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ORIGINAL ARTICLE

Anxiolytic effects of hippocampal neurosteroids in normal and neuropathic rats with spared nerve injury

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Abstract

Neurosteroids are synthesized in the nervous system from cholesterol or steroidal precursors imported from peripheral sources. These compounds are important allosteric modulators of $GABA_A$ receptors, which play a vital role in modulating hippocampal functions. Chronic pain is accompanied by increased neurosteroid production in the spinal cord and thalamus. We hypothesize that hippocampal neurosteroids participate in pain or pain-associated emotions, which we tested with high-performance liquid chromatography/tandem mass spectrometry and pharmacological behavioral tests. We observed increased levels of hippocampal neurosteroids (pregnenolone, progesterone, deoxycorticosterone, and allopregnanolone) in rats with chronic neuropathic pain (28 days after spared nerve injury). Meanwhile, the expression of the translocator protein, the upstream steroidogenesis rate-limiting enzyme, increased in the ventral but not dorsal hippocampus of neuropathic rats. In both naïve and neuropathic rats, *in vivo* stereotaxic microinjection of PK 11195, the translocator protein inhibitor, into the ventral hippocampus exacerbated anxiety-like behaviors. These results indicate anxiolytic effects of hippocampal neurosteroids in both normal and neuropathic rats. Neurosteroids could be considered as agents for treatment of general and pain-related anxiety disorders.

Keywords: anxiety, hippocampus, neurosteroids, pain, spared nerve injury, translocator protein.

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Steroids had been considered to be produced only in endocrine glands such as the adrenals, gonads, and placenta until the 1980s, when Baulieu and colleagues reported higher concentrations of steroids including pregnenolone, dehydroepiandrosterone and their sulfates and lipoidal esters in the nervous system than in the plasma (Corpechot *et al.* 1981). In addition, steroids remain in the brain long after gonadectomy or adrenalectomy (Corpechot *et al.* 1983). Later, a series of enzymes for steroidogenesis have been

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Abbreviations used: AP, allopregnanolone; DOC, deoxycorticosterone; HPLC/MS, high-performance liquid chromatography/tandem mass spectrometry; MT, methyltestosterone; PREG, pregnenolone; PROG, progesterone; PWT, paw withdrawal threshold; SNI, spared nerve injury; THDOC, tetrahydrodeoxycorticosterone; TSPO, translocator protein.

detected in the nervous system (Robel and Baulieu 1995; Compagnone and Mellon 2000; Tsutsui *et al.* 2000), indicating local synthesis of steroids. These 'neurosteroids' include pregnenolone (PREG) and dehydroepiandrosterone as well as their sulfates, progesterone (PROG), deoxycorticosterone (DOC), the reduced metabolites such as allopregnanolone (AP) and tetrahydrodeoxycorticosterone (THDOC). Translocator protein (TSPO, 18 kDa) is the rate-limiting enzyme of steroidogenesis. It locates predominantly in the outer mitochondrial membrane (Garnier *et al.* 1993; Culty *et al.* 1999), and is particularly abundant in contact sites of outer and inner mitochondrial membranes in steroid-synthesizing tissues, including the brain. TSPO translocates the substrate cholesterol from the outer to the inner mitochondrial membrane (Papadopoulos *et al.* 1997, 2006a), where it is metabolized to PREG by the mitochondrial cholesterol side-chain cleavage enzyme P450scc (Robel and Baulieu 1995; Compagnone and Mellon 2000). Neurosteroids regulate a variety of physiological responses including anxiety, stress, reproductive, and sexual behaviors (Robel and Baulieu 1995; Compagnone and Mellon 2000). In general, neurosteroids do not exert their functions through classic steroid hormone nuclear receptors, but ion-gated neurotransmitter receptors (Lambert *et al.* 1995; MacKenzie and Maguire 2013). Neurosteroids bind directly to $GABA_A$ receptors, extrasynaptic δ subunit-containing $GABA_A$ receptors in particular, resulting in the potentiation of $GABA_A$ receptor-mediated inhibitory currents (Mihalek *et al.* 1999; Stell *et al.* 2003).

Pain is a multidimensional experience including sensory-discriminative, emotional-affective, and cognitive components (Millan 1999). Patients and animal models with chronic pain are susceptible to psychiatric disorders such as anxiety and depression, which eventually reduce the quality of life (Kontinen *et al.* 1999; Monassi *et al.* 2003; Demyttenaere *et al.* 2007; Jiang *et al.* 2014; Wang *et al.* 2015). In chronic pain conditions, increased neurosteroid production was observed in the spinal cord and the thalamus (Patte-Mensah *et al.* 2006; Zhang *et al.* 2016). Neurosteroids have strong anxiolytic effects in both animals and human beings (Bitran *et al.* 2000; Modol *et al.* 2011; MacKenzie and Maguire 2013; Schule *et al.* 2014). It is known that the hippocampus participates in pain and anxiety-related behaviors (Kjelstrup *et al.* 2002; Zhang *et al.* 2014). Key steroidogenesis enzymes, as well as synaptic and extrasynaptic $GABA_A$ receptors, are widely expressed in the hippocampus (Robel and Baulieu 1995; Sperk *et al.* 1997; Compagnone and Mellon 2000; Pirker *et al.* 2000). However, whether hippocampal neurosteroids participate in pain remains unclear.

In this study, we examined the hippocampal levels of five neurosteroids (PREG, PROG, DOC, AP and THDOC) and the steroidogenesis enzyme TSPO under physiological and neuropathic pain states, and investigated the possible role of neurosteroids in pain and anxiety-like behaviors in rats.

Materials and methods

Animals

Adult male Sprague–Dawley rats (240 ± 30 g) were provided by the Department of Experimental Animal Sciences, Peking University Health Science Center (Beijing, China). Animals were housed in standard cages with food and water *ad libitum* under a light–dark cycle of 12 h. All experimental procedures were approved by the Animal Care and Use Committee of our University (LA2016061), according to the guidelines of the International Association for the Study of Pain.

Chemicals and drugs

PREG (purity 98%), PROG (purity 99%), DOC (purity 97%), AP (purity 98%), and THDOC (purity 95%) were from Sigma-Aldrich (St. Louis, MO, USA). 17-alpha-Methyltestosterone (MT, purity 99%) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). AC-5216 (purity 99%) was a gift from Professor Yun-Feng Li in the Beijing Institute of Pharmacology and Toxicology. PK 11195 (purity 98%) was purchased from Cayman (Ann Arbor, MI, USA).

Spared nerve injury model of neuropathic pain in rats

The neuropathic pain model of spared nerve injury (SNI) rats was established as previously described (Decosterd and Woolf 2000; Li *et al.* 2013). In brief, the left common peroneal and tibial nerves were tightly ligated with 5.0 silk sutures, and sectioned distal to the ligation with removal of 2–4 mm nerve stump, leaving the sural nerve left intact. Sham-surgery rats experienced all surgical procedures except for nerve injury.

Mechanical allodynia measured as 50% paw withdrawal threshold

Rats were habituated in a transparent plastic box on a metal mesh floor before testing. Von Frey filaments (0.41–15.1 g; North Coast, Gilroy, CA. USA) were applied to the lateral plantar surface of the hind paws (i.e. to the receptive field of the sural nerve). The 50% paw withdrawal threshold (PWT) was calculated by the 'up and down' method as previously described (Chaplan *et al.* 1994). These behavioral tests were designed and carried out single-blindly by another person who was blind of animal groups.

Mechanical allodynia was measured 1 day before, and 3, 7, 14, 21, and 28 days after SNI or sham surgery.

Open-field test

The apparatus for the open-field test (Cunha and Masur 1978; Jiang *et al.* 2014) was a box (100 × 100 × 50 cm) made of opaque plastic materials (Shanghai Mobiledatum Information Technology Co., Shanghai, China). The test was conducted in a quiet room (~ 50 lux) in the morning (8:00–12:00 am). Each rat was placed in the center and its behavior was recorded for 10 min. The open-field area was partitioned into 25 equal-size squares. The time spent and distance travelled in the central nine squares were two indexes for anxiety-like behaviors. The total distance travelled was calculated to indicate exploratory behaviors. The box was cleaned with 10%

ethanol after each test. Rats in different groups were examined on the same day according to a random number table.

