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SPINAL TRANSLOCATOR PROTEIN (TSPO) MODULATES PAIN BEHAVIOR IN RATS WITH CFA-INDUCED MONOARTHRITIS

Hayley Hernstadt^{1,2}, Shuxing Wang², Grewo Lim², and Jianren Mao²

¹Faculty of Medicine, Dentistry and Health Sciences, University of Melbourne, Victoria 3010, Australia

²MGH Center for Translational Pain Research, Department of Anesthesia and Critical Care, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

Abstract

Translocator protein 18kDa (TSPO), previously known as the peripheral benzodiazepine receptor (PBR), is predominantly located in the mitochondrial outer membrane and plays an important role in steroidogenesis, immunomodulation, cell survival and proliferation. Previous studies have shown an increased expression of TSPO centrally in neuropathology, as well as in injured nerves. TSPO has also been implicated in modulation of nociception. In the present study, we examined the hypothesis that TSPO is involved in the initiation and maintenance of inflammatory pain using a rat model of Complete Freund's Adjuvant (CFA)-induced monoarthritis of the tibio-tarsal joint. Immunohistochemistry was performed using Iba-1 (microglia), NeuN (neurons), anti-Glial Fibrillary Acidic Protein, GFAP (astrocytes) and anti-PBR (TSPO) on day 1, 7 and 14 after CFA-induced arthritis. Rats with CFA-induced monoarthritis showed mechanical allodynia and thermal hyperalgesia on the ipsilateral hindpaw, which correlated with the increased TSPO expression in ipsilateral lamina I-II on all experimental days. Iba-1 expression in the ipsilateral dorsal horn was also increased on Day 7 and 14. Moreover, TSPO was co-localized with Iba-1, GFAP and NeuN within the spinal cord dorsal horn. The TSPO agonist Ro5-4864, given intrathecally, dose-dependently retarded or prevented the development of mechanical allodynia and thermal hyperalgesia in rats with CFA-induced monoarthritis. These findings provide evidence that spinal TSPO is involved in the development and maintenance of inflammatory pain behaviors in rats. Thus, spinal TSPO may present a central target as a complementary therapy to reduce inflammatory pain.

Keywords

Translocator protein 18kDa; TSPO; Peripheral benzodiazepine receptor; pain; inflammation; inflammatory pain; arthritis; spinal cord

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Correspondence to: Jianren Mao, M.D., Ph.D., MGH Center for Translational Pain Research, Department of Anesthesia and Critical Care, WACC 324, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, Phone: 617-726-2338, Fax: 617-724-8844, Email: jmiao@partners.org.

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Introduction

Inflammatory pain mechanisms feature in many chronic pain states, including osteoarthritis (OA) and rheumatoid arthritis (RA), wherein inflammation-induced plasticity within the peripheral and central nervous system contributes to the persistence of pain. Arthritis, like other conditions producing chronic inflammatory pain, is characterized by a heightened painful response to noxious (hyperalgesia) or innocuous (allodynia) stimulation, and pain at rest (spontaneous pain). The inflammation within the joint results in an increased efficacy of synaptic transmission between primary afferent fibres and dorsal horn neurons, known as peripheral sensitization, which corresponds with the development of central sensitization, whereby neurons within the spinal cord also become hyperexcitable with an increased response to peripheral stimulation (Melzack and Wall, 1999). While the pathophysiology of arthritic joint destruction has been researched in detail, the central mechanisms contributing to arthritic pain production and maintenance have not been thoroughly elucidated.

The translocator protein (TSPO, 18kDa) was discovered in 1977 as a peripheral receptor for benzodiazepine (Braestrup and Squires, 1977; Papadopoulos et al., 2006a). It is similar to the central benzodiazepine receptor (CBR) in that both bind benzodiazepine although their location, distribution and action are quite different. TSPO is found in peripheral, non-neuronal tissue, as well as centrally, which led to its initial name of peripheral benzodiazepine receptor (Anholt et al., 1986). Other names of this molecule have included mitochondrial benzodiazepine receptor (Krueger, 1995) and mitochondrial DBI receptor complex (Romeo et al., 1992) before its new nomenclature (TSPO) was instituted in 2006 (Papadopoulos et al., 2006a).

