Expression of 18 KDa translocator protein by microglia in spinal cord and hippocampus in rats with neuropathic pain

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Abstract: Translocator protein of 18 Kda (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. TSPO is involved in neuroinflammatory processes and also implicated in the modulation of nociception. We examined the hypothesis that TSPO is involved in the initiation and maintenance of neuropathic pain in a rat model of chronic constrictive nerve injury. Mechanical pain withdrawal threshold and thermal withdrawal latency were measured before surgery, and 3, 7, 14, and 28 d after surgery. Injured rats showed mechanical allodynia and thermal hyperalgesia in the ipsilateral hind paw, longer mean escape latency in the Morris water maze, and longer escape latency lower in the platform quadrant in the spatial probe test, which were significantly correlated with increased TSPO expression in the spinal dorsal horn and hippocampus. A marker of microglia expression (Iba-1) was significantly increased in the spinal dorsal horn and hippocampus on days 7 and 14 and co-localized with TSPO. These findings provide evidence that spinal and hippocampal TSPO might be involved in the development and maintenance of neuropathic pain in rats and could be a target of complementary therapy to ameliorate pain.

Keywords: Translocator protein 18 kDa, neuropathic pain, microglia, spinal cord, hippocampus

Introduction

Neuropathic pain (NPP) is spontaneous pain that derives from injury to nervous tissue. The damage changes the structure and function of the nervous system, often leading to a decrease in pain threshold, enhancement of pain reaction and spontaneous pain [1]. It can be accompanied by anxiety, depression, mood changes and cognitive decline [2]. There are many reasons for NPP, including trauma, infection, ischemia, metabolism, and nutritional diseases [3]. Patients with chronic NPP suffer from severe changes such as pathological and physiological reactions, which can in turn increase mortality and disability rates [4]. The translocator protein (TSPO, 18 kDa) was discovered as a peripheral receptor for benzodiazepine in 1977 [5]. It is similar to the central benzodiazepine receptor (CBR) due to their affinity to benzodiazepine, although their location, distribution and actions are quite different. TSPO is distributed throughout the body, particularly in steroidogenic tissues [6]. Renal and myocardial tissues contain intermediate levels of TSPO, while liver, brain and spinal cord express comparatively low levels. In the central nervous system (CNS), TSPO is expressed in microglia and astrocytes and has also been found in cultured cortical neurons and dorsal root ganglion (DRG) neurons in vivo after peripheral nerve injury [7]. TSPO ligands, isoquinoline carboxamide (PK11195) and 4’-chloro derivative of diazepam (Ro5-4864), are often used to investigate the roles of TSPO as both bind to TSPO with high affinity. TSPO has been implicated in chronic inflammatory pain because TSPO ligands (PK11195 or Ro5-4864) dose-dependently inhibit inflammatory responses in various mouse models of inflammation [8].
It has been shown that expression of microglia cells within spinal cord plays an important role in the induction and maintenance of pathological pain [9], and the level of TSPO expression may be related to glial expression, although glial expression may differ under various inflammatory conditions and be influenced by glial markers. TSPO is expressed centrally in neuro-pathological tissue and in injured nerves. This has been found mainly by positron emission topography studies [10]. One report showed that although the upregulation of TSPO in AD is still inconclusive, TSPO appears to be involved in neuroinflammatory processes and has been shown to involve substantial inflammation [11]. TSPO is also implicated in the modulation of nociception and thus may be involved in cognitive impairment after NPP, playing an important regulating role in its pathogenesis [12]. It is therefore necessary to provide experimental and theoretical evidence of the role of TSPO to improve clinical drug research and development. Using a chronic constriction injury of the sciatica nerve in a rat model, this study explores the possible mechanism between TSPO expression and cognitive function. We also investigate changes in TSPO protein expression levels in the dorsal horn of lumbar spinal cord and microglia and hippocampus with the view to provide a new basis for NPP therapy.