Elevated plus-maze test

Anxiety-like behaviors were also evaluated by the elevated plus-maze test (Rivlin and Tator 1977; Jiang *et al.* 2014) 2 days after the open-field test in the same room. The apparatus consisted of two open and two closed arms (48 × 8 × 40 cm each arm) (Shanghai Mobiledatum Information Technology Co., Ltd). Each rat was placed in the center of the elevated plus-maze facing one of the open arms. The time spent in the open or closed arms was recorded during a 10-min test period. The elevated plus-maze was carefully cleaned with 10% ethanol before each animal was placed onto the equipment. Rats in different groups were examined on the same day according to a random number table.

Measurement of neurosteroid levels by high performance liquid chromatography/tandem mass spectrometry

Under isoflurane anesthesia, rats were quickly decapitated between 16:00 and 18:00. Brains were removed rapidly and placed on ice. The bilateral hippocampi (120–140 mg in wet weight) were isolated and stored in liquid nitrogen. After being weighted, one hippocampal sample was homogenized on ice in 500 μL double-distilled water with 10 μL MT as an internal standard (0.3 pg/mL in methanol). The homogenate was extracted with 1200 μL of ethyl acetate/hexane (9 : 1, V/V), and vortexed for 5 min. After centrifugation at 12 400 g for 5 min, the clear supernatant was transferred to another Eppendorf tube, dried under nitrogen, and re-suspended with 100 μL of 0.1% formic acid aqueous/methanol (1 : 1, V/V). The sample was centrifuged at 12 400 g for 15 min and the supernatant was transferred to an autosampler vial for further HPLC/MS analysis.

PREG, PROG, DOC, AP, and THDOC levels were measured by HPLC/MS as previously described (Zhang *et al.* 2016). The brain extracts were analyzed on a Zorbax SB-C18 column (50 × 4.6 mm, 1.8 μm; Agilent, Santa Clara, CA, USA). The mobile phase was composed of 5 mM ammonium acetate in water (A) and methanol (B) using the following gradient program: 0–2.5 min, isocratic at 60% B; 2.5–5.5 min, linear gradient from 60% to 95% B; 5.5–7.0 min, isocratic at 95% B; 7.0–7.1 min, linear gradient from 95% to 60% B; 7.1–10.0 min, isocratic at 60% B. The flow-rate was 0.50 mL/min, and the column temperature was 45°C. The injection volume was 10 μL. MT was used as the internal standard for quantitative analysis. The chromatographic conditions were optimized by analyzing standard solutions and also spiked blank extracts.

Tandem MS detection was performed using an AB SCIEX QTRAP (6500; Applied Biosystems, Foster City, CA, USA) triple quadrupole mass analyzer equipped with an ESI ion source operating at 25°C in the positive mode. Detection and quantitation of all analytes were accomplished by using multiple reaction monitoring with two transitions monitored per analyte. Analyst Software (Applied Biosystems) was used for instrument control, data acquisition, qualitative and quantitative data analysis. The analytical MS parameters of the target compounds are listed in Table 1.

Neurosteroid levels were measured 7, 14, and 28 days after SNI or sham surgery.

Hippocampal microinjection

Rats were deeply anesthetized with 1% pentobarbital sodium in saline (50 mg/kg, *i.p.*). Infusion guide cannulae (internal diameter, 0.38 mm; RWD Life Science, Shenzhen, China) were stereotaxically implanted above bilateral ventral hippocampi (AP −5.4 mm, ML ± 4.8 mm, and DV 1.0 mm) (Paxinos and Watson 2005). A sterile obturator was left inside each cannula until drug delivery. Rats were allowed to recover for at least 7 days before further experiments.

For drug delivery, the injection needle (internal diameter, 0.20 mm; 6.0 mm longer than the matched cannula) was introduced through the guide cannula until its lower end was 6.0 mm below the cannula, right into the ventral hippocampus. The TSPO activator AC-5216 was prepared as a suspension in tragacanth gum (0.5% aqueous final solution). PK 11195 was dissolved in 100% dimethylsulfoxide and stored in sterile aliquots at −40°C. Immediately prior to administration, aliquots were thawed and diluted to the final concentration of 4.0 μg/μL in artificial cerebrospinal fluid (4% dimethylsulfoxide in final solution). Drugs or vehicle solutions (1 μL) were microinjected into the bilateral ventral hippocampi using the polyethylene catheter (PE-10) connected to a 2-μL syringe needle (Gaoge, Shanghai, China) over 2 min. The needle was left *in situ* for 1 min to maximize drug diffusion. The 50% PWTs, open-field test or elevated plus-maze test was carried out 1 h (AC-5216) or 4 h (PK 11195) later after drug delivery by another experimenter who was single-blinded to drugs and animal grouping. Vehicle injections served as controls. Drugs were microinjected into naïve rats or rats 28 days after SNI.

Immunofluorescence

Rats were deeply anesthetized with sodium pentobarbital in saline (50 mg/kg, *i.p.*) and perfused intracardially with 400 mL of 0.9% saline, followed by 400 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2–7.4, 4°C). The brains were removed, post-fixed overnight at 4°C, and kept in 30% sucrose in 0.1 M phosphate-buffered saline (PBS) for dehydration. Coronal sections (30 μm in thickness) were cut on a cryostat and kept in anti-freezing fluid (30% ethanediol, 20% propanetriol, and 50% 0.01 M PBS) at −20°C. Sections were washed twice in 0.01 M PBS for 10 min each and incubated in 0.01 M citrate buffer (pH 6.0) for 30 min at 37°C for antigen retrieval. After washing, sections were incubated in 0.3% Triton X-100 for 30 min at 25°C. They were next blocked for 1 h in 3% bovine serum albumin (0.01 M PBS with 0.3% Triton X-100). All cryostat sections were incubated overnight at 4°C with monoclonal rabbit anti-TSPO antibody (1 : 200; Abcam, Cambridge, UK). After washing, sections were incubated for 1 h at 25°C with the Cy3- or FITC-conjugated secondary antibody (1 : 500; Abcam). For double immunofluorescence staining, sections were incubated with a mixture of rabbit anti-TSPO antibody and mouse monoclonal anti-neuronal specific nuclear protein (NeuN, neuronal marker, 1 : 200; Millipore, Temecula, CA, USA), mouse monoclonal anti-glial fibrillary acidic protein (astrocyte marker, 1 : 500; Cell Signaling Technology, Danvers, MA, USA), or a goat polyclonal anti-ionized calcium-binding adaptor molecule 1 (Iba1, microglia marker, 1 : 500; Abcam) overnight at 4°C. The sections were then incubated with a mixture of FITC- and Cy3-conjugated secondary antibodies for 1 h at 25°C. The stained sections were examined with a fluorescence microscope (200× magnifications;

Table 1 Mass spectrometric parameters for target neurosteroids

Analytes	Parent ion (m/z)	Product ion (m/z)	Retention time (min)	Declustering potential (V)	Collision energy (eV)
Pregnenolone (PREG)	317.2	281.2	7.0	36	16.4
		159.1[a]			30.8
Progesterone (PROG)	315.5	109.1	6.7	76	34.0
		97.0[a]			26.6
11-Deoxycorticosterone (DOC)	331.5	109.0	5.6	79	26.0
		97.0[a]			27.0
Allopregnanolone (AP)	319.2	301.2[a]	7.4	25	13.5
		213.2			32.8
Tetrahydrodeoxycorticosterone (THDOC)	352.2	317.2[a]	6.7	40	21.2
		335.2			27.4
17-alpha-Methyltestosterone (MT)	303.5	109.1	6.2	66	37.8
		97.0			33.9

[a]Quantitative ion.

