Antinociceptive Effect of Tetrandrine on LPS-Induced Hyperalgesia via the Inhibition of IKKβ Phosphorylation and the COX-2/PGE2 Pathway in Mice

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Abstract

Tetrandrine (TET) is a bisbenzylisoquinoline alkaloid that is isolated from the Stephania Tetrandra. It is known to possess anti-inflammatory and immunomodulatory effects. We have shown that TET can effectively suppress the production of bacterial lipopolysaccharide (LPS)-induced inflammatory mediators, including cyclooxygenases (COXs), in macrophages. However, whether TET has an antinociceptive effect on LPS-induced hyperalgesia is unknown. In the present study, we investigated the potential antinociceptive effects of TET and the mechanisms by which it elicits its effects on LPS-induced hyperalgesia. LPS effectively evoked hyperalgesia and induced the production of PGE2 in the sera, brain tissues, and cultured astroglia. TET pretreatment attenuated all of these effects. LPS also activated inhibitor of NF-κB (IKKβ) and its downstream components in the IκB/nuclear factor (NF)-κB signaling pathway, including COX-2; the increase in expression levels of these components was significantly abolished by TET. Furthermore, in primary astroglia, knockdown of IKKβ, but not IKKα, reversed the effects of TET on the LPS-induced increase in IκB phosphorylation, P65 phosphorylation, and COX-2. Our results suggest that TET can effectively exert antinociceptive effects on LPS-induced hyperalgesia in mice by inhibiting IKKβ phosphorylation, which leads to the reduction in the production of important pain mediators, such as PGE2 and COX-2, via the IKKβ/IκB/NF-κB pathway.

Introduction

Inflammatory mediators, such as prostaglandins (PGs), PG syntheses, and cyclooxygenases (COXs), can cause abnormal neuronal activity, which leads to pain hypersensitivity [1]. Over the past decade, many studies have focused on the roles of these mediators in the regulation of hypersensitivity that is induced by environmental stimuli and pro-inflammatory factors, such as bacterial lipopolysaccharide (LPS) [2].

In the central nervous system (CNS), treatment of astrocytes and microglia with low concentrations of LPS can produce PGE2 via Toll-like receptor 4-dependent manners [3,4]. PGE2 can directly trigger pain-sensitive neurons to induce nociception [5,6]. At the same time, PGE2 receptors that are located in peripheral tissues trigger pain-sensitive neurons to induce nociception [5,6]. At the same time, PGE2 receptors that are located in peripheral tissues trigger pain-sensitive neurons to induce nociception. At the same time, PGE2 receptors that are located in peripheral tissues trigger pain-sensitive neurons to induce nociception. At the same time, PGE2 receptors that are located in peripheral tissues trigger pain-sensitive neurons to induce nociception.

Moreover, in inflammatory pain conditions, COX-2 itself can act as a nociceptive stimulator to directly cause pain. COX-2 is regulated by nuclear factor (NF)-κB, which is a well-known transcription factor that is involved in inflammation or injury. Recent reports revealed that NF-κB is also implicated in hyperalgesia [10–11], which is regulated by a series of adaptors. Under normal conditions, NF-κB is inactive, and it is bound to inhibitor IκB via its subunits, P65 and P50, in the cytoplasm. Upon IκB kinase (IKK) activation, IκB is phosphorylated, thus resulting in its ubiquitination and subsequent degradation by the 26S proteasome. NF-κB then translocates into the nucleus to regulate the transcription of genes that code for inflammatory cytokines and nociceptive substances [12,13].

Tetrandrine (TET) is an important bisbenzylisoquinoline alkaloid that is isolated from Stephania Tetrandra (Fig. 1A). It is traditionally used in China and Korea to treat patients with arthritis. Previous studies have shown that it possesses anti-arrhythmic [14], anti-hypertensive [15], cardio-protective [16], anti-tumorigenic [17], and anti-inflammatory effects [18]. We have demonstrated that TET exhibits anti-inflammatory and hepatoprotective effects in mice [19,20], and it inhibits IL-6 and TNF-α.
production in macrophages. However, whether it is involved in the inflammatory processes of nociception is unknown. In this study, we tested the role of TET on LPS-induced hyperalgesia in mice and investigated the potential mechanisms by which TET elicits its effects.

**Materials and Methods**

2.1 Animals

BALB/C mice (6–8 weeks old, 20–22 g) were obtained from the Laboratory Animal Center of Chongqing Medical University (Chongqing, China). All mice received humane care, and all studies were performed with approval from the Animal Care and Use Committee of Chongqing Medical University (approval #SCXK20070001). The mice were maintained in a SPF-grade facility under controlled conditions (22°C, 55% humidity, and 12 h day/night rhythm) and fed standard laboratory chow. After each experiment, mice were sacrificed under anesthesia with isoflurane and decapitated to ameliorate any suffering.

