., 1997. Pharmacology of cannabinoid CB1 and CB2 receptors. Pharmacol. Ther. 74, 129–180.

Pertwee, R.G., Ross, R.A., 2002. Cannabinoid receptors and their ligands. Prostaglandins Leukot. Essent. Fat. Acids 66, 101–121.

Piomelli, D., Beltramo, M., Giuffrida, A., Stella, N., 1998. Endogenous cannabinoid signalling. Neurobiol. Dis. 5, 462–473.

Rice, A.S.C., 2001. Cannabinoids and pain. Curr. Opin. Investig. Drugs 2, 399–414.

Richardson, J.D., Kilo, S., Hargreaves, K.M., 1998. Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral and CB1 receptors. Pain 75, 111–119.

Rodella, L., Rezzani, R., Gioia, M., Tredici, G., Bianchi, R., 1998. Expression of Fos immunoreactivity in the rat supraspinal regions following noxious visceral stimulation. Brain Res. Bull. 47, 357–366.

Rodella, L., Borsani, E., Rezzani, R., Lanzi, R., Bianchi, R., 2001. Local pentoxifilline administration decreases the formalin Fos expression in rat spinal cord. Neurosci. Res. Comm. 29, 155–162.

Ross, R.A., 2003. Anandamide and vanilloid TRPV1 receptors. Br. J. Pharmacol. 140, 790–801.

Salio, C., Fischer, J., Franzoni, M.F., Mackie, K., Kaneko, T., Conrath, M., 2001. CB1-cannabinoid and μ-opioid receptor colocalization on postsynaptic target in the rat dorsal horn. NeuroReport 12, 3689–3692.

Salio, C., Doly, S., Fischer, J., Franzoni, M.F., Conrath, M., 2002a. Neuronal and astrocytic localization of the cannabinoid receptor-1 in the dorsal horn of the rat spinal cord. Neurosci. Lett. 329, 13–16.

Salio, C., Fischer, J., Franzoni, M.F., Conrath, M., 2002b. Pre- and postsynaptic localizations of the CB1 cannabinoid receptor in the dorsal horn of the rat spinal cord. Neuroscience 110, 755–764.

Sanudo-Pena, M.C., Strangman, N.M., Mackie, K., Walker, J.M., Tsou, K., 1999. CB1 receptor localization in rat spinal cord and roots, dorsal root ganglion and peripheral nerve. Acta Pharmacol. Sin. 20, 1115–1120.

Sarker, K.P., Maruyama, I., 2003. Anandamide induces cell death independently of cannabinoid receptors or vanilloid receptor 1: possible involvement of lipid rafts. Cell. Mol. Life Sci. 60, 1200–1208.

Scott, D.A., Wright, C.E., Angus, J.A., 2004. Evidence that CB1 and CB2 receptors mediate antinociception in neuropathic pain in the rat. Pain 109, 124–131.

Skaper, S.D., Buriani, A., Del Toso, R., Petrelli, L., Romanello, S., Facci, L., Leon, A., 1996. The ALIAmide palmitoylethanolamide and cannabinoids, but not anandamide, are protective in a delayed postglutamate paradigm of excitotoxic death in cerebellar granule neurons. Proc. Natl. Acad. Sci. U. S. A. 93, 3984–3989.

Tsou, K., Lowitz, K.A., Hohmann, A.G., Martin, W.J., Hathawai, C.B., Bereiter, D.A., Walker, J.M., 1996. Suppression of noxious stimulus-evoked expression of Fos protein-like immunoreactivity in rat spinal cord by a selective cannabinoid agonist. Neuroscience 70, 791–798.

Tsou, K., Brown, S., Sanudo-Pena, M.C., Mackie, K., Walker, J.M., 1998. Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. Neuroscience 83, 393–411.

Wilson, R.I., Nicoll, R.A., 2002. Endocannabinoid signaling in the brain. Science 296, 678–682.

Woolf, C.J., Shortland, P., Coggeshall, R.E., 1992. Peripheral nerve injury triggers central sprouting of myelinated afferents. Nature 355, 75–78.

Zhang, J., Hoffert, C., Vu, H.K., Groblewski, T., Ahmad, S., O'Donnell, D., 2003. Induction of CB2 receptor expression in the rat spinal cord of neuropathic but not inflammatory chronic pain models. Eur. J. Neurosci. 17, 2750–2754.

Zygmunt, P.M., Chuang, H., Movahed, P., Julius, D., Hogestatt, E.D., 2000. The anandamide transport inhibitor AM404 activates vanilloid receptors. Eur. J. Pharmacol. 396, 39–42.