Leica DMI 4000B, Wetzlar, Germany), and images were captured with a CCD spot camera. The fluorescent density was analyzed using Image-Pro Plus Version 6 (Media Cybernetics, Rockville, MD, USA).

TSPO expression was detected in sham-surgery and SNI rats on 7, 14, and 28 days post-surgery.

Nissl staining for microinjection sites

After behavioral experiments, 0.5 μL 2.5% Evans blue in the saline solution was injected to verify the location of the needle tip. Each rat was deeply anesthetized and perfused with 4% paraformaldehyde in phosphate buffer. The brain was removed and stored in a paraformaldehyde solution. Sections were cut coronally through the hippocampus at 30 μm in thickness on a cryostat microtome and then mounted on gelatin-coated glass slices. After drying, the sections were stained in 1% cresyl violet solution for 10 min and washed in a gradient of alcohols. The location of the needle tip on the slide was inspected and photographed under a light microscope (Leica DMI 4000B).

Statistics

Data were presented as mean ± SEM. The 50% PWTs among groups were analyzed by two-way repeated measures ANOVA with groups and time points as independent factors, followed by Bonferroni *post hoc* comparisons. Immunofluorescence data were analyzed by Student's *t*-test for independent-samples or one-way ANOVA according to group conditions. Data from open-field and elevated plus-maze tests were analyzed by one-way ANOVA, followed by Bonferroni *post hoc* comparisons. The level of statistical significance was set at 5% ($p < 0.05$) in all analyses.

Results

Elevated hippocampal neurosteroids in rats with chronic neuropathic pain

Spared nerve injury induced significant and persistent mechanical allodynia in ipsilateral (group: $F_{1,30} = 148.40$, $p < 0.001$; time points: $F_{5,150} = 24.29$, $p < 0.001$; interaction: $F_{5,150} = 15.35$, $p < 0.001$, $n = 16$ per group, Fig. 1a) but not contralateral hindpaws (group: $F_{1,30} = 0.02$, $p = 0.895$; time: $F_{5,140} = 3.42$, $p = 0.006$; interaction: $F_{5,140} = 0.14$, $p = 0.983$, repeated measures of two-way ANOVA followed by Bonferroni *post hoc* test, Fig. 1b). Five neurosteroids (PREG, PROG, DOC, AP, and THDOC, illustrated in Fig. 1c) in the hippocampus were measured on days 7, 14, and 28 after surgery by HPLC/MS. The analytical mass spectrometric parameters of target compounds were shown in Table 1. The contents of these neurosteroids were comparable between SNI and sham-surgery groups (Fig. 1d and e) until 28 days, when PREG ($t_{26} = 2.19$, $p = 0.038$), PROG ($t_{26} = 3.80$, $p = 0.001$), DOC ($t_{26} = 2.39$, $p = 0.024$) and AP ($t_{26} = 2.04$, $p = 0.050$) showed significant increases in the SNI group (Fig. 1f). THDOC showed an increase trend though not statistically significant ($t_{26} = 1.57$, $p = 0.129$, Student's *t*-test). These results demonstrate increased hippocampal neurosteroid contents in chronic neuropathic pain rats.

Up-regulated TSPO expression in the ventral hippocampus in rats with chronic neuropathic pain

We next examined hippocampal expression of TSPO, the upstream rate-limiting enzyme for steroidogenesis (Papadopoulos *et al.* 1997, 2006a), with immunofluorescence staining. TSPO expression in dorsal and ventral poles of the hippocampus was explored separately given their functional distinction (Fanselow and Dong 2010). TSPO was mainly observed in the pyramidal layer of the hippocampus, with the most extensive expression in the CA3 region (Figs 2 and 3). TSPO expression in the ventral (ipsilateral CA3: $F_{3,15} = 5.70$, $p = 0.008$; contralateral CA3: $F_{3,15} = 6.38$, $p = 0.005$, Fig. 3), but not dorsal, CA3 region (ipsilateral CA3: $F_{3,15} = 0.33$, $p = 0.804$; contralateral CA3: $F_{3,16} = 1.97$, $p = 0.159$, one-way ANOVA followed by Bonferroni *post hoc* test, Fig. 2) showed a significant increase on

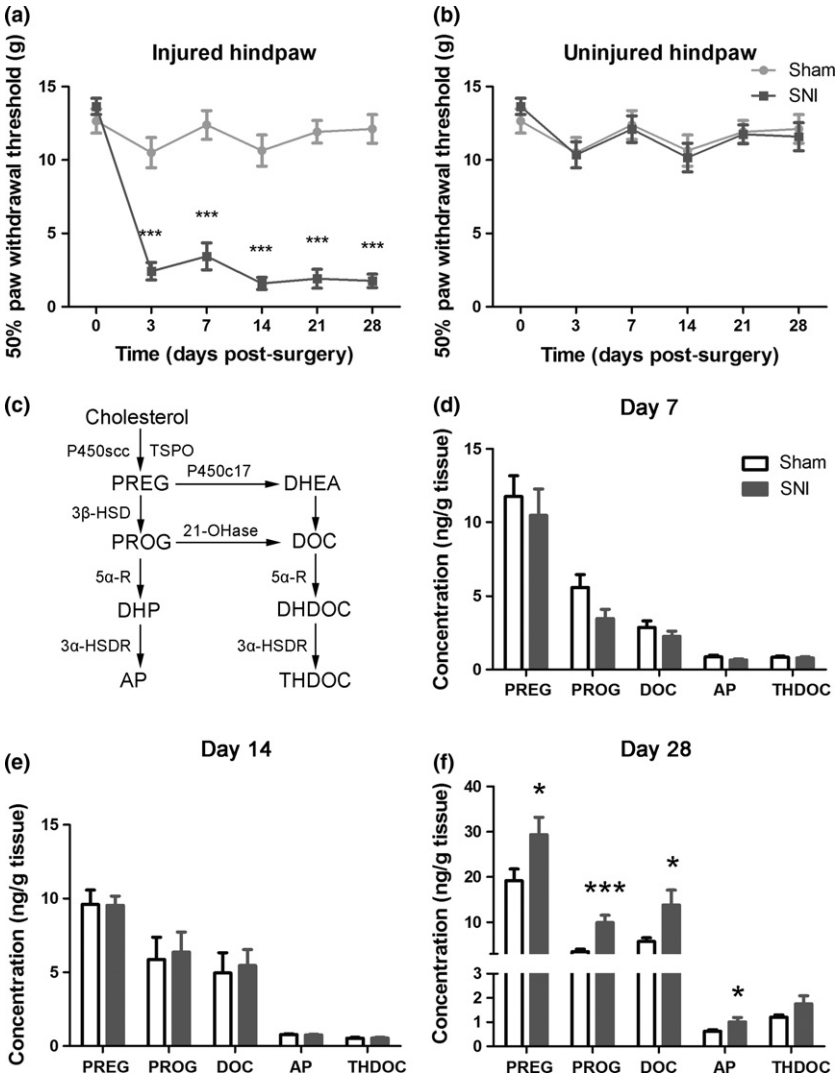

Fig. 1 Elevated hippocampal neurosteroids in rats with chronic neuropathic pain. (a) and (b) 50% paw withdrawal thresholds of injured (a) or uninjured (b) hindpaws ($n = 16$ animals/group). (c) Schematic illustration of the biosynthetic steps for major neurosteroids. (d, e and f) Concentrations of five neurosteroids in the hippocampus on days 7, 14, and 28 after spared nerve injury (SNI) surgery ($n = 15$–21 animals/group). *$p < 0.05$, ***$p < 0.001$ compared with the Sham-surgery group, two-way ANOVA with repeated measures or Student's *t*-test of independent-samples.

day 28 after SNI but not earlier. No significant differences were detected between bilateral hippocampi (data not shown). Taken together, TSPO expression increases in the ventral hippocampus in chronic neuropathic pain, which parallels increased expression of neurosteroids.

