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## Involvement of subtype 1 metabotropic glutamate receptors in apoptosis and caspase-7 over-expression in spinal cord of neuropathic rats

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### Abstract

The effect of the non-selective, 1-aminoindan-1,5-dicarboxylic acid (AIDA), and selective (3,4-dihydro-2H-pyrido[2,3-b]quinolin-7-yl)-(cis-4-methoxycyclohexyl) methanone (JNJ16259685), metabotropic glutamate subtype 1 (mGlu1) receptor antagonists, on rat sciatic nerve chronic constrictive injury (CCI)- induced hyperalgesia, allodynia, spinal dorsal horn apoptosis, and gliosis was examined at 3 and 7 days post-injury. RT-PCR analysis showed increased expression of bax, apoptotic protease-activating factor-1 (apaf-1), nestin, GFAP, and caspase-7 mRNA in the dorsal horn spinal cord by 3 days post-CCI. At 7 days post-CCI, only over-expression of bcl-2, nestin and GFAP mRNA was observed. Administration of AIDA reduced thermal hyperalgesia and mechanical allodynia at 3 and 7 days post-CCI; administration of JNJ16259685 reduced thermal hyperalgesia at 3 and 7 days post-CCI, but not mechanical allodynia. AIDA decreased the mRNA levels of bax, apaf-1, GFAP and caspase-7 genes. JNJ16259685 increased the mRNA levels of bcl-2 and GFAP gene, and decreased APAF-1 and caspases-7 genes. Inhibiting mGlu1 receptors also reduced TUNEL-positive profiles and immunohistochemical reactivity for caspase-7. We report here that despite inhibiting CCI-induced over-expression of pro-apoptotic genes in the spinal cord dorsal horn, the selective mGlu1 receptor antagonist JNJ16259685 exerted only a slight and transient allodynic effect. Moreover, JNJ16259685, but not the non-selective AIDA, increased astrogliosis which may account for its decreased analgesic efficacy. This study provides evidence that the contemporary and partial blockade of group I and likely ionotropic glutamate receptors may be a more suitable therapy than selective blockade of mGlu1 subtype receptors condition to decrease neuropathic pain symptoms.

### Keywords

neuropathic pain; metabotropic glutamate receptor; apoptosis; caspase-7

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## Introduction

Neuropathic pain is caused by an injury or dysfunction in the peripheral or central nervous system [1,2,3]. In neuropathic pain conditions, noxious stimuli are perceived as more painful (hyperalgesia), and normal, harmless stimuli may elicit pain (allodynia) [1]. The mechanisms that centrally control such spread of pain stem from neurochemical and functional changes, and therefore, neuropathic pain should be considered a neuropathological condition. Evidence for this classification is offered by several animal models of chronic pain showing that a sustained release of glutamate, cytokines and neurotrophic factors (i.e. neurokinins, BDNF, TNF-alpha, etc.) induces sensitisation at both dorsal root ganglion neurons and second-order neurons in the dorsal horn [4,5,6]. Indeed, there is evidence that synaptic facilitation requires the release of neurokinins, glutamate, and tropomyosin-related kinase (TRK) family of neurotrophin receptors. Spinal N-methyl-D-aspartate (NMDA) glutamate receptor contributes to triggering intracellular signals that induce long lasting effects at the transcriptional level [3,7,8,9,10,11,12].

Moreover, not only central sensitisation, but also a disinhibitory process associated either with excitotoxic-induced neuronal death [2,10,13,14] or with a rearrangement in the pathophysiology of the endogenous antinociceptive pathway [15], has been suggested to be required for the induction of dorsal horn circuitry sensitization. Morphological studies suggested the occurrence of neuronal apoptosis in the spinal cord following peripheral nerve insult [16,17]. Nevertheless, there is also evidence that astrocytes undergo apoptosis in the spinal cord of neuropathic rat [18]. We have shown that the occurrence of glutamate-dependent apoptosis may be an early and rapid event, as pro-apoptotic bax and bcl-xS mRNA levels increased in the spinal cord of rats within 2–3 days after sciatic nerve chronic constrictive injury (CCI) [19,20]. Many studies have highlighted the involvement of metabotropic glutamate (mGlu) receptors in nociception control [21,22,23,24,25,26]. Group I metabotropic glutamate receptors (mGluRs) (mGlu1 and -5) have been implicated in the processes of central sensitization and persistent nociception [27]. Treatment with selective antagonists for mGlu1 and mGlu5 receptors attenuated the development of mechanical allodynia and decreased extracellular glutamate in the spinal cord [28,29]. We have previously shown that mGlu5 receptor blockade was transiently anti-allodynic and reduced spinal cord apoptosis in neuropathic rats [20], although an analysis of terminal caspase activation was not carried out in that study.

Caspases, a family of cysteine proteases, play a critical role in the execution phase of apoptosis and are responsible for many of the biochemical and morphological changes associated with apoptosis [30]. Procaspase-9 has been proposed as an initiator caspase that activates the effector caspases-3, -6, and -7 [31]. This apoptotic step is implicated in motorneuron degeneration produced by mutant superoxide dismutase-1 [32], in models of peripheral neuropathy [33], Alzheimer disease [34,35,36], Huntington disease [37], and neuropathic pain, suggesting a critical role for these proteases in numerous neurological conditions. To our knowledge, however, there is no study aimed at identifying the possible relationship between mGlu1 receptors, the activation of specific spinal caspases and the development of hyperalgesia/allodynia in neuropathic pain models.

In this study, we have addressed the issue of whether the blockade of mGlu1 receptors might modify early over-expression of pro-apoptotic and gliosis genes in the spinal cord dorsal horn, together with allodynia and hyperalgesia development by CCI of the sciatic nerve.

## Materials and Methods

### Animals

Male Wistar rats (Harlan, Udine, Italy) (250–300 g) were housed 3 per cage under controlled illumination (12:12 h light:dark cycle; light on 06.00 h) and environmental conditions (room temperature 20–22°C, humidity 55–60%) for at least 1 week before the commencement of experiments. Rat chow and tap water were available *ad libitum*. Behavioural testing was performed before surgery to establish a baseline for comparison with post-surgical values. CCI and sham rats were assessed for thermal and mechanical hyperalgesia before being used for further study in other tests. Rats were divided into multiple groups:

1. Naïve rats (n = 5);
2. Sham-operated rats treated for 3 days with vehicle (n = 5) or 1-aminoindan-1,5-dicarboxylic acid (AIDA) (2 mg/Kg i.p.) (n = 5); or 3,4-dihydro-2H-pyrano[2,3-b]quinolin-7-yl)-(cis-4-methoxycyclohexyl) methanone (JNJ16259685) (5 mg/Kg i.p.) (n = 5);
3. CCI rats treated for 3 days with vehicle (n = 5) or AIDA (2 mg/Kg i.p.) (n = 5); or JNJ16259685 (5 mg/Kg i.p.) (n = 5);
4. Sham-operated rats treated for 7 days with vehicle (n = 5) or AIDA (2 mg/Kg i.p.) (n = 5); or JNJ16259685 (5 mg/Kg i.p.) (n = 5);
5. CCI rats treated for 7 days with vehicle (n = 5) or AIDA (2 mg/Kg i.p.) (n = 5); or JNJ16259685 (5 mg/Kg i.p.) (n = 5).

In this study, a minimal still effective dose of AIDA (2 mg/kg) has been chosen based on previous evidence that it does not affect motor coordination [38] and poorly interacts with other glutamate receptor subtypes. Moreover, JNJ16259685 has been used because it is about 6000 times more potent than CPCCOEt [39].