Materials and methods

Experimental animals and groups

Adult male Wistar rats (200-300 g) were used in these experiments. Rats were purchased from Weifang Medical Experimental Animal Center, Weifang, China. During the experiment, rats were housed under standard conditions (temperature 22-26°C, 12 h: 12 h light-dark cycles) with free access to food and water. All animal handling and experimental procedures were in compliance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals were randomly divided into two groups: sham operated group (n = 6) or chronic constriction injury (CCI) surgery group (n = 24). The CCI operated rats were divided into four time points: CCI 3 day group (n = 6), CCI 7 day group (n = 6), CCI 14 day group (n = 6) and CCI 28 day group (n = 6). Based on the literature, we established a NPP model in rats through CCI of the sciatic nerve. Rats in the sham group had the sciatic nerve exposed, but were not ligated. Mechanical pain threshold (MWT) and thermal withdrawal latency (TWL) were measured before surgery and 3, 7, 14, and 28 d after surgery. Animals were tested in a spatial reference memory task before and after the experiment; the sham-operated group were tested at the same time as experimental counterparts.

Chronic constriction nerve injury model

The pain models in this study are widely used and experimental rats were prepared as previously described [13]. The rats were put under 10% chloral hydrate anesthesia (40 mg/kg, i.p.), and the right sciatic nerve was exposed, surrounded by neural stem with 4-0 chromic catgut respectively, for four mild ligation ring, 1 mm pitch, ligation strength to cause a small leg muscles can be mild tremor. We then layered suture muscle and skin, and to prevent wound infection, the post-operative wound was coated with penicillin then bandaged with sterile gauze. In addition to not being ligated, the animals in the sham surgery group and experimental group received the same treatments. For each pain model, spinal cords were harvested at times routinely used for assessment of pain-associated behaviors. Spinal cords from CCI rats were harvested at additional times post CCI injury in order to preliminary assess time course changes in TSPO and glial cell in this model.

Behavioral tests

All rats were tested for mechanical and thermal hypersensitivity of the plantar surface of the hind paws before surgery, and then at 3, 7, 14 and 28 d after surgery. On each testing day, rats were brought into the behavioral room at least 30 min before the test session. Mechanical sensitivity was assessed with the up-down method, as described previously, using a set of von Frey hairs [14] with logarithmically increasing stiffness ranging from 3.61 (0.41 g) to 5.18 (15.14 g). The 2.04 g stimulus, in the middle of the series, was applied first. Briefly, rats were placed inside acrylic cages on a wire mesh grid floor. The probe was applied to the middle of the left or right hind paw to determine the stimulus intensity threshold stiffness, and there was a 5 min wait before the next stimulus was applied. Quick withdrawal in response to
the stimulus was considered a positive response. In the event of the absence of paw withdrawal, the next stronger stimulus was chosen. On the contrary, a weaker stimulus was applied.

Heat hypersensitivity was tested using a plantar test (7370, Ugobasile, Commeria, Italy) according to the method described by Hargreaves [15]. Rats were placed into individual plastic cages with glass floors, and a radiant heat source was aimed at the plantar surface of the hind paw. The hind paw was tested alternately with greater than 5-min intervals between consecutive tests. A maximal cut-off of 25 s was used to prevent tissue damage. The three measurements of latency per side were averaged as the result of per test.

**Morris water maze**

The spatial reference memory version of Morris water maze [16] is a standard task used to assess hippocampal dependent spatial learning in rodents. Spatial acquisition and retention was assessed using a water maze navigational task. Baseline cognitive performance and retrieval of pre-surgically acquired memory were determined in the MWM as described before [17]. A round water-filled maze (140 cm ID, 26 ± 1°C) was surrounded by visual cues and divided in 4 quadrants. An invisible platform was placed 1 cm below the water surface in quadrant 4 (target quadrant). MWM training was performed on 4 days, consisting of two trials in which the rat was randomly placed in quadrants 1, 2, or 3. The trial stopped when the animal found the platform and sat on it for 10 s. The rat was guided manually to the platform if it failed to find the platform within 1 min. Average escape latency to the platform for each training day was determined to assess learning performance. The day after the last training and at postoperative days 28, spatial memory was tested. Before the test, the platform was removed from the pool. The rat was placed in quadrants 1, 2 or 3 and the time in each quadrant and swimming speed were tracked for 1 min with EthoVision 3.0 (Noldus Information Technology, Wageningen, Netherlands). Time spent in the target quadrant was determined to assess memory retrieval. Each test was followed by one training trial to avoid confounding effects of the MWM test on subsequent performance in the MWM.