Cellular distribution of hippocampal TSPO

To determine the types of cells expressing TSPO, double immunofluorescence staining of TSPO was performed on hippocampal sections with three cell-specific markers: NeuN (a marker for neurons), glial fibrillary acidic protein (a marker for astrocytes), and Iba1 (a marker for microglia). As shown in Fig. 4, TSPO was mainly expressed in neurons (Fig. 4a and d) and microglial cells (Fig. 4c and f), but not in astrocytes (Fig. 4b and e). Compared with the sham-surgery group, the percentage of neurons ($t_7 = 4.97$, $p = 0.002$, Fig. 4g) and microglia ($t_7 = 2.78$, $p = 0.027$, Student's *t*-test, Fig. 4i) that expressed TSPO increased on day 28 after surgery in the SNI group, suggesting that the increased expression of TSPO might be derived from neurons and microglia, but not astrocytes, in the ventral hippocampus.

Lack of effects of activation or inhibition of TSPO on mechanical pain

We next examined whether hippocampal neurosteroids affected pain behaviors. Since increased expression of TSPO was found mainly in the ventral hippocampus, we microinjected the TSPO activator (AC-5216) (Kita *et al.* 2004; Papadopoulos *et al.* 2006a) or inhibitor (PK 11195) (Le Fur *et al.* 1983) into the ventral hippocampus (Fig. 5). Our previous work showed that local microinjection of AC-5216 (4.0 μg/μL, 1 μL per side) induced a 2–3 fold increase of neurosteroids in the brain, whereas PK 11195 (4.0 μg/μL, 1 μL per side) induced a significant decrease (Zhang *et al.* 2016). However, neither AC-5216 nor PK 11195 affected the mechanical 50% PWTs of naïve or neuropathic rats (Fig. 6), indicating limited effects of hippocampal neurosteroids on the sensory aspect of pain.

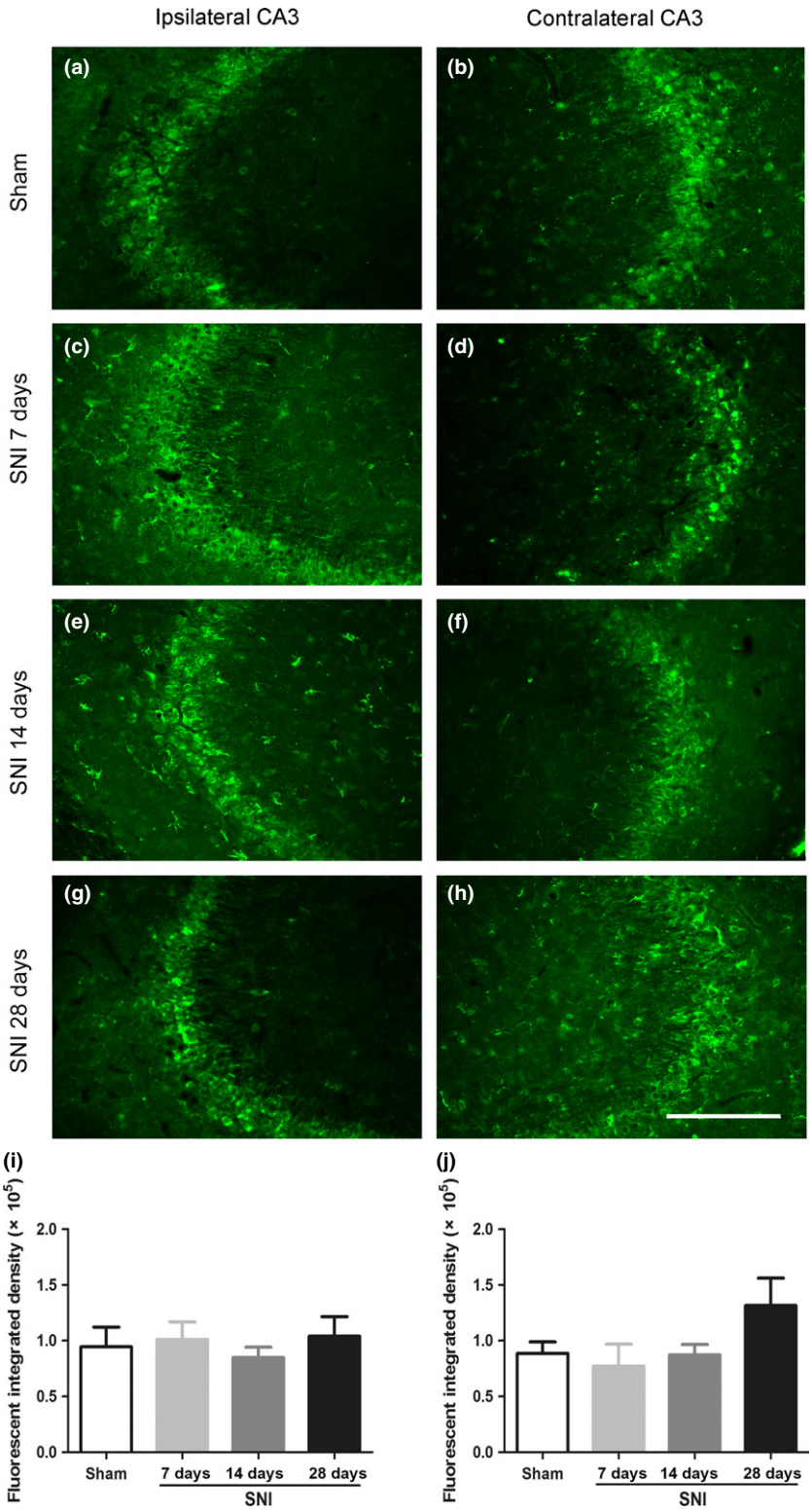

Fig. 2 Similar levels of translocator protein (TSPO) expression in the CA3 region of bilateral dorsal hippocampi after spared nerve injury (SNI) surgery. (a–h) Representative images showing TSPO expression. (i and j) Quantification of TSPO expression levels. $n = 5$ animals/group. *Scale bar* = 200 μm.

Anxiolytic effects of hippocampal neurosteroids in normal rats

We next asked whether hippocampal neurosteroids modulated anxiety-like behaviors, which depend on the ventral hippocampus (Kjelstrup *et al.* 2002; Fanselow and Dong 2010). Open-field and elevated plus-maze tests are frequently used to examine anxiety-like and exploratory behaviors in rats (Jiang *et al.* 2014). In normal rats, AC-5216 microinjection significantly increased the time spent