The experimental procedures were approved by the Animal Ethics Committee of the Second University of Naples. Animal care was in compliance with the IASP and European Community (E.C. L358/1 18/12/86) guidelines on the use and protection of animals in experimental research. All efforts were made to minimise animal suffering and the number of animals used.

### Drugs

The mGlu1 glutamate receptor antagonist AIDA (Tocris, Bristol, UK) was dissolved in saline with NaOH. Few drops of NaOH were supplemented until the solution was clear. The mGlu1 glutamate receptor antagonist JNJ16259685 (Johnson and Johnson Pharmaceutical Research, Janssen Pharmaceutica, Beerse, Belgium) was dissolved in Ringer solution. Vehicle was saline solution for AIDA, and the Ringer solution for JNJ16259685.

### Sciatic nerve chronic constriction injury

Mononeuropathy was induced according to the method of Bennet and Xie (1988). Rats were anaesthetised with sodium pentobarbital (60 mg/kg, i.p.). The sciatic nerve was exposed at mid-thigh and freed of connective tissue, four chromic gut ligatures (4–0) were loosely tied around the nerve. Sham rats were anaesthetised, the sciatic nerve was exposed at the same level, but not ligated.

### Nociceptive Behaviour

Thermal hyperalgesia was evaluated by using a Plantar Test Apparatus (Ugo Basile, Varese, Italy). On the day of the experiment each animal was placed in a plastic cage (22cm × 17cm

× 14cm; length × width × height) with a glass floor. After a 30 min habituation period, the plantar surface of the hind paw was exposed to a beam of radiant heat through the glass floor. The radiant heat source consisted of an infrared bulb (Osram halogen-halophot bulb; 8 V, 50 W). A photoelectric cell detected light reflected from the paw and turned off the lamp when paw movement interrupted the reflected light. The paw withdrawal latency was automatically displayed to the nearest 0.1 sec; the cut-off time was 30 s in order to prevent tissue damage.

Mechanical allodynia was measured by using Dynamic Plantar Anesthesiometer (Ugo Basile, Varese, Italy). Rats were allowed to move freely in one of the two compartments of the enclosure positioned on the metal mesh surface. Rats were adapted to the testing environment before any measurements were taken. After that the mechanical stimulus was delivered to the plantar surface of the hindpaw of the rat from below the floor of the test chamber by an automated testing device. A steel rod (2 mm) was pushed with electrical ascending force (0–30 g in 10 sec). When the animal withdrew its hindpaw, the mechanical stimulus was automatically withdrawn and the force recorded to the nearest 0.1 g. Nociceptive responses for thermal and mechanical sensitivity were expressed as thermal paw withdrawal latency (PWL) in seconds and mechanical paw withdrawal threshold (PWT) in grams.

Sham and CCI rats were treated with AIDA or JNJ16259685 (AIDA: 2 mg/kg i.p., twice a day; JNJ16259685: 5 mg/Kg i.p.). Each rat served as its own control, the responses being measured both before and after surgical procedures.

### RNA extraction and RT-PCR

Total RNA was extracted from homogenized dorsal sides of lumbar L4–L5 spinal cord using an RNA Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's protocol. The extracted RNA was subjected to *DNase I* treatment at 37° C for 30 min. The total RNA concentration was determined by UV spectrophotometer. The mRNA levels of the genes under analysis were measured by RT-PCR amplification, as previously reported [40]. RT minus controls were carried out to check potential genomic DNA contamination. These RT minus controls were performed without using the reverse transcriptase enzyme in the reaction mix. Sequences for the rat mRNAs from GeneBank (DNASTAR INC., Madison, WI) were used to design primer pairs for RT-PCRs (OLIGO 4.05 software, National Biosciences Inc., Plymouth, MN). Each RT-PCR was repeated at least four times to achieve best reproducibility data. A semiquantitative analysis of mRNA levels was carried out by the "Gel Doc 2000 UV System" (Bio-Rad, Hercules, CA). The measured mRNA levels were normalised with respect to hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), chosen as housekeeping gene. The *HPRT* gene expression did not change in several experimental conditions [41]. To our knowledge there is no molecular evidence for variation in *HPRT* mRNA-levels in CCI neuropathic pain model. The gene expression values were expressed as arbitrary units ±SE. Amplification of genes of interest and *HPRT* were performed simultaneously.

### Immunohistochemistry

The animals were perfused transcardially with 150 ml of saline solution (0.9% NaCl), and 250 ml of Bouin's fixative (picric acid, 40% formaldehyde, acetic acid glacial). Lumbar spinal cord at the L4–L5 level was taken out and kept in the fixative for 24 hours followed by 75% ethanol for another 24 hours. The tissue was then dehydrated in graded ethanols and embedded in paraffin resin. Serial 5 µm sections of the lumbar segments of the spinal cord were sliced. Sections were deparaffinized by further passages in Citric Solvent (Fisher Scientific, Pittsburgh, PA) and in isopropanol. Citrate antigen retrieval was performed by placing slides in citrate buffer (0.1 M citric acid monohydrate, and 0.1 M sodium citrate, Sigma) in a water filled steamer for 20 min. After cooling, endogenous peroxidase activity was quenched by

incubating sections for 15 min in 0.3% hydrogen peroxide in PBS and non-specific antibody binding was inhibited by incubation for 30 min in blocking solution (1% BSA, 0.2% powdered skim milk, 0.3% Triton-X 100 in PBS). Primary antibodies were diluted in PBS blocking buffer and slides were incubated overnight at 4°C in primary antibodies to rabbit anti-GFAP (1:10,000; DAKO, CA) and rabbit anti-caspase-7 (1:100; Cell Signaling, CA). The rabbit anti-cleaved caspase-7 antibody detects endogenous levels of the large fragment of caspases-7 resulting from cleavage at aspartic acid 198. The antibody does not cross-react with full length inactive caspases-7 or with other caspases. HRP-labelled secondary antibodies (1:1,000; Jackson ImmunoResearch Laboratories, West Grove, PA) specific to the IgG species used as a primary antibody and fluorescein labeled tyramide amplification (TSA; Perkin Elmer Life Science Products, Boston, MA) were used to locate the specific antigens in each section. Sections were counterstained with bisbenzimidazole (Hoechst 33258) and mounted with PBS:glycerol (1:1). For GFAP-optical detection, streptavidin-labeled secondary antibodies (1:200; Molecular Probes, Eugene, OR) specific to the IgG species used as a primary antibody and DAB reaction were used to locate the specific antigens in each section. Sections were counterstained with Mayer's hematoxyline, dehydrated in graded alcohols, fixed in xylene, and mounted with Entellan (EMS, Hatfield, PA). The staining was visualized using an optical microscope (Leica DMLB, Cambridge, UK). Positive profiles were quantified within the selected areas (498.7 mm<sup>2</sup>) in the dorsal horn by using digital system IM500 Leika.

Fluorescently labelled sections were viewed with a Zeiss Axioskop (Zeiss, Germany) epifluorescence microscope and the images were captured with a Zeiss AxioCam and AxioVision software.

We used different secondary antibodies for GFAP immunostaining in order to better clarify whether JNJ16259685 treatment was able to affect astrocyte morphology and activation rather than a quantification of increased positive cell number.