TSPO is distributed throughout the body, particularly in steroidogenic tissues (Anholt et al., 1985). Renal and myocardial tissue contain intermediate levels of TSPO, while the liver, brain and spinal cord express comparatively low levels (Anholt et al., 1985). Within the central nervous system (CNS), TSPO is expressed in microglia and astrocytes (Gavish et al., 1992), and has also been found in cultured cortical neurons and DRG neurons in vivo after peripheral nerve injury (Jayakumar et al., 2002; Karchewski et al., 2004; Mills et al., 2005; Xiao et al., 2002). Subcellularly, TSPO is located mainly on the outer mitochondrial membrane, being particularly abundant at the contact sites of outer and inner mitochondrial membranes (Anholt et al., 1985; Anholt et al., 1986; Basile and Skolnick, 1986; Culty et al., 1999; Garnier et al., 1993). It is also present on plasma membranes of blood leukocyte subsets (Canat et al., 1993; Olson et al., 1988), the inner mitochondrial membrane (Mukherjee and Das, 1989), and nuclear membranes (Hardwick et al., 1999; Kuhlmann and Guilarte, 2000).

TSPO has a range of functions, including steroidogenesis (Besman et al., 1989; Papadopoulos et al., 1997), heme biosynthesis (Krueger, 1995; Snyder et al., 1987; Verma et al., 1987), regulation of cell proliferation (Hardwick et al., 1999; Kletsas et al., 2004), and immunomodulation (Bessler et al., 1992). TSPO often forms a trimeric complex with 32kDa voltage-dependent anion channel (VDAC) and 30kDa adenine nucleotide carrier (ANC) to make up the mitochondrial permeability transition pore (MPTP) (Casellas et al., 2002; Weizman and Gavish, 1993), which is important in maintaining the inner mitochondrial membrane potential and the matrix pH (Casellas et al., 2002). Thus, TSPO also has roles in anion transport (Beavis, 1989; Gavish et al., 1999), mitochondrial respiration (Hirsch et al., 1989), apoptosis (McEnery et al., 1992; Veenman et al., 2007; Veenman et al., 2008), and peripheral nerve degeneration and regeneration (Karchewski et al., 2004).

TSPO ligands, isoquinoline carboxamide (PK11195) and 4'-chloro derivative of diazepam (Ro5-4864), are often used to investigate TSPO as both bind to TSPO with high affinity (Braestrup and Squires, 1977; Le Fur et al., 1983). They have a variety of effects such as

promotion of steroidogenesis in central and peripheral tissues (Besman et al., 1989; Lacor et al., 1999; Papadopoulos et al., 1997), anti-inflammation and anti-nociception (Bressan et al., 2003; Farges, 2003; Torres et al., 1999; Torres et al., 2000), and modulation of immune function (Bessler et al., 1992). TSPO has been implicated in chronic inflammatory pain conditions because TSPO ligands (PK11195 or Ro5-4864) dose-dependently inhibit inflammation in various mouse models of inflammation (Torres et al., 2000).

It has been shown that glial expression within the spinal cord plays an important role in the induction and maintenance of pathological pain, and the level of TSPO expression may be related to the glial expression (Ledeboer et al., 2005; Milligan and Watkins, 2009; Watkins et al., 2001; Watkins and Maier, 2002), although the glial expression may differ under various inflammatory conditions and be influenced by glial markers (Clark et al., 2007; Honore et al., 2000; Lin et al., 2007; Raghavendra et al., 2004). In this study, we examined the time course of TSPO and microglia expression within the spinal cord following CFA-induced mono-arthritis as well as the involvement of TSPO in arthritic pain behaviors using the TSPO ligand Ro5-4864.

Results

Pain behaviors in CFA-induced right tibio-tarsal joint inflammation

Animals subjected to intra-articular CFA injection showed an ipsilateral inflammatory reaction with redness, swelling and guarding of the injected hindpaw, which persisted for the duration of the experiment. These rats did not show any lesions outside of the injected joint, nor did they show signs of distress, suffering, weight loss, and/or self-mutilation.