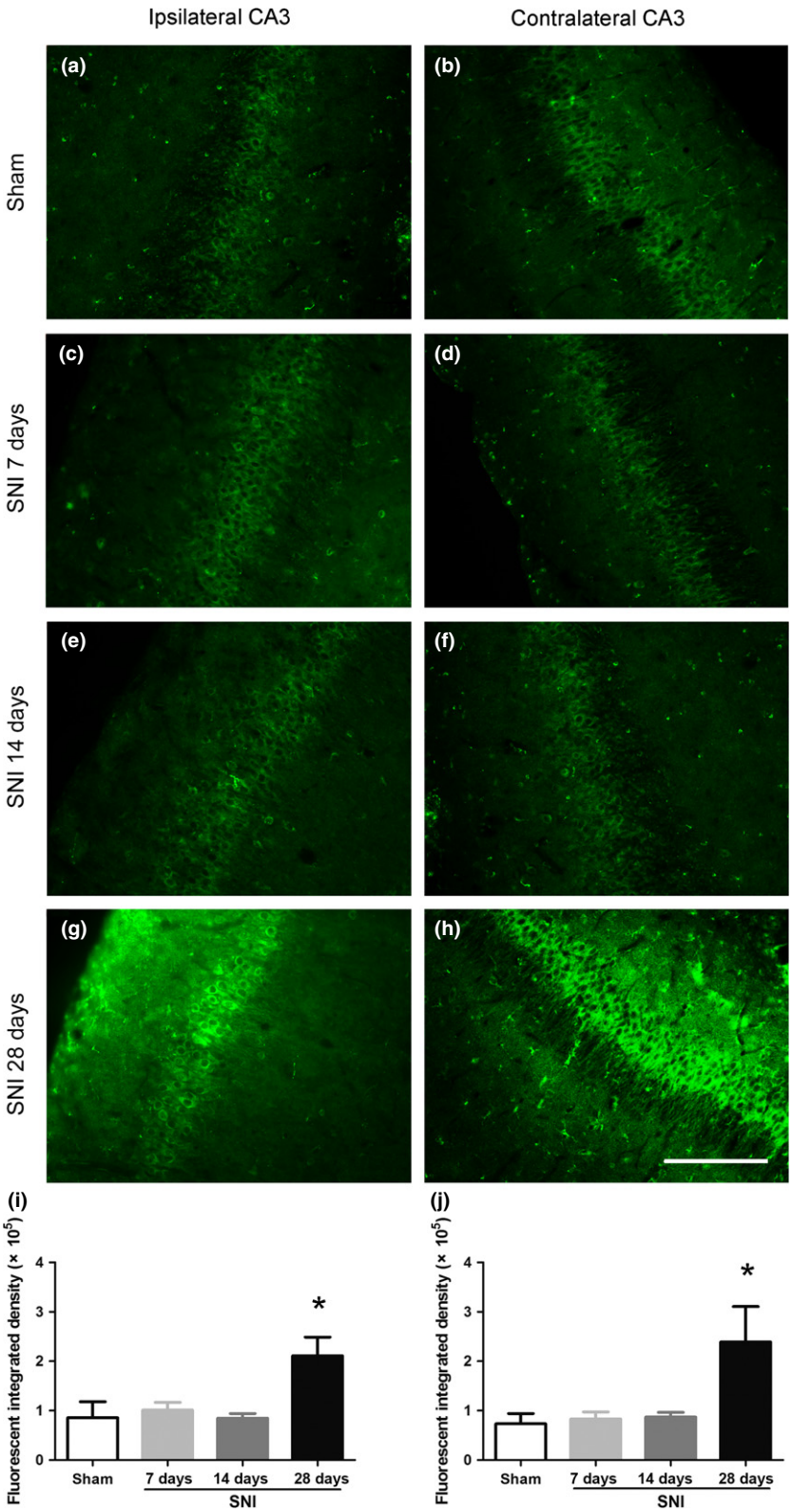

Fig. 3 Increased translocator protein (TSPO) expression in CA3 regions of bilateral ventral hippocampi 28 days after SNI. (a–h) Representative images showing TSPO expression. (i and j) Quantification of TSPO expression levels. *$p < 0.05$ compared with Sham-surgery group, one-way ANOVA followed by Bonferroni *post hoc* test, $n = 5$ animals/group. *Scale bar* = 200 μm.

($F_{2,24} = 11.90$, $p < 0.001$, Fig. 7d) and entries into the open arms ($F_{2,24} = 13.80$, $p < 0.001$, Fig. 7e) in the elevated plus-maze test. The time spent and distance travelled in the center of the open-field test (Fig. 7a and b) showed similar trend though not statistical significance. By contrast, PK 11195 significantly decreased the time spent and the distance

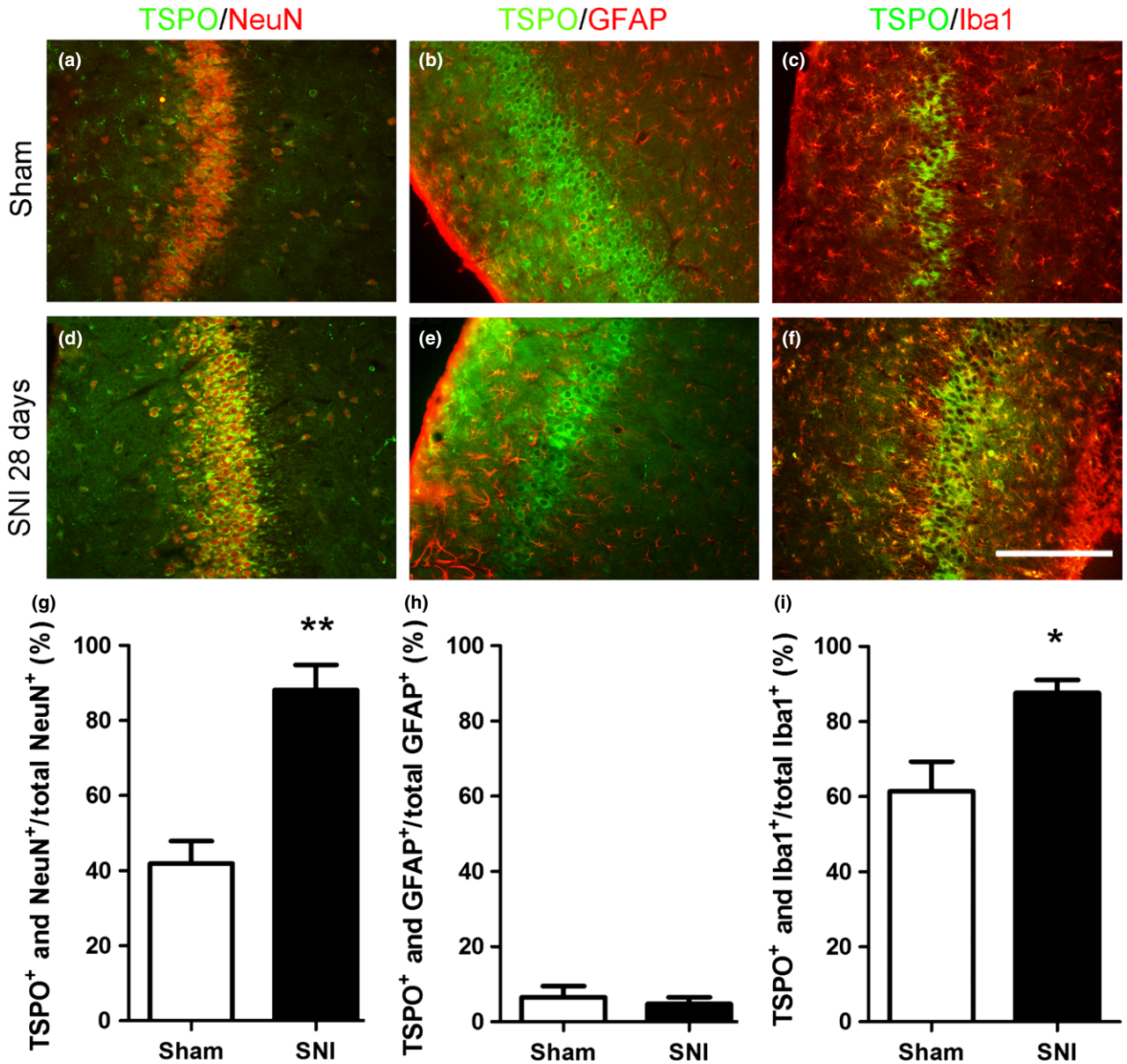

Fig. 4 Cellular distribution of translocator protein (TSPO) in the hippocampus. Double immunofluorescence staining of TSPO (green) with NeuN (marker for neuron, a and d), glial fibrillary acidic protein (GFAP) (red, marker for astrocyte, b and e) and Iba1 (red, marker for microglia, c and f) showed co-localization of TSPO with NeuN and Iba1, but not with GFAP. (g–i) Proportions of TSPO-positive neurons, astrocytes and microglia were shown in (g), (h) and (i), respectively. $*p < 0.05$, $**p < 0.01$ compared with Sham-surgery group, Student's *t*-test of independent-samples, $n = 5$ animals/group. *Scale bar* = 200 μm.

travelled in the center of the open field (time: $F_{2,24} = 10.15$, $p < 0.001$; distance: $F_{2,24} = 11.07$, $p < 0.001$, one-way ANOVA followed by Bonferroni *post hoc* test, Fig. 7a and b). The time spent and entries into open arms in the elevated plus-maze test (Fig. 7d and e) also showed an obvious decrease trend though not reaching statistical significance. Total distance travelled in the open field ($F_{2,24} = 1.03$, $p = 0.373$, Fig. 7c) and the entries into closed arms ($F_{2,24} = 1.26$, $p = 0.302$, one-way ANOVA followed by Bonferroni *post hoc* test, Fig. 7f), both reflecting locomotive activities, remained unaffected. These results indicate anxiolytic effects of hippocampal neurosteroids in normal rats.