For Caspase-7 fluorescence-staining, digitised images were obtained from 5 non consecutive sections per every L4–L5 lumbar spinal cord (total sections per group = 25), using an instrument connected to an Axioskop Zeiss microscope. Quantification of numbers of Caspase-7 profiles in the superficial dorsal horn was performed with NeuroLucida Software (MicroBrightField, Colchester, VT) using a 20X plan apochromat objective by an observer blind to the treatment. The areas to be analysed were outlined using NIH Image Software, and the integrate optical density (IOD) of staining in each outlined area was measured. Next, measurements from corresponding areas of the dorsal horn were compared. Staining on the control side always resembled that in sham animals; thus, a percentage difference in the IOD was calculated as  $100 \times (\text{IOD}_{\text{CCI}} - \text{IOD}_{\text{control}}) / \text{IOD}_{\text{control}}$ . This measure is positive when the staining on the CCI side exceeds that on the control side and is negative when the opposite is the case. For GFAP staining quantitative assessment was carried out by determining the intensity within fixed area of the dorsal horn. A square measuring 10<sup>4</sup> μm<sup>2</sup> was placed onto areas of the lateral, central, and medial dorsal horn. The intensity was recorded within these areas.

### Detection of DNA cleavage

To analyse fragmented DNA, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labelling (TUNEL) was performed using an in situ cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany). The kit contains terminal deoxynucleotidyl transferase, which catalyses polymerisation of fluorescein dUTP to free 3'-OH DNA ends in a template-independent manner. Incorporated fluorescein is detected by sheep anti-fluorescein Fab fragments, conjugated with horseradish peroxidase (POD). After substrate reaction, the slides were counterstained with haematoxylin. TUNEL-positive cells were identified directly by light microscopic examination. Positive cells in five non consecutive sections (10 μm),



randomly selected from each L4–L5 lumbar spinal cord, were quantified by an experimenter blinded to the treatment and expressed as means  $\pm$ SE per group.

## Statistics

Behavioural data are represented as means  $\pm$  SE. ANOVA, followed by Sheffe's *post hoc* test, was used to determine the statistical significance between mean behavioural difference scores pre- and post-surgery in sciatic nerve-constricted and sham-operated animals. Histological data are shown as the means  $\pm$  SE. ANOVA and Dunnett's *post hoc* test was used to determine the statistical significance between groups. Molecular data are shown as means  $\pm$  SE. ANOVA, followed by Student-Neuman-Keuls *post hoc* test was used to determine the statistical significance between groups.  $P < 0.05$  was considered statistically significant.

## Results

### **AIDA prevents thermal hyperalgesia and mechanical allodynia by 3 and 7 days CCI, JNJ16259685 prevents thermal hyperalgesia, but not mechanical allodynia**

Post-surgery mean difference scores for thermal hyperalgesia were significantly lower for the CCI sciatic nerve groups at 3 and 7 days (Fig. 1 A, B) than sham rats. The increased sensitivity on the CCI sides was not present on the contralateral sides (data not shown). Similarly, sciatic nerve CCI rats showed mechanical allodynia only on the ipsilateral sides of the nerve ligation at 3 and 7 days post-surgery (Fig. 1 C, D). Administration of AIDA, a preferential mGlu1 receptor antagonist, was effective in preventing the appearance of thermal hyperalgesia and mechanical allodynia at both 3 and 7 days post-CCI (Fig. 1 A, C). Administration of JNJ16259685, a selective mGlu1 receptor antagonist, prevented the appearance of thermal hyperalgesia at both 3 and 7 days post-CCI (Fig. 1B), but not mechanical allodynia (Fig. 1D).

### **AIDA prevents the over-expression of *bax*, *APAF-1* and *caspase-7* genes, and increases the expression of *bcl-2* gene by 3 days CCI**

The semiquantitative analysis of mRNA levels measured by RT-PCR amplification, at the L4–L5 dorsal spinal cord of rats, showed an increase in the pro-apoptotic *bax* gene but no change in the anti-apoptotic *bcl-2* gene 3 days after sciatic nerve CCI (Table 1). Indeed, at this stage the *bax/bcl-2* ratio was significantly increased (mean  $\pm$  SE of arbitrary units:  $1.89 \pm 0.17$  vs  $0.40 \pm 0.08$  in CCI with respect to the sham rats, respectively) in CCI rats. Seven days post-CCI, no change in the *bax/bcl-2* ratio was observed (mean  $\pm$  SE of arbitrary units:  $0.30 \pm 0.06$  vs  $0.40 \pm 0.08$  in CCI with respect to the sham rats, respectively) (Table 1). In sham rats, AIDA increased the expression of pro-apoptotic *bax* after 3 days of treatment, but not after 7 days. This fact could be due because the spinal cord mGlu receptors may potentially have a constitutive, tonic neurotrophic role, as we demonstrated in our previous work [20]. In fact, a number of factors (e.g. exposure time to drugs or ambient glutamate, NMDA receptor functional overactivation, function of astrocytes clearing extracellular glutamate and production of neurotoxic/neuroprotective factors) must be considered when examining the dual role (neurotoxicity and neuroprotection) that group I mGlu receptors play in the CNS. Moreover, AIDA also increased baseline expression of the anti-apoptotic *bcl-2* after 3 days of treatment (Table 1). In CCI rats, AIDA prevented *bax* over-expression and increased the anti-apoptotic *bcl-2* expression 3 days by sciatic nerve injury (Table 1).

To check activation of the apoptosome complex, expression of the apoptotic protease-activating factor-1 (*APAF-1*) was analyzed. The mRNA levels of *APAF-1* increased strongly 3 days after CCI (mean  $\pm$  SE of arbitrary units:  $1.62 \pm 0.32$  vs  $0.63 \pm 0.11$  in CCI with respect to the sham rats, respectively) (Table 1). Consistently, the expression level of the apoptotic executor gene *caspase-7* was also increased 3 days following nerve injury (mean  $\pm$  SE of arbitrary units:  $1.64 \pm 0.08$  vs  $1.02 \pm 0.06$  in CCI with respect to the sham rats, respectively)

(Table 1). Treatment with AIDA did not modify baseline *APAF-1* or *caspase-7* mRNA levels after 3 and 7 days of treatment; however, it reduced expression of both genes 3 days following CCI. AIDA inhibited the *APAF-1* over-expression in 7 day CCI spinal cord (mean  $\pm$  SE of arbitrary units:  $0.93 \pm 0.06$  vs  $1.62 \pm 0.32$  in CCI + treated and CCI rats, respectively). In sham rats, 3 days-treatment with AIDA increased the baseline level of *APAF-1* mRNA expression (mean  $\pm$  SE of arbitrary units:  $1.04 \pm 0.17$  vs  $0.63 \pm 0.11$ ) in treated and sham rats, respectively) (Table 1); however, this effect was not apparent after 7 days of treatment. For all genes examined, sham and naïve rat spinal cord showed similar levels of expression.

### **AIDA prevents the incidence of TUNEL-positive profiles in the dorsal horn spinal cord by 3 days post-CCI**

Consistent with biomolecular data, sciatic nerve CCI induced a marked increase in the incidence of TUNEL-positive profiles by 3 days post-CCI on the side ipsilateral to the nerve injury (means  $\pm$  SE:  $12 \pm 2.5$  profiles) (Fig. 2). Treatment with AIDA in CCI, but not in sham rats, caused a significant decrease in the number of TUNEL-positive profiles in the dorsal horn spinal cord at 3 (means  $\pm$  SE:  $4 \pm 0.8$  profiles) (Fig. 2), but not 7 days after CCI (data not shown).