**Immunofluorescence**

Rats were deeply anesthetized with 10% chloral hydrate (40 mg/kg, i.p.), perfused intracardially with 300 ml of 0.9% saline and followed by 200-300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.2-7.4, 4°C). The L4-5 spinal segments and hippocampus tissue was placed in 4% paraformaldehyde liquid for 3 h, then placed in 30% sucrose overnight. The spinal cord and hippocampus were cut into 10 μm in a cryostat (LEICACM1900). The biopsy tissue was coated with 10% goat serum in 0.3% TritonX-100 for 1 h at room temperature and incubated in rabbit polyclonal antibody PBR (1:200, Santa Cruz) and mouse monoclonal antibody to Iba-1 (1:200, Santa Cruz) at 4°C overnight. The tissue was then washed three times with 0.01 M PBS every 10 min and the frozen sections were incubated at room temperature for 2 h with the secondary antibody FITC (1:250, ZSGB-BIO) and TRITC (1:250, ZSGB-BIO). The stained sections were examined with fluorescence microscopy and images were analyzed using image J software.

**Western blotting**

Rats were anesthetized with 10% chloral hydrate, decapitated, and the hippocampus and lumbar spinal cord tissues were rapidly removed and homogenized in lysate buffer (300 μl RIPA, PMSF and aprotinin) and centrifuged at 13000 g for 30 min at 4°C. The supernatant containing tissue lysate protein samples were stored at -20°C. 100 μg of each sample was taken and an equal volume of 4 x loading buffer was added and samples boiled in the water bath for 3-5 mins. The sample proteins were separated by SDS-PAGE gel electrophoresis (stacking gel 5%, 75v; separating gel 15%, 115v) and transferred to PVDF membranes (80 mA, 90 min). The membranes were incubated in 5% skimmed milk powder in Tris-Buffered Saline and Tween 20 (TBST) for 1 h at room temperature and then incubated with primary antibodies rabbit polyclonal anti-PBR antibody (1:500), mouse monoclonal anti-Iba-1 antibody (1:500) and mouse monoclonal β-actin antibody (1:2000), respectively, overnight at 4°C. The membranes were then washed three times in TBST for 10 min each time, and then incubated with secondary antibody HRP-labeled goat anti-rabbit IgG (1:2000) or goat anti-
18 kDa translocator protein in rats with neuropathic pain

mouse IgG (1:2000) for 2 h at room temperature. After washing three times for 10 min in TBST, proteins were detected by electrochemical and exposed to X-ray film (Kodak). Films were scanned under grayscale and image analyzed.

**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 are presented as mean ± S.E.M.

**Results**

**Behavioral test to estimate the effect of CCI on alldynia**

All rats were tested for mechanical and thermal hypersensitivity of the plantar surface of the hind paws before surgery and then at 3, 7, 14, and 28 d after CCI injury. The mechanical paw withdrawal threshold (MWT) significantly decreased in the CCI group (P < 0.05) compared with the pre-operative baseline. As shown in Table 1, compared with sham group, the CCI group rats developed robust mechanical allodynia at the same time (P < 0.05). Consistent with mechanical allodynia and nerve injury induced thermal hypersensitivity, the thermal withdrawal latency (TWL) significantly decreased in the CCI group (P < 0.05) as compared with sham group (Table 1). Both mechanical and thermal allodynia persisted for at least 3 weeks. In contrast, sham-operated rats showed no apparent response to mechanical stimuli at any of the time points, indicating an absence of allodynia. These observations suggested mechanical and thermal hypersensitivity were produced under nerve injury.

**MWM test to identify the effect of CCI on spatial learning and memory**

The MWM test evaluates the memory and spatial learning functions of animals by measuring the average escape latency and the numbers of traversing flat. After surgery, the average escape latencies in CCI rats became significantly longer from 3 to 28 d, compared with the sham group (P < 0.05, Table 2). A similar response was seen in the numbers of traversing flat testing. There were significant decreased times in the CCI group (Table 2). Sham-operated rats showed no apparent changes at any of the time points in the MWM. These results indicated that CCI induced a significant impairment in spatial learning and memory performance in rats.