Administration of CFA to the right tibio-tarsal joint resulted in a decreased paw withdrawal latency (seconds) to noxious thermal stimuli. ANOVA testing with post-hoc Student-Newman-Keuls tests showed a statistically significant difference between CFA and naïve rats in the ipsilateral hind paw at all time points (up to day 14), and a statistically significant difference between CFA and vehicle-treated (sham) rats at all time points except on Day 1 (Fig. 1). Sham rats exhibited “pain” behaviors on the first day possibly due to the irritation induced by the vehicle injected into the joint space. Contralateral paw withdrawal latencies for all rats (CFA, sham or naïve) showed no statistically significant difference to baseline measures on all days.

Similarly, CFA rats showed a significant difference in paw withdrawal threshold to Von Frey fiber stimulation, as compared to that of their own baseline, naïve rats, and sham rats on all experimental days (up to day 14) except on Day 1 (Fig. 1). Similar to thermal withdrawal latencies, sham rats showed a significant difference in paw withdrawal threshold on Day 1 as compared to their own baseline (Fig. 1). Contralateral paw withdrawal thresholds for CFA-treated, sham rats and naïve rats did not show any statistically significant change. These results indicate that intra-articular CFA injection reliably induced lasting pain behaviors (thermal hyperalgesia and mechanical allodynia), distinguishable from both sham rats and naïve rats.

Altered spinal expression of TSPO in CFA rats

We then examined whether the spinal expression of TSPO would differ between CFA rats and sham rats using immunohistochemistry. There was a low level of basal expression of TSPO within the spinal cord dorsal horn, which was substantially increased within lamina I and II of the lumbar spinal cord dorsal horn on Day 1 following CFA (Fig. 2). This increase reached its peak on Day 7 and was maintained up to Day 14 in CFA rats (Fig. 2). In contrast, while an increase in contralateral expression was observed on days 7 and 14, this was not significantly different to baseline (Fig. 2). Fluorescent density analysis of the ipsilateral dorsal horn on day 1, 7 and 14 after CFA injection showed a statistically significant increase of the TSPO

expression as compared to the baseline, contralateral dorsal horn, and sham rats (Fig. 3). These results demonstrate that the TSPO expression was selectively upregulated within the spinal cord dorsal horn of CFA rats.

Co-expression of TSPO with neuronal and glial markers

The spinal TSPO expression was colocalized with NeuN (a neuronal marker), GFAP (an astrocyte marker), and Iba-1 (a microglial marker) in CFA rats (Fig. 4), which is consistent with previous findings that TSPO is expressed in neurons, astrocytes and microglia (Gavish et al., 1992; Jayakumar et al., 2002; Karchewski et al., 2004; Mills et al., 2005; Xiao et al., 2002). Both TSPO and Iba-1 expression increased over the 14 days, but fluorescent density analysis indicated TSPO reached its peak earlier on Day 7, as compared to Day 14 for Iba-1 (Fig. 5). While the Iba-1 immunoreactivity was barely detectable in the naïve rat, a slight bilateral increase in the Iba-1 expression within the lumbar spinal cord dorsal horn was observed after CFA (Fig. 6), though changes in the contralateral dorsal horn were not found to be statistically significant. Collectively, the immunohistochemical results indicate that the TSPO expression was detected in both neuronal and glial cells within the spinal cord dorsal horn.

Ro5-4864 dose-dependently prevented pain behaviors after CFA

In order to examine whether TSPO played a role in the development of pain behaviors after CFA, Ro5-4864 was given intrathecally twice daily for five consecutive days after CFA. The onset of mechanical allodynia was delayed in CFA rats treated with 2.5 µg Ro5-4864 (but not 0.5 µg Ro5-4864 or vehicle), such that their ipsilateral withdrawal latency remained close to baseline when examined on day 4 as compared with CFA rats given vehicle (Fig. 5). Moreover, thermal hyperalgesia was prevented in CFA rats treated with 2.5 µg Ro5-4864 (but not vehicle) and the effect of Ro5-4864 lasted for at least 5 days (i.e., till day 10 after CFA) after the treatment was discontinued on day 6 after CFA (Fig. 7). Animals given 0.5 µg Ro5-4864 showed a similar decrease in thermal hyperalgesia, albeit less effective as compared with CFA rats treated with 2.5 µg Ro5-4864 (Fig. 7). TSPO did not alter the baseline thermal or mechanical nociceptive response in the contralateral paw of either CFA or sham rats, nor did it produce the change in the motor function (e.g., gait) at the current doses. These results indicate that intrathecal Ro5-4864 dose-dependently diminished mechanical allodynia and thermal hyperalgesia in CFA rats.