### **JNJ16259685 increases expression of *bcl-2* mRNA by 3 and 7 days CCI, without affecting *bax* gene over-expression, and prevents the over-expression of *APAF-1* and *caspases-7* genes**

In CCI rats, JNJ16259685 treatment increased the anti-apoptotic *bcl-2* expression at 3 and 7 days by sciatic nerve injury (Table 2), without affecting pro-apoptotic *bax* over-expression. The *bax/bcl-2* ratio was decreased both at 3 days (mean  $\pm$  SE of arbitrary units:  $0.54 \pm 0.09$  vs  $1.89 \pm 0.13$  in CCI/treated and CCI rats, respectively) and 7 days by sciatic nerve CCI (mean  $\pm$  SE of arbitrary units:  $0.13 \pm 0.09$  vs  $0.30 \pm 0.07$  in CCI/treated and CCI rats, respectively).

JNJ16259685 inhibited the *APAF-1* and *caspase-7* gene over-expression in 3 days CCI spinal cord (*APAF-1*: mean  $\pm$  SE of arbitrary units:  $0.53 \pm 0.2$  vs  $1.62 \pm 0.32$  in CCI/treated and CCI rats, respectively), (*caspase-7*: mean  $\pm$  SE of arbitrary units:  $0.84 \pm 0.04$  vs  $1.64 \pm 0.08$  in CCI/treated and CCI rats, respectively). In 7 days CCI spinal cord, JNJ16259685 inhibited the *APAF-1* gene over-expression (*APAF-1*: mean  $\pm$  SE of arbitrary units:  $0.69 \pm 0.10$  vs  $0.98 \pm 0.13$  in CCI/treated and CCI rats, respectively), but not *caspase-7* gene expression which was not modified at this time as compared to the sham operated rats (*caspase-7*: mean  $\pm$  SE of arbitrary units:  $1.30 \pm 0.06$  vs  $0.83 \pm 0.09$  in CCI/treated and CCI rats, respectively).

### **JNJ16259685 prevents the incidence of TUNEL-positive profiles in the dorsal horn spinal cord by 3 days post-CCI**

Treatment with JNJ16259685 in CCI, but not in sham rats, caused a significant decrease in the number of TUNEL-positive profiles in the dorsal horn spinal cord at 3 (means  $\pm$  SE:  $6 \pm 0.7$  profiles) (Fig. 2), but not 7 days after CCI (data not shown).

### **AIDA prevents the over-expression of *nestin* and *GFAP* genes by 3 and 7 days CCI**

To check for the presence of gliosis, changes in the expression of *GFAP* and *nestin* reactive astrocytosis marker genes were analyzed. The mRNA levels of the *GFAP* gene were increased at 3 and 7 days post-CCI (Table 1). Similarly, *nestin* mRNA levels were increased at 3 and 7 days after CCI (Table 1). These molecular data are consistent with the changes in the size and shape of the astrocyte cell bodies (reactive gliosis) in the superficial laminae ipsilateral to CCI that we observed previously [20] and confirmed in this study (fig. 3). In sham rats, AIDA increased the baseline expression of *nestin* and reduced *GFAP* (mean  $\pm$ SE of arbitrary units:  $3.77 \pm 0.34$  vs  $5.21 \pm 0.31$  in treated and untreated sham rats, respectively) after 3 days of treatment (Table 1). These changes were still apparent in sham rats after 7 days of treatment

with AIDA (table 1). In CCI rats, AIDA completely abolished *GFAP* over-expression and also decreased *nestin* levels at 3 days CCI as compared to CCI untreated rats (table 1). Moreover, AIDA normalized both *nestin* and *GFAP* mRNA levels 7 days after CCI (table 1).

Treatment with AIDA produced a reduction of GFAP immunoreactivity in the superficial dorsal horn of the spinal cord 7 days after CCI (GFAP immunoreactivity in arbitrary units  $\pm$  SEM:  $60.60 \pm 1.89$  vs  $44.70 \pm 1.44$ , in CCI-rats and CCI-AIDA treated rats, respectively;  $P < 0.003$ ) (Fig. 3B).

### **JNJ16259685 increases the expression of GFAP gene by 3 days CCI**

JNJ16259685 increased the mRNA levels of GFAP gene at 3 days by sciatic nerve CCI (mean  $\pm$  SE of arbitrary units:  $19.79 \pm 0.35$  vs  $13.02 \pm 0.60$  in CCI/treated and CCI rats, respectively)

Treatment with JNJ16259685 produced a change in GFAP positive-cell morphology in the superficial dorsal horn of the spinal cord 7 days after CCI (Fig. 4).

### **CCI increases caspase-7 immunoreactivity**

We investigated the location of the active form of caspase-7 expression in the dorsal spinal cord following sciatic nerve injury and after treatment with AIDA or JNJ16259685 by using immunohistochemistry. As shown in fig. 5, constitutive immunohistochemical reactivity for the active form of caspase-7 was found in the superficial laminae of dorsal horn of the spinal cord of sham or naïve rats. However, a strong immunohistochemical reactivity for the active form of caspase-7 was observed at 3 (Fig. 5B), and 7 days after CCI (data not shown). The IOD of caspase-7 immunostaining was increased by  $38 \pm 6\%$  in 3 days CCI-rats vs sham rats. Seven days CCI-rats showed increased IOD of caspases-7 immunostaining by  $20 \pm 5\%$  vs sham rats. At this time point, caspase-7 enzyme activity was still present, although caspases-7 mRNA levels did not increase. The antibody used is direct toward the active form of Caspase-7, and this evidence may suggest that 7 days CCI Caspase-7 is still active in the spinal cord, with a possible role in pain maintenance. Consistent with RT-PCR analysis, AIDA reduced the staining for caspase-7 immunoreactivity in the superficial laminae of the spinal cord at 3 days post-CCI (Fig. 5C)

## **Discussion**

It has been shown that increased expression of group I mGlu receptors parallels the development of thermal hyperalgesia and mechanical allodynia, and this change has been suggested to contribute to the development and maintenance of chronic central pain syndrome [22,24,29,43]. This study provides molecular evidence that, in the model of sciatic nerve ligature in the rat [40], neuropathic pain is associated with marked activation of caspase-7 together with its transient increase in expression, and apoptosis in the superficial laminae of the spinal cord.

In our previous work [19] we found that a massive neuro-protective machinery might be activated in the spinal cord dorsal horn of neuropathic rats. Fourteen days after injury we observed no sign of an active apoptotic process, as determined by TUNEL and Hoechst analysis. In addition, in our previous work [20] we showed a possible dual role for bcl-2 family gene after sciatic nerve CCI. In the first few days post-CCI, the bax/bcl-2 ratio increased massively favouring a pro-apoptotic condition. We found that apoptosis occurred transiently within the substantia gelatinosa 3 days post-CCI. These bcl-2 family genes showed an inversed pattern of expression at later stages. Thus, there was a significant lowering in bax/bcl-2 ratio over time as a consequence of increased expression of anti-apoptotic bcl-2 gene. This fact could be the same in the case of caspases-7 gene expression found in the current study. Apoptosis



and neuropathic pain develop together just in the early phase of induction and maintenance of pain. Afterward, the apoptotic process appears to be slowed and then blocked. This fact could be due to the own neuroprotection mechanisms of the nervous system [20].