Anxiolytic effects of hippocampal neurosteroids in neuropathic rats

Anxiety frequently accompanies chronic pain (Kontinen *et al.* 1999; Monassi *et al.* 2003; Demyttenaere *et al.* 2007; Jiang *et al.* 2014; Wang *et al.* 2015). We next asked whether hippocampal neurosteroids alleviated pain-induced anxiety-like behaviors. Rats with chronic neuropathic pain (day 28)

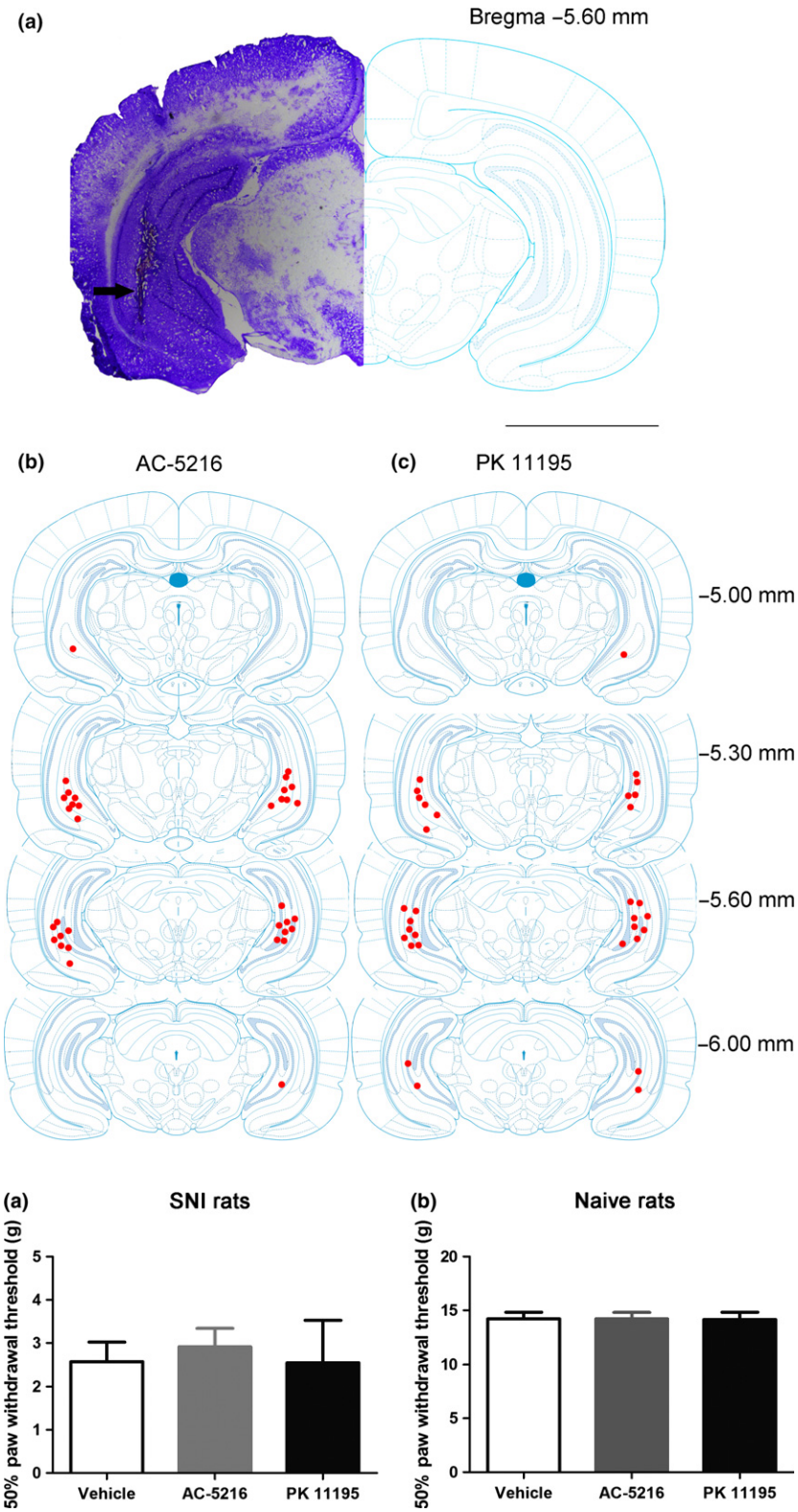

Fig. 5 Histological confirmation of bilateral injection sites in the ventral hippocampus. (a) A representative Nissl staining image for the localization of the injection needle tip (black arrow). Injection locations of AC-5216 (b) or PK 11195 (c) were illustrated as red dots within coronal sections of the rat brain. Images were adapted from the atlas of Paxions and Watson (2005). *Scale bar* = 4 mm. Only data from rats with correct localization were included in the following analysis.

Fig. 6 Altering neurosteroid contents through the translocator protein activator (AC-5216) or inhibitor (PK 11195) did not affect mechanical thresholds of spared nerve injury (SNI) (a) or naïve rats (b). One-way ANOVA, n = 7 - 10 animals/group.

developed anxiety-like behaviors, indicated by decrease time spent (t_{18} = 2.89, p = 0.010, Fig. 8a) and distance travelled (t_{18} = 3.02, p = 0.007, Fig. 8b) in the central area of the open field, as well as decreased time spent (t_{18} = 4.58, p < 0.001, Fig. 8d) and number of entries into open arms (t_{18} = 4.479, p < 0.001, Fig. 8e) in the elevated plus-maze

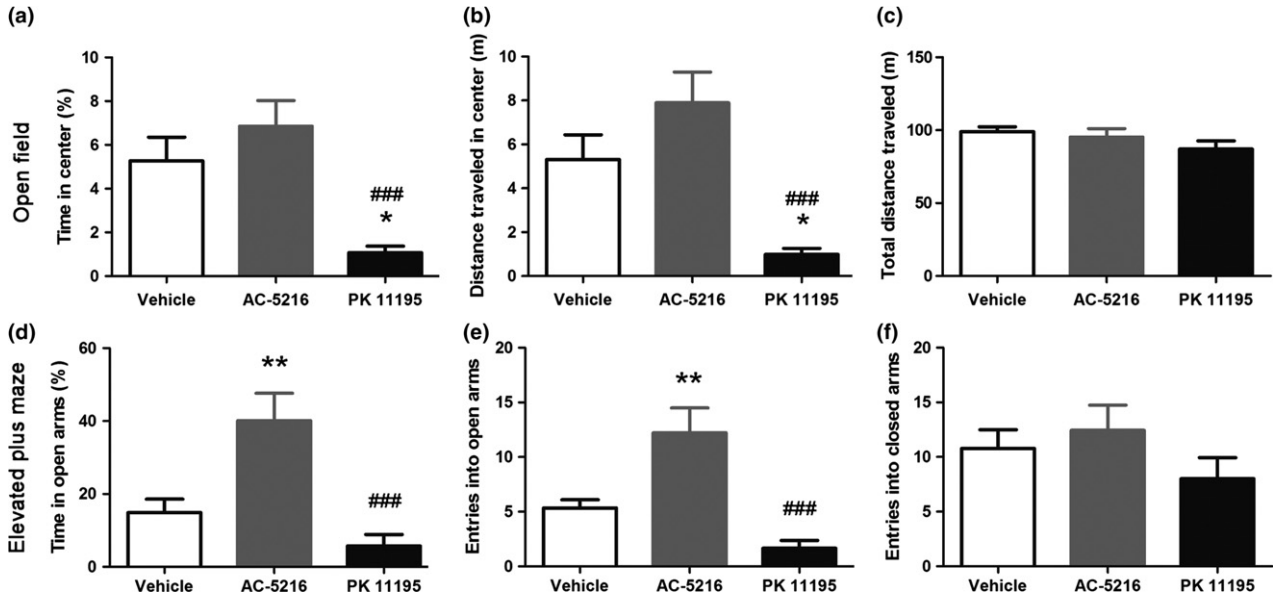

Fig. 7 Anxiogenic effects of translocator protein (TSPO) inhibition in normal rats. Microinjection of AC-5216 (TSPO activator) into the ventral hippocampus relieved, whereas PK 11195 (TSPO inhibitor) exacerbated, anxiety-like behaviors in the open-field (a and b) and elevated plus-maze tests (d and e), without affecting exploratory behaviors (c and d). $*p < 0.05$, $**p < 0.01$ compared with Vehicle injection group, $^{###}p < 0.001$ compared with AC-5216 injection group, one-way ANOVA followed by Bonferroni *post hoc* test, $n = 7$–9 animals/group.