2.2 Materials and Drug Preparations

TET (C₃₈H₄₂O₆N₂, molecular weight: 622.8 g/mol) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and its purity was determined by HPLC, as previously described[19,20]. LPS (Escherichia coli, 0111:B4), morphine hydrochloride, and indomethacin were purchased from Sigma (MO, USA). All drugs were freshly prepared on the day of experiments. TET powder was dissolved in 0.01 M hydrochloric acid, and the pH was adjusted to 5.5 with 0.01 M NaOH. The LPS stock solution was...
reconstituted to a final concentration of 10 μg/ml. Indomethacin solutions for intraperitoneal injections were prepared fresh in 0.01 M sodium carbonate, pH 7.2, at a final concentration of 0.2 mg/ml.

2.3 Induction of hyperalgesia in mice

To induce hyperalgesia, mice were randomly divided into the control group (0.1 ml/10 g pyrogen-free sterile saline, intraperitoneal injections [i.p.]), LPS-stimulated group (100 μg/kg, i.p.), low TET+LPS-treated group (15 mg/kg TET, 100 μg/kg LPS, i.p.), moderate TET+LPS-treated group (30 mg/kg TET, 100 μg/kg LPS, i.p.), high TET+LPS-treated group (45 mg/kg TET, 100 μg/kg LPS, i.p.), indomethacin-treated group (5 mg/kg, 100 μg/kg LPS, i.p.), and morphine-treated group (10 mg/kg, 100 μg/kg LPS, i.p.). TET, indomethacin, or morphine was administered 30 min prior to LPS injections. Each group was repeated at least by five individuals.

The hot-plate and acetic acid-induced abdominal constriction (writhing) tests were performed to measure the hyperalgesic responses to LPS in the presence or absence of TET, indomethacin, or morphine. The hot plates (RB200, Chengdu TME, China) were maintained at 55±1°C, and induction time was determined by measuring the latency of paw licking every 2 h until 8 h after treatment. Due to its long-term effects, acetic acid was administered only once for each individual mouse at different time points (0, 2, 4, 6, and 8 h) and concentrations. The acetic acid-saline solution (0.1 ml/10 g of 0.7% acetic acid-saline) was intraperitoneally injected, and the frequency of abdominal constrictions was counted for 20 min. Writhe was defined as the contraction of forelimbs and elongation of the body.

2.4 Treatment and culture of astroglia

To prepare mouse cerebral astrocytes, cerebral cortices from P1 neonatal BALB/C mice were mechanically dissociated in astrocyte culture medium (Dulbecco’s Modified Eagle Medium [DMEM] with 10% fetal bovine serum [FBS] and antibiotics). After filtering through a 70 μm cell strainer, the cells were seeded in cell culture flasks. To obtain astroglia, confluent cultures were shaken at 250 rpm overnight at 37°C. The purity of astrocytes was checked by immunostaining for GFAP (Abcam, MA, USA), and the threshold was set at >95%[21,22]. When the primary cells reached 80–90% confluency, they were digested by 0.25% trypsin and plated in 12-well tissue-culture plates at a density of 1.0–1.5×10⁶ cells/well. When the cells in the 2nd passage were close to confluence, the culture medium was replaced with FBS-free DMEM. Cell treatments were performed according to following groups: control group (only the FBS-free DMEM); LPS-stimulated group (1 μg/ml); low, moderate, and high TET-treated groups (1 μg/ml LPS with TET [1×10⁻⁹ mol/l, 1×10⁻⁷ mol/l, and 1×10⁻⁶ mol/l, respectively]). Treated cells were incubated for 6 h, after which the cells and supernatants were harvested for various experiments.

2.5 Measurement of cell viability

The TET toxicity of cultured cells was determined using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, according to the manufacturer’s instructions. In brief, cells were seeded in 96-well plates and treated with increasing concentrations of TET [1×10⁻⁶ mol/l to 1×10⁻⁴ mol/l] for 15 min. MTT (terminal concentration: 0.5 g/L) was added into each well and incubated for 4 h. The optical density (OD) was measured at 570 nm by microplate UV spectrometer (SpectraMax 384 Plus).

2.6 Enzyme Immunoassay (EIA)

PGE₂ levels in purified plasma, brain homogenates, and cell culture supernatants were evaluated using a commercial EIA kit (Cayman, Michigan, USA), according to the manufacturer’s protocol. Samples were added to a plate that was pre-coated with goat anti-mouse IgG antibodies. PGE₂ monoclonal antibodies were then added to each well, and the plates were incubated for 1 h at 4°C. Afterwards, Ellman’s Reagent substrate was added to each well. The optical density of each sample was read at 412 nm. The standard curve was plotted, and the final concentrations of PGE₂ in the samples were calculated using the equations that were obtained from the curve.