We have shown in a previous study that the selective blockade of mGlu5 receptors with MPEP had anti-hyperalgesic and acute anti-allodynic effects in neuropathic rats [20]. In the current study, we show that the selective mGlu1 receptor antagonist JNJ16259685 is able to prevent thermal hyperalgesia, but not the development of mechanical allodynia in rats. On the other hand, we show that the preferential mGlu1 receptor antagonist AIDA prevents the development of the mechanical allodynia in rats, suggesting that mGlu1, opposite to mGlu5 receptor [20], appears to be less involved in mediating abnormal mechanical responses after nerve injury. It is worthy of note that AIDA may also antagonize NMDA and AMPA ionotropic receptors [44], and that group I mGlu receptor agonists produce potentiation of the responses to NMDA and AMPA receptors [45,46]. Given that, it is likely that combined involvement of group I metabotropic and ionotropic glutamate receptors takes part in neuropathic pain development and maintenance [47]. Our current study confirms this hypothesis. The fact that JNJ16259685 is able to increase *bcl-2* expression, without affecting pro-apoptotic *bax* expression, might mean that blockade of mGlu1 receptors could be protective only in the early phase of neuropathic pain induction. Stimulation of these receptors up-regulates *c-jun* and *c-fos* gene expression [48], in this way activating multiple downstream signalling regulating several functions including programmed cell death.

In addition to confirming the over-expression of the pro-apoptotic *bax* gene [20,42], we found that *APAF-1* gene expression was significantly increased in the first 2–3 days following CCI. This protein has a key role in the transactivation of the downstream effectors caspases-3 and -7 [32,49,50,51,52,53]. Neuronal apoptosis is typically mediated by caspases, and up-regulation of caspase gene expression and protein activity have been reported in rodent models of neurological diseases [32,54,55]. Caspase signalling pathways may also contribute to pain-related behaviour in several models of neuropathies [33].

We have previously shown caspases-3 activation in a mouse model of CCI neuropathic pain [48]. To confirm a role for caspase signalling pathways in pain-related behaviour associated with sciatic nerve CCI, we quantified expression and localization of these proteases in the dorsal horn in neuropathic rats. We found activation of caspase-7 in the superficial laminae of the spinal cord 3 days after sciatic nerve CCI. Consistent with our observation, a few previous reports have shown highly localized expression of caspase-7 in other brain injury paradigms [36,56].

These findings suggest that specific effector caspases may play selective roles in neurological diseases, as well as in cancer cell lines [36,57]. It has been shown that neuronal death can proceed in the absence of caspase-3 [58] or by activation of caspase-6 and caspase-7 [59]. Caspase-7 is present in the brain and its transient (2–4 days) up-regulation and activation after traumatic brain injury has been reported [56]. Although caspase-3 and caspase-7 are both effector caspases, caspase-7 has a different preferred enzymatic site (specific substrates) as compared to caspase-3 [60]. Therefore, caspase-7 could have either a complementary or even independent role relative to caspase-3 [56]. Separate from their role as apoptotic inducers, caspases may be involved in other neural functions including synaptic plasticity. For example, caspase-3 has been found constitutively active in neurons [61], and there is evidence that caspase-3 may be involved in the long-term potentiation (LTP) phenomenon since some LTP key players are caspase-3 substrates [61]. In addition, it has been shown that blocking caspase activity is able to prevent trans-synaptic neuronal apoptosis and the loss of inhibition in lamina II of the dorsal horn after peripheral nerve injury [62]. Given that, we can not exclude a novel role for caspase-7 in controlling neuro-adaptive processes, such as learning and memory [63].

In this report, we have found constitutive activation of caspase-7 in the outer laminae of spinal cord of neuropathic rats suggesting that it may play non-apoptotic roles in the nervous system. Our result showing that JNJ16259685 is able to block caspases-7 gene expression only at 3 days post-injury enhances this statement.

We have previously shown that in neuropathic rats the cells that over-express pro-apoptotic *bax* gene and undergo apoptosis are neurons [20]. It is likely, therefore, that also in this study the cells over-expressing caspase-7 may be neurons; however, we can not exclude caspase-7 over-expression in astrocytes [56].

Opposite to AIDA, the blockade of mGlu1 receptors with JNJ16259685, while having the same anti-apoptotic effect, triggered over-expression of gliosis associated genes. These data confirm an active involvement of astrocytes, which have high expression of mGlu1 receptors, in the maintenance of pain [64,65]. Astrocytes, through their mGlu1 receptors, can maintain neuronal homeostasis by controlling the extracellular glutamate levels [66]. Activation of spinal cord astrocytes participates in neuronal plasticity, as well as in many neurodegenerative diseases [67]. Activated glia enhance pain transmission by releasing several neuroactive substances (e.g. proinflammatory/pro-allodynic cytokines, glutamate, reactive oxygen species, nitric oxide) [68,69], which may serve as a driving force for triggering and maintaining pain [6,68,70]. There are also evidence to support a role of spinal astrocytes in maintaining chronic pain. In particular, c-Jun N-terminal kinase (JNK) is activated persistently in spinal astrocytes in a neuropathic pain condition produced by spinal nerve ligation. This activation is required for the maintenance of neuropathic pain [71]. Thus, the presence of activated glia could explain the mechanical allodynia that is still present with JNJ16259685 treatment. Besides astrocytes, activated spinal cord microglia show altered morphology, gene expression and function, and have an ongoing role in pain-associated behaviours [72,73,74]. p38 MAPK is activated in spinal microglia after nerve injury and contributes importantly to neuropathic pain development and maintenance [75]. It has been recently demonstrated that disinhibition of neurons in the superficial spinal dorsal horn, via microglia - neuron signaling leading to disruption of chloride homeostasis, is a potential cellular substrate for neuropathic pain [76]. The fact that we found here a decrease in reactive astrocytes with AIDA, together with a reduction in mechanical allodynia, might suggest a pivotal role for these cells in pain processing and maintenance. Spinal cord microglia involved in the nociceptive pathway could potentially offer a novel target for pain treatment

In conclusion, this study provides evidence that the contemporary and partial blockade of group I and likely ionotropic glutamate receptors may be a more suitable therapy than selective blockade of mGlu1 subtype receptors to decrease neuropathic pain symptoms. In the early phase of pain induction, caspase-7 could be a novel therapeutic target to reduce the pain-associated apoptotic process.