Discussion

This study demonstrates that the TSPO expression is significantly increased within the ipsilateral spinal cord dorsal horn in rats with CFA-induced mono-arthritis and this change is temporally associated with the development and maintenance of allodynia and hyperalgesia. This is the first report of the central involvement of TSPO in persistent inflammatory pain. These results indicate that TSPO likely contributes to allodynia and hyperalgesia in this animal model of mono-arthritis. The modulation of TSPO by intrathecal administration of TSPO ligand Ro5-4864 significantly prevented or delayed both mechanical allodynia and thermal hyperalgesia. It is possible that the increased expression of TSPO represents an endogenous attempt to counter the overwhelming pro-nociceptive inflammatory state. Thus, spinal TSPO may present a way to exploit local compensatory mechanisms in the treatment of persistent inflammatory pain.

The increased TSPO expression in CFA rats is consistent with previous findings indicating the involvement of TSPO in a wide range of pathological conditions including inflammation (Wilms et al., 2003), gliomas (Cornu et al., 1992), brain injury (Chen et al., 2004; Chen and Guilarte, 2008; Wilms et al., 2003), Alzheimer's disease (Ji et al., 2008), Parkinson's disease (Schoemaker et al., 1982), multiple sclerosis (Vowinckel et al., 1997) and peripheral nerve

degeneration and regeneration (Mills et al., 2008; Mills et al., 2005). Moreover, Karchewski et al showed an increase in TSPO expression in lamina I and II of the spinal cord dorsal horn at three days after sciatic nerve transection (SNT), which was maintained till three weeks post-SNT (Karchewski et al., 2004). SNT and sciatic nerve crush injury also caused the slow induction of TSPO within the dorsal root ganglion (DRG), which reached high levels of expression two weeks post-injury (Karchewski et al., 2004).

TSPO expression was colocalized with GFAP, Iba-1 and NeuN, markers for astrocytes, microglia and neurons respectively which is consistent with previous findings (Chen and Guilarte, 2008; Gavish et al., 1992; Jayakumar et al., 2002; Karchewski et al., 2004; Xiao et al., 2002). There was a significant upregulation of Iba-1 immunoreactivity within the ipsilateral dorsal horn. Upregulation of spinal cord glia has been reported in most animal pain models including neuropathic, inflammatory, and bone cancer pain (Cao and Zhang, 2008; Honore et al., 2000; Moalem and Travey, 2006; Sun et al., 2007). In inflammation models, there was a bilateral increase of OX-42 immunoreactivity within the spinal cord, and upregulation of inflammatory mediators on both sides of the spinal cord despite the insult being unilateral (Sun et al., 2007; Verge et al., 2004). It should be noted that microglia are also capable of reacting to peripheral inflammation without overt morphological changes (Hua et al., 2005; Nimmerjahn et al., 2005).

The subcellular location of TSPO should be studied in further detail. TSPO can be found in many subcellular regions including the outer and inner mitochondrial membrane (Anholt et al., 1986; Basile and Skolnick, 1986; Garnier et al., 1993), plasma membrane (Canat et al., 1993; Olson et al., 1988), and nuclear membrane (Hardwick et al., 1999). NeuN staining locates the nuclear membrane of neurons and there is co-localization of TSPO with neuronal nuclear membranes in our experiment, a finding consistent with another study indicating that TSPO is implicated in aggressive phenotype breast cancer cell proliferation (Hardwick et al., 1999).