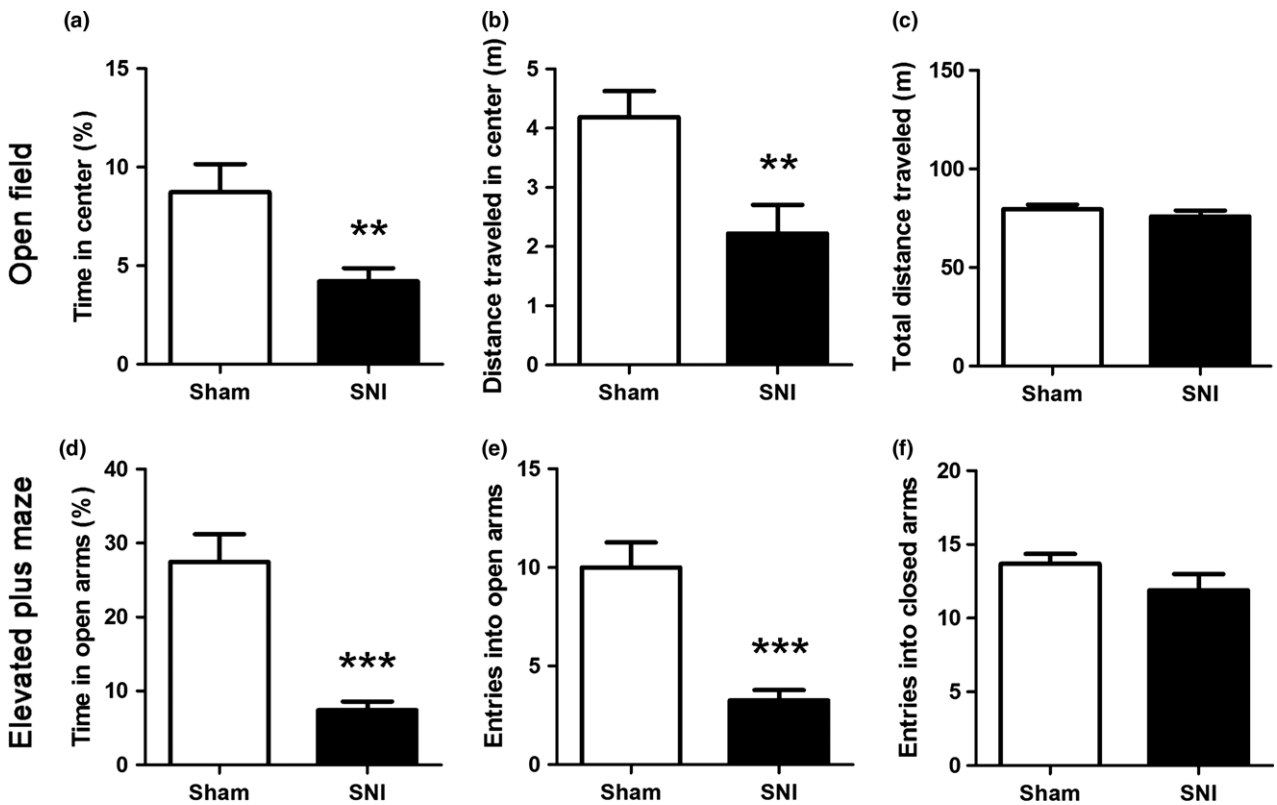

Fig. 8 Anxiety-like behaviors in rats with chronic neuropathic pain. Neuropathic pain was accompanied by decreased time (a) and distance travelled in the central area (b) but similar total distance travelled (c) in the open field in the open-field test, and decreased time (d) and number of entries (e) into open arms but similar close arm entries (f) in the elevated plus-maze. $**p < 0.01$, $***p < 0.001$ compared with the Sham-surgery group, Student's *t*-test of independent-samples, $n = 10$ animals/group.

test. The total distance travelled in the open field (t_{18} = 0.96, p = 0.349, Fig. 8c) and the number of entries into closed arms in the elevated plus-maze test (t_{18} = 1.42, p = 0.173, Student's t-test, Fig. 8f) remained unchanged.

In vivo microinjection of PK 11195 into bilateral ventral hippocampi significantly decreased the time spent in open arms and the number of open arm entries of elevated plus maze test (time: $F_{2,18}$ = 8.81, p = 0.002; entries: $F_{2,18}$ = 11.93, p < 0.001, Fig. 9d and e). The time spent and the distance travelled in the center of the open-field test (time: $F_{2,18}$ = 4.71, p = 0.023; distance: $F_{2,18}$ = 3.40, p = 0.056, Fig. 9a and b) also showed a decrease trend. Total distance travelled ($F_{2,18}$ = 0.59, p = 0.564, Fig. 9c) and the number of entries into closed arms ($F_{2,18}$ = 2.50, p = 0.110, one-way ANOVA followed by Bonferroni *post hoc* test, Fig. 9f) showed similar locomotor abilities. Microinjection of AC-5216 produced insignificant changes, possibly due to ceiling effects of elevated neurosteroids. Overall, these findings indicate anxiolytic effects of hippocampal neurosteroids in neuropathic pain.

Discussion

In this study, we found that the contents of four neurosteroids (PREG, PROG, DOC, and AP) and their rate-limiting enzyme TSPO in the hippocampus increased in chronic neuropathic pain (28 days after SNI surgery). Pharmacological intervention with TSPO activators and inhibitors indicated anxiolytic effects of hippocampal neurosteroids in naïve and neuropathic rats.

Increased hippocampal neurosteroids and TSPO in chronic neuropathic pain

Pain-induced elevation of neurosteroids has been reported in the spinal cord (Poisbeau *et al.* 2005; Mensah-Nyagan *et al.* 2008), the brainstem (Patte-Mensah *et al.* 2004), the whole brain (Kawano *et al.* 2011), and the lateral thalamus (Zhang *et al.* 2016). In addition to the sensory component, chronic pain also involves the activation of mood-affective and emotion-cognitive control (Millan 1999). The hippocampus is closely related to negative emotions such as depression, anxiety, catastrophizing and stress (Kjelstrup *et al.* 2002), as well as the affective dimension of pain (Derbyshire 2000; Ploghaus *et al.* 2001; Liu and Chen 2009; Gondo *et al.* 2012). In this study, we observed increased hippocampal neurosteroids on day 28 after SNI. This time point is much later compared with previous studies on neurosteroid changes in other regions, for example, 10 or 14 days after spinal nerve ligation in the spinal cord (Patte-Mensah *et al.* 2004; Kawano *et al.* 2011). In addition to the different regions examined, this variation might also result from differences in pain models (spinal nerve ligation vs. SNI), or analytical methods (radioimmunoassay vs. our HPLC/MS).