<p>| Table 1. Comparison of mechanical allodynia between the two groups of rats (X ± s) |
|---------------------------------|--------|--------|--------|--------|--------|--------|</p>
<table>
<thead>
<tr>
<th>Project</th>
<th>Groups</th>
<th>Pre-operation</th>
<th>3 d</th>
<th>7 d</th>
<th>14 d</th>
<th>28 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>WMT</td>
<td>Sham group</td>
<td>46.91 ± 3.15</td>
<td>43.85 ± 3.15</td>
<td>43.60 ± 3.18</td>
<td>43.96 ± 2.96</td>
<td>45.06 ± 2.18</td>
</tr>
<tr>
<td></td>
<td>CCI group</td>
<td>45.14 ± 3.00</td>
<td>32.20 ± 3.26</td>
<td>22.79 ± 3.23</td>
<td>16.77 ± 1.25</td>
<td>24.20 ± 3.16</td>
</tr>
<tr>
<td>PWTL</td>
<td>Sham group</td>
<td>7.72 ± 0.67</td>
<td>7.50 ± 0.75</td>
<td>7.12 ± 0.70</td>
<td>6.86 ± 0.79</td>
<td>7.35 ± 0.98</td>
</tr>
<tr>
<td></td>
<td>CCI group</td>
<td>7.48 ± 0.92</td>
<td>5.16 ± 0.87</td>
<td>4.19 ± 0.65</td>
<td>2.99 ± 0.71</td>
<td>4.05 ± 0.67</td>
</tr>
</tbody>
</table>

Paw withdrawal threshold (gram) using stimuli with von Frey filament at pre-operative 1 day, 3, 7, 14 and 28 days after nerve injury. Data are presented as withdrawal threshold means ± SD. *P < 0.05 vs pre-operative; †P < 0.05 vs sham group at the same time.

<p>| Table 2. Comparison of the results of the Morris water maze between the two groups of rats (X ± s) |
|---------------------------------|--------|--------|--------|--------|--------|--------|</p>
<table>
<thead>
<tr>
<th>Project</th>
<th>Groups</th>
<th>Pre 1 d</th>
<th>3 d</th>
<th>7 d</th>
<th>14 d</th>
<th>28 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>The escape latency</td>
<td>Sham group</td>
<td>36.0 ± 4.1</td>
<td>36.4 ± 5.1</td>
<td>33.5 ± 6.5</td>
<td>33.5 ± 6.3</td>
<td>31.3 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>CCI group</td>
<td>33.9 ± 4.6</td>
<td>45.4 ± 4.5</td>
<td>46.9 ± 3.2</td>
<td>53.5 ± 3.9</td>
<td>51.3 ± 6.1</td>
</tr>
<tr>
<td>Frequency</td>
<td>Sham group</td>
<td>4.1 ± 0.8</td>
<td>3.9 ± 0.5</td>
<td>3.5 ± 0.4</td>
<td>3.3 ± 0.4</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>CCI group</td>
<td>4.4 ± 0.3</td>
<td>2.4 ± 0.4</td>
<td>2.0 ± 0.6</td>
<td>1.6 ± 0.4</td>
<td>2.1 ± 0.3</td>
</tr>
</tbody>
</table>

Changes of the escape latency and the time across the original platform of the rats following CCI (X ± s). *P < 0.05 vs pre-operative; †P < 0.05 vs sham group at the same time.
Upregulation of TSPO in the spinal dorsal horn is closely correlated with the activation of microglia in neuropathic pain following CCI

To determine the expression of TSPO and microglia, we carried out western blotting and double immunofluorescence staining of TSPO (using PBR marker) and microglia (using Iba-1 marker). Compared with the sham group, the morphology of glial cells became hypertrophic in rays (Figure 1) in CCI rats from the third postoperative day, indicating that the microglia cells were activated. After surgery, the expression of TSPO changed with both time and the activation of microglia in the spinal dorsal horn. TSPO expression reached a peak on day 14 (P < 0.05) and then returned to baseline levels in the CCI group, but after 28 d it was still significantly higher than that in the sham group (P < 0.05).

Figure 1. Co-localization between TSPO and Iba-1. The expression of spinal TSPO was detected with PBR marker and microglia were detected with Iba-1. Scale bar: 50 μm. The microglia marker was upregulated at 3 days (*P < 0.05, compared with baseline values) and peaked at 14 days. The expression of TSPO was upregulated at 7 days (*P < 0.05, as compared with baseline values) and reached a peak at 14 days, the same time as microglia.

Discussion

TSPO is widely distributed in central and peripheral tissue cells on the mitochondrial outer membrane. It has many physiological functions including cell growth and proliferation, activation of microglia, synthesis of steroids and regulation of mitochondrial respiration. Its specific ligands R-PK11195 (PK) and Ro5-4864 (RO) cause proliferation and phagocytosis of microglia, mainly by increasing the production of reactive oxygen species (ROS), an effect mediated by mitochondrial NADPH oxidase [18].