The exact mechanism of the TSPO involvement in persistent inflammatory pain remains to be elucidated. TSPO is responsible for the rate-determining step of steroidogenesis, the translocation of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane (Casellas et al., 2002; Papadopoulos, 1993; Papadopoulos et al., 1997). Cholesterol is then transformed into pregnenolone by the C27 cholesterol side-chain cleavage cytochrome P450 enzyme (P450_{scc}) and electron transferring proteins, located on the inner mitochondrial membrane (IMM) (Catt et al., 1980). It is hypothesized that TSPO may act by increasing the production of endogenous neurosteroids due to its role in cholesterol translocation, which then attenuate pain sensitivity through their action on other cellular elements (Mitchell et al., 2008; Papadopoulos et al., 2006b; Pathirathna et al., 2005; Schlichter et al., 2006). Previous studies have shown that intraperitoneally administered Ro5-4864 and PK11195 attenuated arthritis-associated nociception, and this effect may be dependent on steroid formation (Bressan et al., 2003; DalBo et al., 2004). However, these results are difficult to interpret as systemic administration of TSPO ligands affects TSPO throughout the body in its many functions (Bessler et al., 1992; Hardwick et al., 1999; Kletsas et al., 2004; Veenman et al., 2007; Veenman et al., 2008).

A rise in production of endogenous 5 α -neurosteroids in the presence of inflammation has been noted, and is thought to increase synaptic inhibition within the substantia gelatinosa (Poisbeau et al., 2005). These progesterone-derived neurosteroids were found to exert a fast non-genomic effect through the allosteric modulation of GABA_AR, thereby decreasing nociception (Charlet et al., 2008). However, there have been reports suggesting changes to GABA_AR activity in the spinal cord may contribute to inflammatory hyperalgesia (Anseloni and Gold, 2008; Lin et al., 1996). The effect of TSPO modulation on GABA_AR, and their contribution to inflammatory pain should be investigated in greater detail. Centrally produced steroids may facilitate

inhibition through GABA_AR and *N*-methyl-D-aspartate receptors (Baulieu, 1997; Eser et al., 2008; Malayev et al., 2002), thereby modulating pain-related comorbid behaviors including anxiety, cognition, sleep and reward-seeking, and depression (Birzniece et al., 2006; Dhir and Kulkarni, 2008; Dubrovsky, 2000; Engel and Grant, 2001; Eser et al., 2008; Mitchell et al., 2008). The role of TSPO in steroidogenesis and immunomodulation may also direct communication between astrocytes, microglia and neurons (Garcia-Ovejero, D., et al., 2005; Melcangi, R.C., et al., 1994; Koenig, H.L., et al., 1995). Collectively, it is possible that TSPO ligands may play a role in pain mechanisms through regulation of endogenous neurosteroid production which modulates downstream receptors and cellular components. Future studies should further explore such possibilities.

In conclusion, the present study demonstrated that the TSPO expression was significantly increased within the lumbar spinal cord after CFA-induced monoarthritis with a concurrent increase in spinal microglia expression, with intrathecal administration of Ro5-4864 delaying the onset of pain behaviors in CFA rats. Thus, modulation of TSPO function may present a potential target for the drug development in the treatment of pain.

Method

Experimental animals

Male Sprague-Dawley rats (Charles River) weighing 225-300g were used. Animals with intrathecal catheter insertion were housed individually. The standard 12h light-dark cycle (lights on between 7am and 7pm) was in effect, with food and water available *ad libitum*. Experiments were performed during the light cycle. The animal protocol was approved through the Massachusetts General Hospital Institutional Animal Care and Use Committee. CFA or sham injections were into the right tibio-tarsal joint of the rat. The day of surgery is referred to as Day 0.

The first experiment involved three experimental groups: (i) CFA-injection into right tibio-tarsal joint, (ii) vehicle injection into right tibio-tarsal joint (sham), (iii) naïve rats. The second experiment involved four experimental groups: (i) CFA-injection into right tibio-tarsal joint with vehicle intrathecally (i.t.), (ii) vehicle injection into right tibio-tarsal joint with 2.5 µg Ro5-4864 i.t., (iii) CFA injection into right tibio-tarsal joint with 2.5 µg Ro5-4864 i.t., and (iv) CFA injection into right tibio-tarsal joint with 0.5 µg Ro5-4864 i.t.