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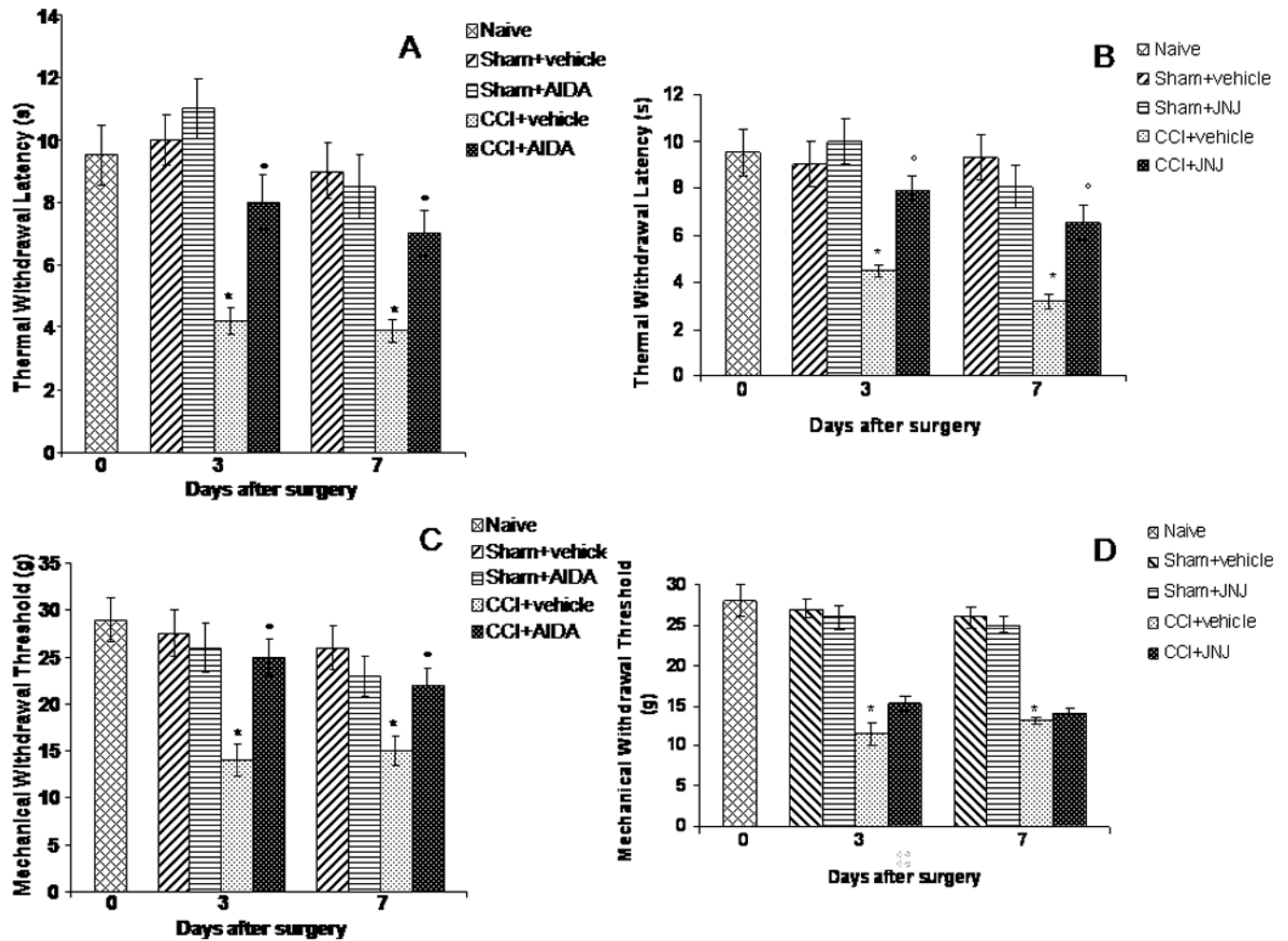
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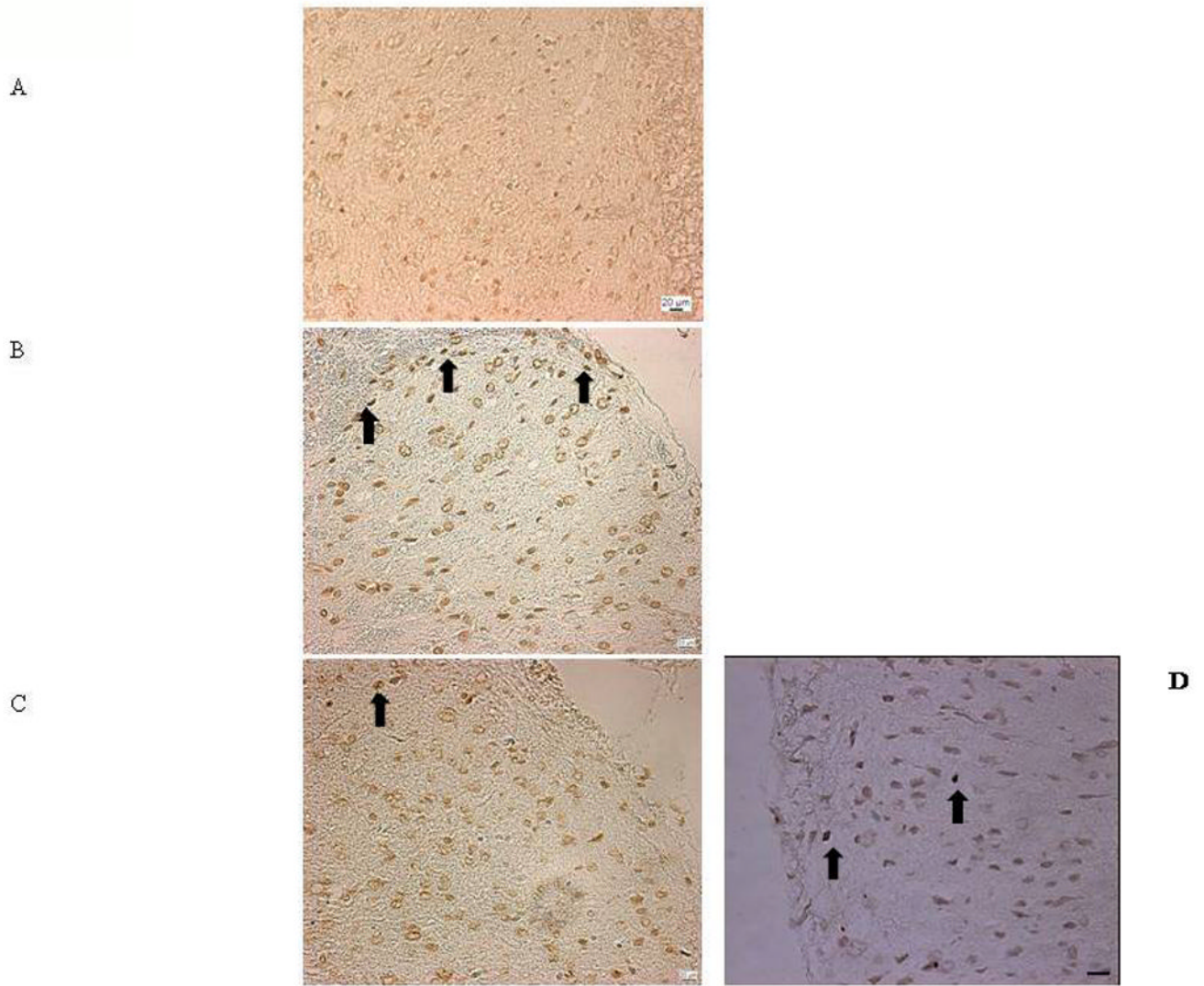
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**Figure 1.**