TSPO exists in small amounts in the central nervous system in both neurons and glial cells [19, 20]. Microglial cells are activated when our
bodies suffer from trauma, inflammation, infection and other pathological situations, and TSPO expression increases in a dose-dependent manner at the same time. Many studies have confirmed that TSPO levels increase in affected areas of CNS diseases such as the Alzheimer’s disease, Parkinson’s disease, ischemic stroke, and peripheral nerve degeneration and regeneration [21]. As raised TSPO levels are also detected in many brain injury models, we realized that it can also be used as a marker of microglia activation. This is consistent with its physiological functions, and TSPO can also promote cholesterol to glial cells, mitochondria were synthesized neural steroids (e.g. four hydrogen progesterone), strengthen the function of GABA receptors, inhibit brain hippocampus LTP, affect the plasticity of synaptic transmission, affect the body to the outside environment of learning and memory ability, close connection and cognition.

The CCI model used in this experiment was established in 1988 by Bennett [22] and has been recognized as the classical model for neuropathic pain. We found that after sciatic nerve ligation in rats, thermal pain threshold and mechanical pain threshold gradually reduced after 2 d, and then significantly reduced at 14 d, which explained why CCI rat limbs had different degrees of postoperative hyperalgesia.

A number of studies have confirmed that the activation of spinal microglia participate in the occurrence of NPP [23, 24]. After peripheral nerve injury, spinal cord dorsal horn of sensory nerve endings release adenosine triphosphate (ATP), attract microglia activation and release many inflammatory factors. Using immunofluorescence labeling, we showed that 3-14 d after ligation of the sciatic nerve microglia are activated in the spinal dorsal horn. There is also a change in morphology as protuberant characteristics cause a larger cell volume. At 28 d after ligation, positive expression of microglia decreased significantly. Our injury model confirmed microglia activation and induced thermal pain threshold and mechanical pain threshold [25]. This study shows that the mental derangement rational pain in the rat spinal cord dorsal horn microglia activation is associated with increasing TSPO, a dose-dependent increase consistent with microglia...
activation. When microglial cells are activated, TSPO can promote inflammatory genes by reducing the expression of COX-2, IL-6 and IL-1 cytokines and TNF alpha [26], and promote apoptosis by reactive oxygen specificity ligand effect in the removal of the microglia. TSPO ligands can be produced through endogenous neural steroidal lower adjustment and adjustment of the receptor and reduce pain sensitivity [27].

With increasing global incidences of chronic pain and its related diseases, many data have showed that chronic pain is also associated with depression and cognitive impairment. The spinal dorsal horn is a crucial area in the body that receives and accepts stimuli and integrates information; it not only receives nociceptive information stimulation, but also transmits signals up to brain areas. Local inflammation caused by peripheral nerve injury can induce damage to tissue and sensory nerve endings to release a larger number of endogenous substances such as substance P, excitatory amino acids, serotonin, slow excitation peptide, ATP, resulting in activated spinal microglia. Continuous stimulation by inflammatory factors can lead to a special communication between glial cells via calcium waves [28]. It can also result in disorders of the internal environment, immune responses, nerve inflammation and increased TSPO in rat hippocampus and activation of microglia. It is known that the intracellular calcium ion concentration increases between glial cells, and between neurons and glial cells; that calcium ions can be passed from one cell to another cell, and the regulation of intracellular calcium signals plays an important role in the process and participates in the integration process of the nervous system.

This study suggested that mental derangement rational pain in the rat spinal cord dorsal horn and hippocampus activated microglia. The behavioral experiments verified that chronic pain is caused by an emotional response and cognitive change, leading to increased microglia activation in the dorsal horn of the spinal cord and hippocampus and increased expression of TSPO. Thus, TSPO specific ligands R-PK11195 (PK) and Ro5-4864 (RO) can be used in the dorsal horn of the spinal cord to promote nerve growth and axon regeneration and repair of brain injury and thus may be used in treatment of NPP [12, 29]. TSPO can be viewed as a kind of regulatory protein, and should be considered as a potential target for the treatment of cognitive impairment caused by pain, inflammatory peripheral nerve injury and cognitive impairment.

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Disclosure of conflict of interest

None.

Abbreviations

TSPO, Translocator protein 18 kDa; PBR, peripheral benzodiazepine receptor; CCI, Chronic constriction injury of the sciatica nerve; NPP, Neuropathic pain; MWT, Mechanical paw withdrawal threshold; TWL, Thermal withdrawal latency; CNS, central nervous system; MWM, Morris water maze.

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