Drugs

Ro5-4864 (4'-chlorodiazepam) was purchased from Sigma-Aldrich. Ro5-4864 was dissolved in 100% DMSO (Sigma-Aldrich) at a concentration of 5 µg/µl and stored in sterile aliquots at 4°C. Immediately prior to administration, aliquots were thawed and diluted to concentrations of either 2.5 µg/µl or 0.5 µg/µl in 100% DMSO. Of note, few studies have used TSPO ligands intrathecally and there was little information in the literature regarding their intrathecal dosage (DalBo et al., 2004). As TSPO ligands only dissolve in polar solvents, DMSO was chosen as the drug solution such that the TSPO ligand was dissolved in 1 µl of 100% DMSO and then flushed through the catheter with 10 µl of saline. This approach allowed us to wash the ligand into the intrathecal space with a flush of saline to ensure the ligand in DMSO solution was diluted and not precipitated. The same approach was used for the vehicle group except that no TSPO ligand was added.

Surgery and intra-articular microinjection

CFA injection—Monoarthritis was induced by an injection of complete Freund's adjuvant (CFA, Sigma) into the right tibio-tarsal joint using the method of Butler et al, while the rat was briefly anesthetized with 2% isoflurane (Butler et al., 1992). The skin around the site of

injection was sterilized with 75% alcohol. The right leg was held and the fossa of the lateral malleolus of the fibula located. A 28-gauge needle was inserted vertically to penetrate the skin and turned distally to insert into the articular cavity at the gap between the tibiofibular and tarsus bone until a distinct loss of resistance was felt. A volume of 50 μ l of CFA was then be injected. Control (sham) animals underwent the same procedure but were injected with a vehicle. Naïve rats did not undergo any procedure but their behavior was tested at the same time points as the other rats.

Intrathecal catheters—For repeated intrathecal injections, a catheter was for each rat implanted as described by Yaksh and Rudy (Yaksh and Rudy, 1976). A polyethylene catheter (PE-10), 8.5 cm long, was inserted intrathecally through the cisternal membrane with the catheter tip aimed at the dorsal surface of the spinal cord, such that the tip of the catheter was placed at the lumbar enlargement. Animals were allowed to rest for two days prior to beginning the experiment, and rats with neurological deficits (e.g., paralysis) were excluded from the experiment. The indwelling catheters were used to microinject drugs or vehicle into the cerebrospinal fluid (CSF) space surrounding the lumbosacral spinal cord. Drugs were administered twice a day for five days starting one hour prior to the CFA or sham injection.

Behavior testing

Animals were habituated to the test setting two days prior to beginning the experiment. Behavior tests were carried out in a quiet room between 8:30 AM and 1:00 PM wherein the withdrawal threshold to thermal and mechanical stimulation was elicited and recorded. Testers were blinded to animal conditions. Ipsilateral and contralateral hindpaws were measured prior (baseline) to CFA injection, sham injection, and intrathecal catheter placement, and again at 1, 4, 7, 10, and 14 days afterwards. Rats were given at least 20 minutes to acclimatize prior to the testing.

For mechanical withdrawal threshold, rats were placed individually in transparent plastic boxes with a wire mesh floor. A single calibrated von Frey fiber was applied to the plantar surface five times with an inter-stimulation interval of at least 5 seconds. One clear withdrawal response out of five applications was considered a positive response. Thermal paw withdrawal threshold was assessed using the Hargreaves apparatus (Hargreaves et al., 1988). A radiant thermal stimulus was applied to the lateral plantar surface of the hindpaw through the glass plate. The latency (seconds) for the withdrawal of the paw from the heat source was recorded, and rats had a minimum break of 5 minutes between each test. The heat source shut off automatically at 20 seconds to prevent tissue injury. Two trials were performed on each hindpaw.

Immunohistochemistry

Rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.), perfused intracardially with 300ml of 0.9% saline, and followed by 200-300ml of 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.2-7.4, 4°C). Lumbar spinal segments were removed, postfixed overnight 4°C, then kept in 30% sucrose in 0.1M phosphate-buffered saline (PBS) at 4°C. Dissected tissue was mounted in OCT compound and frozen at -20°C. Transverse spinal cord sections (10 μ m) were cut using a cryostat, then placed in PBS. Sections were washed in 0.01M PBS twice for 10 minutes then blocked for one hour in 5% bovine serum albumin and 0.1% Triton X-100. Free floating tissue sections were then incubated overnight for approximately 16 hours at 4°C on a rocker with one of the following primary antibodies: PBR (TSPO; R&D systems): 1:300, rabbit polyclonal; NeuN (Chemico; Millipore): 1:1000, mouse monoclonal; GFAP (BD Biosciences – Pharmingen): 1:1000, mouse monoclonal; Iba-1 (Abcam): 1:50 mouse monoclonal. Tissue samples were then washed twice with PBS for 8 minutes each and incubated with FITC- or Cy3- conjugated antibody (1:300, Jackson ImmunoResearch Laboratories Inc.) in blocking solution without Triton X-100 for 1 hour at