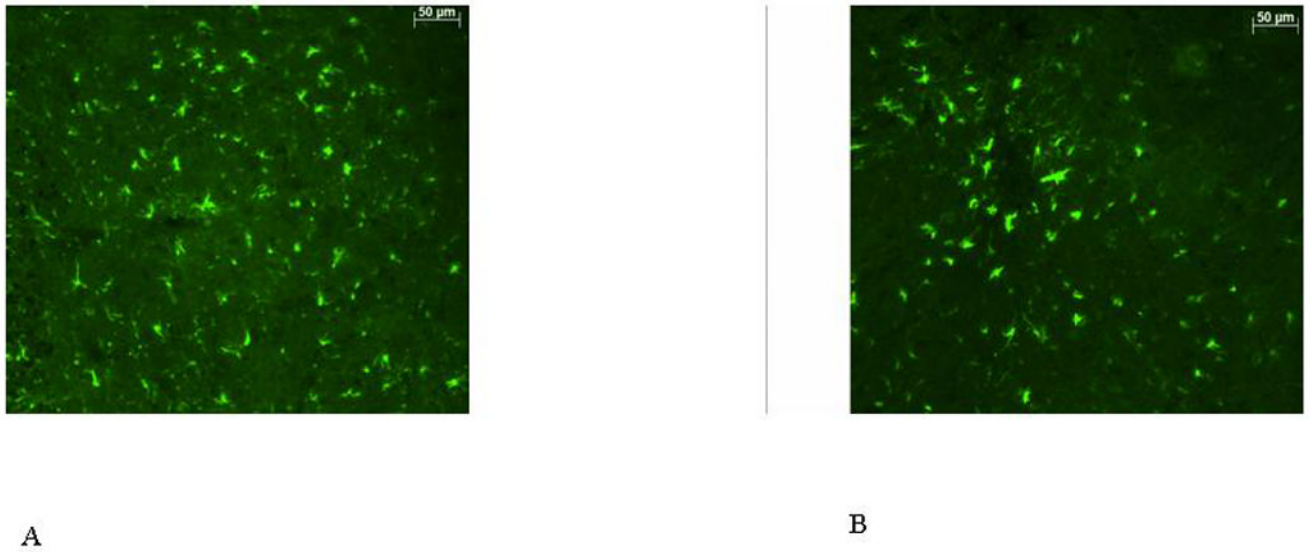
Effects of AIDA and JNJ16259685 on reflex withdrawal responses to noxious thermal and mechanical stimuli in CCI rats. Data represent the mean of thermal PWL (sec  $\pm$  s.e.) and the mechanical PWT (g  $\pm$  s.e.), for thermal hyperalgesia and mechanical allodynia, respectively, of 5 randomly selected rats for each histogram. The onset of CCI-induced thermal hyperalgesia was evaluated in ipsilateral (A, B) and contralateral (data not shown) sides.

Post-surgery mean difference scores for thermal hyperalgesia were significantly lower for the CCI sciatic nerve groups at 3 and 7 days (Fig. 1 A, B). The increased sensitivity on the CCI sides was not present on the contralateral sides. Similarly, sciatic nerve CCI rats showed mechanical allodynia only the ipsilateral sides of the nerve ligature at 3 and 7 days post-surgery (Fig. 1 C, D). Administration of AIDA (2 mg/Kg i.p. twice, daily), a preferential mGlu1 receptors antagonist, was effective in preventing the appearance of thermal hyperalgesia and mechanical allodynia at both 3 and 7 days post-CCI (Fig. 1 A, C). Administration of JNJ16259685 (5 mg/Kg i.p., daily), a selective mGlu1 receptors antagonist, prevented the appearance of thermal hyperalgesia at both 3 and 7 days post-CCI (Fig. 1B), but not mechanical allodynia (Fig. 1D). Vehicle is ringer solution (0.9 % NaCl). \* $p < 0.05$  vs sham operated rats or control rats,  $^{\circ}p < 0.05$  vs CCI rats.



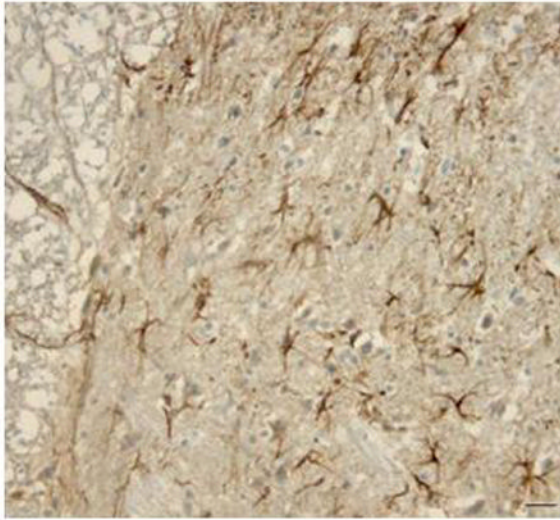
**Figure 2.**

TUNEL-positive profiles in dorsal horn spinal cord of rats 3 days after sciatic nerve CCI. CCI induced a marked increase in the incidence of TUNEL-positive profiles by 3 days post-surgery in the ipsilateral CCI (B), as compared to sham-operated rats (A), as shown by arrows. AIDA treatment caused a reduction of the TUNEL-positive profiles by 3 days post-CCI (C). The same effect was observed after JNJ16259685 treatment (D). Scale bars: 20 μm.

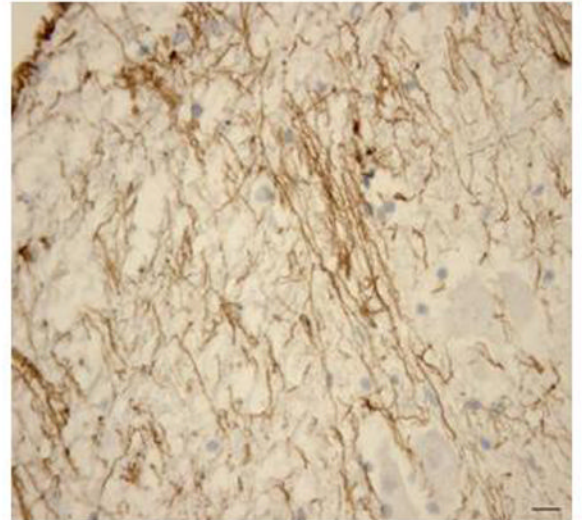


**Figure 3.** AIDA partially reduced reactive gliosis in the lumbar spinal cord of rats with CCI. GFAP immunoreactivity in 5 μm-thick nonadjacent sections from L4–L5 lumbar spinal cord from 7 days sciatic nerve CCI rats before (A) and after (B) AIDA treatment. Astrocyte morphology was characterized by changes in cell sizes after 7 days CCI. This reactive gliosis was attenuated by AIDA. Scale bar: 50 μm