The increased neurosteroids most possibly result from the increased expression of steroidogenesis enzymes including TSPO (Papadopoulos *et al.* 1997, 2006a,b). Our previous work has confirmed that microinjection of the TSPO activator AC-5216 up-regulated five neurosteroids in the thalamus, whereas the TSPO inhibitor PK 11195 down-regulated them (Zhang *et al.* 2016). In this study, TSPO expression increased

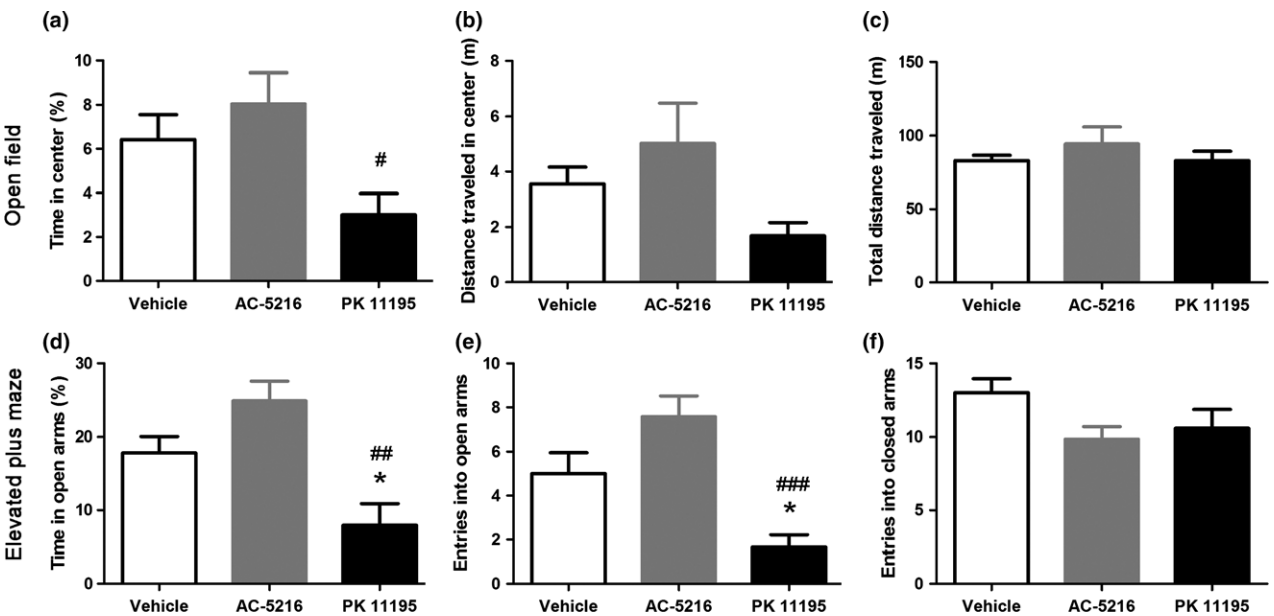

Fig. 9 Anxiogenic effects of translocator protein inhibition in neuropathic rats. (a–c) Time in the central area (a), distance travelled in the central area (b), and total distance travelled (c) in the open field. (d–f) Time spent (d), number of entries (e) into open arms, and number of entries into closed arms (f) in the elevated plus-maze. *p < 0.05 compared with Vehicle injection group, $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 compared with AC-5216 injection group, one-way ANOVA followed by Bonferroni *post hoc* test, n = 7–8 animals/group.

in the ventral hippocampus simultaneously with the elevation of neurosteroids after SNI. TSPO is mainly expressed in neurons and microglia, and the percentages of TSPO-positive neurons and microglia increased 28 days after SNI. Consistent with our findings, pyramidal neurons in the hippocampus are immunopositive for 5α-reduced steroids (Saalmann *et al.* 2007; Tokuda *et al.* 2010) and are the primary, if not exclusive, cells that express 5α-reductase (a key synthetic enzyme for 5α-reduced steroids) (Agis-Balboa *et al.* 2006), TSPO (Tokuda *et al.* 2010) and steroidogenic acute regulatory protein (StAR, a molecular shuttle that delivers cholesterol to mitochondria for steroid synthesis) (Wehrenberg *et al.* 2001). In addition, the 5α-reduced steroid AP colocalizes with TSPO in pyramidal neurons, while a specific TSPO activator (2-[2-(4-fluorophenyl)-1H-indol-3-yl]-N,N-dihexylacetamide) enhances steroid immunoreactivities (Tokuda *et al.* 2010). There is also evidence for the expression of TSPO in hippocampal glia (Falchi *et al.* 2007).

Distinction between ventral and dorsal hippocampus

The functional differentiation between dorsal and ventral hippocampus has been well documented (Fanselow and Dong 2010). The dorsal hippocampus, which corresponds to the posterior hippocampus in primates, mediates declarative cognition, whereas the ventral (anterior in primates) pole is more related to stress, emotion, and affection (Kjelstrup *et al.* 2002; Zhang *et al.* 2014). Anatomical basis of this divergence lies in fact that the ventral hippocampus strongly and directly connects to the amygdala and the hypothalamus, key components of brain circuits for anxiety and fear, while the dorsal hippocampus is more closely linked to the entorhinal cortex that is implicated in visuospatial information encodings (Schultz and Engelhardt 2014).

Our immunofluorescent data showed that the TSPO expression increased only in the ventral but not in the dorsal hippocampus. Since the HPLC/MS method required relatively large mass of brain tissues (> 80 mg), we did not make this separation for the measurement of neurosteroid contents. The TSPO expression pattern demonstrates that the increased neurosteroids observed in this study derive most likely from the ventral, but not the dorsal hippocampus. Consistent with this hypothesis, microinjection of bicuculline into ventral, but not dorsal, hippocampus attenuates formalin-induced suppression of pyramidal cell discharges (Zheng and Khanna 2008). In addition, pilot data from our laboratory also show that lesions of the ventral rather than dorsal dentate gyrus of hippocampus exacerbated the chronic complete Freund's adjuvant-induced inflammatory pain (Zheng *et al.* unpublished data).

Anxiolytic effects of hippocampal neurosteroids

A number of studies have reported the analgesic effects of neurosteroids or their analogs in the spinal cord (Poisbeau *et al.* 2005; Patte-Mensah *et al.* 2006) and the thalamus (Zhang *et al.* 2016). However, we failed to detect analgesic effects of hippocampal neurosteroids in either naïve or neuropathic rats. By contrast, this study has revealed anxiolytic role of neurosteroids in the ventral hippocampus. Systematic (*i.p.*) administration of TSPO activators or neurosteroids produces anxiolytic effects in naïve or chronic pain animals (Kita *et al.* 2004; Longone *et al.* 2011; Wang *et al.* 2015). More specifically, activation of TSPO by activators or up-regulation of 3α-hydroxy pregnane neurosteroids in the dorsal hippocampus relieves anxiety-like behaviors (Bitran *et al.* 2000; Modol *et al.* 2011). It is worth noting that the lack of pain-modulatory effects of TSPO manipulations in this study suggests that hippocampal neurosteroids produce direct anxiolytic effects, rather than indirect effects secondary to analgesia.

Further experiments are required to explore mechanisms underlying the anxiolytic effects of hippocampal neurosteroids. It is well known that neurosteroids have potent and selective effects on $GABA_A$ receptors (Lambert *et al.* 1995; Stell *et al.* 2003; MacKenzie and Maguire 2013). PREG, PROG, and DOC are positive modulators of $GABA_A$ receptors in a non-genomic manner, whereas AP and THDOC potentiate synaptic $GABA_A$ receptor functions and activate δ-subunit-containing extrasynaptic $GABA_A$ receptors that mediate tonic currents (Mihalek *et al.* 1999; Stell *et al.* 2003), both of which are widely distributed in the hippocampus (Sperk *et al.* 1997). Alpha-2 containing $GABA_A$ receptor subunits in the ventral hippocampus mediate unconditioned fear or anxiety (McEown and Treit 2013). Thus, $GABA_A$ receptors might be preferential targets for the fast anxiolytic function of these neurosteroids.

In conclusion, this study demonstrates anxiolytic effects of neurosteroids in ventral hippocampus in naïve rats or rats with persistent neuropathic pain. Hippocampal TSPO and the downstream neurosteroids might be potential therapeutic targets for the treatment of anxiety.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

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