room temperature in the dark. Staining for colocalization was done by incubating sections with a second primary antibody and then a second secondary antibody. Controls were performed by omitting either primary antibody or secondary antibody. Fluorescent images on these sections were captured with a digital camera (Olympus), and adjusted using Adobe PhotoShop (Version 7). The fluorescent density was analyzed using a computer software (Image-Pro Plus; Media Cybernetics Version 6).

Statistical analysis

The behavioral test data and the fluorescent density of immunohistochemistry were analyzed by one-way ANOVA followed by the Student-Newman-Keuls test using SigmaStat Pro Software (Version 2.03). The data is presented as mean $\pm$ S.E.M.

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Figure 1. CFA injection into the tibio-tarsal joint induced persistent pain behavior

CFA rats showed a significant difference to baseline measures over the experimental period in Von Frey and Hargreaves testing (**A** and **C**), whereas the contralateral hindpaw showed no significant change (**B** and **D**). On Day 1, sham rats showed a significantly decreased threshold in Von Frey testing as compared to baseline (**A**), but behavioral measures returned to the baseline after Day 1. Data is shown as mean $\pm$ S.E.M. * $P < 0.01$ as compared with baseline (n=6-8).

Figure 2. Immunohistochemical staining of spinal TSPO

There was a time-dependent increase in the TSPO expression within the ipsilateral spinal cord dorsal horn after CFA-induced mono-arthritis. There was also a delayed increase, albeit less robust, of TSPO expression within the contralateral spinal dorsal horn. Scale bar: 160 μ m.

Figure 3. Increase in the spinal TSPO expression

Significant differences in immunoreactivity were present in the ipsilateral spinal cord dorsal horn of CFA rats on Day 1, 7 and 14, as compared to the contralateral dorsal horn of CFA rats as well as naïve or sham rats. Note that the peak TSPO expression occurred on Day 7 within the ipsilateral spinal cord dorsal horn of CFA rats. Data is shown as mean $\pm$ S.E.M. * $P < 0.01$ as compared with the contralateral side of CFA rats or with naïve or sham rats ($n = 5$).

Figure 4. Colocalization between TSPO and NeuN, GFAP and Iba-1
The expression of spinal TSPO was colocalized with markers for neurons (NeuN) (A), astrocytes (GFAP) (B), and microglia (Iba-1) (C). Scale bar: 160 μ m.

Figure 5. Increase in the spinal Iba-1 (microglial) expression

Significant differences in immunoreactivity were present in the ipsilateral spinal cord dorsal horn of CFA rats on Days 7 and 14, as compared to the contralateral dorsal horn of CFA rats as well as naïve or sham rats. Peak Iba-1 expression occurred on Day 14 within the ipsilateral spinal cord dorsal horn of CFA rats (experiment duration was 14 days). Data is shown as mean $\pm$ S.E.M. * $P < 0.01$ as compared with the contralateral side of CFA rats or with naïve or sham rats ($n = 5$).

Figure 6. Immunohistochemical staining of spinal Iba-1

Increase in Iba-1 immunoreactivity within the spinal cord dorsal horn after CFA-induced arthritis, with increased expression ipsilateral to injury. Scale bar: 160 μ m.

Figure 7. Effect of a TSPO ligand, Ro5-4864, on pain behavior

Intrathecal treatment with Ro5-4864 twice daily for 5 days, beginning the day of surgery, delayed or prevented the development of mechanical allodynia (A) and thermal hyperalgesia (C) in the ipsilateral hindpaw of CFA rats. The contralateral hindpaw in all groups did not show a significant change after the Ro5-4864 treatment (B and D). Data is shown as mean $\pm$ S.E.M. * $P < 0.05$ as compared with the vehicle group.