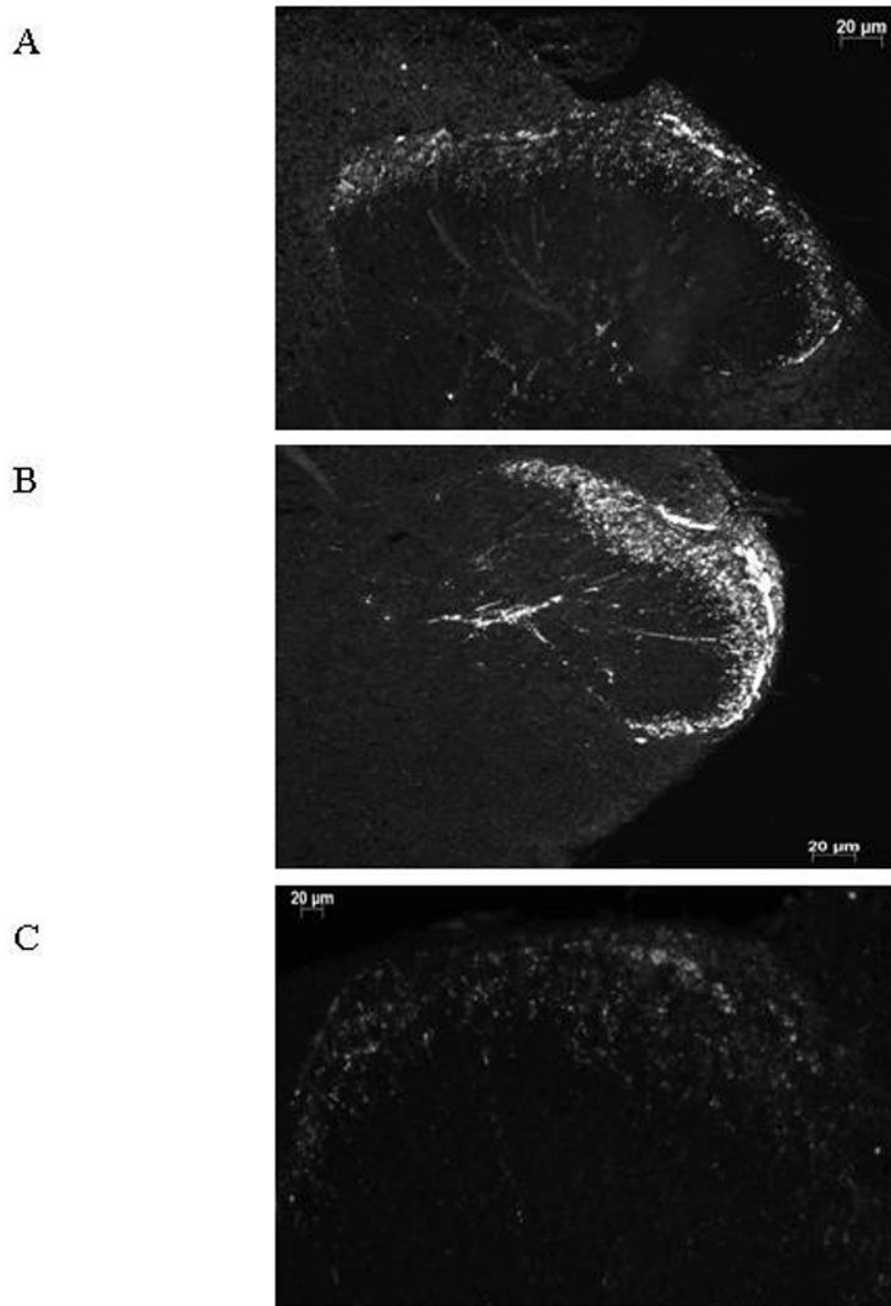
A



B

**Figure 4.** JNJ16259685 increased reactive gliosis in the lumbar spinal cord of rats with CCI. GFAP immunoreactivity in 5  $\mu\text{m}$ -thick nonadjacent sections from L4–L5 lumbar spinal cord from 7 days sciatic nerve CCI rats before (A) and after (B) JNJ16259685 treatment. Astrocyte morphology was characterized by changes in cell sizes after 7 days CCI. Scale bar: 50  $\mu\text{m}$





**Figure 5.** Caspase immunostaining. Caspase-7 immunostaining in lamina II spinal cord of rats 3 days after sciatic nerve CCI. Strong immunohistochemical reactivity for caspase-7 was found in the dorsal horn cells of the spinal cord at 3 days after CCI (A: sham rat; B: CCI-rat). At 3 days post-CCI, AIDA treatment resulted in reduction staining for caspase-7 immunoreactivity in the superficial laminae of the spinal cord (C). Scale bar: 20  $\mu\text{m}$ .

The mRNA levels (mean±SE) of the genes under analysis measured by RT-PCR amplification are reported. Each RT-PCR was repeated at least four times. The semiquantitative analysis of mRNA levels was carried out by the "Gel Doc 2000 UV System" (Bio-Rad, Hercules, CA). The measured mRNA levels were normalised with respect to *HPRT* (housekeeping gene) and gene expression values were expressed as arbitrary units ± SE.

Table 1

| Gene           | Sham      | Sham/AIDA 3 days | CCI 3 days  | CCI/AIDA 3 days         | Sham/AIDA 7 days | CCI 7 days  | CCI/AIDA 7 days         |
|----------------|-----------|------------------|-------------|-------------------------|------------------|-------------|-------------------------|
| bax/HPRT       | 0.17±0.03 | 0.85±0.12*       | 0.68±0.13*  | 0.26±0.04 <sup>o</sup>  | 0.08±0.01        | 0.21±0.02   | 0.04±0.01 <sup>oo</sup> |
| bcl-2/HPRT     | 0.43±0.05 | 0.73±0.037       | 0.36±0.04   | 0.51±0.05 <sup>o</sup>  | 0.42±0.03        | 0.71±0.04*  | 0.25±0.02 <sup>oo</sup> |
| nestin/HPRT    | 1.25±0.07 | 2.11±0.17*       | 5.31±0.37*  | 3.59±0.18 <sup>oo</sup> | 2.14±0.19*       | 2.74±0.27*  | 1.54±0.15 <sup>o</sup>  |
| GFAP/HPRT      | 5.21±0.31 | 3.77±0.34*       | 13.02±0.60* | 5.22±0.36 <sup>o</sup>  | 2.41±0.14*       | 11.04±1.21* | 4.85±0.33 <sup>o</sup>  |
| APAF-1/HPRT    | 0.63±0.11 | 1.04±0.17*       | 1.62±0.32*  | 0.93±0.06 <sup>o</sup>  | 0.54±0.08        | 0.98±0.13*  | 0.83±0.14               |
| caspase-7/HPRT | 1.02±0.06 | 1.15±0.07        | 1.64±0.08*  | 1.17±0.05               | 0.99±0.06        | 0.83±0.09   | 1.04±0.04               |

\* p<0.05 vs the corresponding sham.

<sup>o</sup> p<0.05 vs the CCI AIDA untreated rats, as analysed by ANOVA, followed by Student-Neuman-Keuls test.

The mRNA levels (mean±SE) of the genes under analysis measured by RT-PCR amplification are reported. Each RT-PCR was repeated at least four times. The semiquantitative analysis of mRNA levels was carried out by the "Gel Doc 2000 UV System" (Bio-Rad, Hercules, CA). The measured mRNA levels were normalised with respect to *HPRT* (housekeeping gene) and gene expression values were expressed as arbitrary units ± SE.

Table 2

| Gene           | Sham      | CCI 3 days  | CCI/JNJ16259685 3 days  | CCI 7 days  | CCI/JNJ16259685 7 days |
|----------------|-----------|-------------|-------------------------|-------------|------------------------|
| bax/HPRT       | 0.17±0.03 | 0.68±0.13*  | 0.67±0.20*              | 0.21±0.02   | 0.18±0.02              |
| bcl-2/HPRT     | 0.43±0.05 | 0.36±0.04   | 1.25±0.18 <sup>o</sup>  | 0.71±0.04*  | 1.34±0.27 <sup>o</sup> |
| GFAP/HPRT      | 5.21±0.31 | 13.02±0.60* | 19.79±0.35 <sup>o</sup> | 11.04±1.21* | 5.83±0.18 <sup>o</sup> |
| APAF-1/HPRT    | 0.63±0.11 | 1.62±0.32*  | 0.53±0.02 <sup>o</sup>  | 0.98±0.13*  | 0.69±0.10 <sup>o</sup> |
| caspase-7/HPRT | 1.02±0.06 | 1.64±0.08*  | 0.84±0.04 <sup>o</sup>  | 0.83±0.09   | 1.30±0.06*             |

\* p<0.05 vs the corresponding sham.

<sup>o</sup> p<0.05 vs the CCI JNJ16259685 untreated rats, as analysed by ANOVA, followed by Student-Neuman-Keuls test. JNJ16259685 did not affect behavioural analysis in sham rats. For this reason we did not perform other experiments on JNJ16259685 treatment in sham rats.