2.7 Western blotting

Brain tissues and cultured cells were homogenized in protein extract solution. Protein concentrations were determined using the BCA protein assay kit (Thermo, USA). Samples (40 μg) were loaded onto a 12% polyacrylamide-sodium dodecyl sulfate (SDS) gel and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% (w/v) fat-free milk in Tris-buffered saline (TBS) containing 0.05% tween-20, followed by overnight incubations at 4°C with the primary antibody (1:1000). Afterwards, the membranes were treated with horseradish peroxidase-conjugated secondary antibody (1:5000, Abcam, USA) and visualized using an ECL chemiluminescence system with short exposure to X-ray films (Kodak, USA). The following primary antibodies were used: COX-1, COX-2, P65, phosphorylated P65 (pP65), IκBα, phosphorylated IκBα (pIκBα), IKKα, phosphorylated IKKα (pIKKα), IKKβ, phosphorylated IKKβ (pIKKβ), GAPDH, and tubulin (Abcam, USA).

2.8 Small-interfering RNA (siRNA) transfection

The SignalSilence siRNAs of IKKα, IKKβ, and control siRNA (unconjugated) were obtained from Santa Cruz Biotechnology (CA, USA). Cells were plated onto a 6-well plate at a density of 1.6×10⁵ cells/well. Once they were 60–80% confluent, cells were washed with PBS, and the pre-mixed siRNA transfection solution (including siRNA duplex solution and the dilution reagent) was added directly to the culture medium. Cells were then incubated for 24 h, and the culture medium was changed for another 24 h. The reactions were stopped, and lysis buffer was added to extract proteins from the cells for further experiments.

2.9 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from brain tissues and cultured cells using Trizol reagent, (Invitrogen, USA) according to the manufacturer’s guidelines, followed by further purification using the RNeasy Mini Kits (Qiagen, USA). Purified RNA was reverse-transcribed into cDNA with random hexamers by the SuperScriptTM First-Strand Synthesis System kit (Qiagen, USA) and analyzed by real-time RT-PCR with the Quantitect SYBR Green PCR Kits (Qiagen, USA), using a MJ DNA Engine Opticon2 qPCR System (MJ, USA). The following oligonucleotide primers were used: COX-1 forward: 5’-TGC CCT CGT TAC CCA AAG AC-3’, reverse: 5’-GGA CCC ATC TTC CCA GAG GT-3’; COX-2 forward: 5’-CGG AGA GAG TTC ATC CCT GA-3’; reverse: 5’-ATC CTT GAA AAG GGC GAG T’-3’; GAPDH (control) forward: 5’-CTC ATG ACC ACA GTC CAT GC-3’, reverse: 5’-CAC ATG GGT AGG GAT AAC AC-3’.
2.10 Statistical Analysis

All data were expressed as mean±standard deviation (M±SD) from at least four independent experiments. Results were analyzed by Student’s t test or analysis of variance (ANOVA). P≤0.05 was considered to be statistically significant.

Results

3.1 LPS induced hyperalgesia in mice via time- and dose-dependent manners

The administration of LPS (50, 100, or 200 μg/kg, i.p.) evoked dose-dependent hyperalgesia, as evaluated by the hot-plate and acetic acid-induced abdominal constriction tests (Fig 1). In the hot-plate test, hyperalgesia was assessed by the action of hind-paw licking. A decrease in the latency time of paw licking was observed at 2 h after LPS treatment, and maximal reduction was observed at 6 h (from 17.0 to 9.7, 7.1 and 4.1 seconds at LPS of 50, 100, 200 μg/kg, respectively). This was maintained until 8 h after LPS treatment (Fig 1B). Acetic acid-induced abdominal constriction numbers increased with LPS treatment, and the analogous initial and peak time points were determined (Fig 1C). Based on these results, we chose the 6-h time point for further experiments. In addition, mice that were exposed to the highest concentration of LPS (200 μg/kg) exhibited obvious hyperalgesia. However, this was accompanied by adverse reactions, including cachexia, diarrhea, and sustained trembling, all of which may potentially influence the evaluation of the algesic effect. Alternatively, a lower dose of LPS (100 μg/kg) induced obvious hyperalgesia without the adverse effects. Therefore, this concentration of LPS was used for further experiments.

3.2 Analgesic effects of TET on LPS-induced hyperalgesia in mice

Different doses of TET were administered to mice 30 min prior to LPS treatment. LPS-induced hyperalgesia was significantly repressed, as indicated by the elongated threshold time in hot-plate tests and the decrease in the number of writhing in acetic acid-induced abdominal constriction tests. The percentages of protection at 6 h by TET concentrations of 15, 30, and 45 mg/kg were 11.7%, 27.8%, and 39.6%, respectively, in the hot-plate test (Fig 1D) and 9.7%, 16.8%, and 49.6%, respectively, in the acetic acid-induced abdominal constriction test (Fig 1E). Indomethacin (5 mg/kg) and morphine (10 mg/kg) were used as the positive controls, due to their known antinociceptive effects[23,24]. The percentages of protection by morphine and indomethacin were 81.2% (hot-plate test) and 62.7% (acid-induced abdominal constriction test), respectively. These results indicate that TET may possess both peripheral and central antinociceptive properties on LPS-induced hyperalgesia in mice.

3.3 TET repressed PGE2 production in LPS-induced hyperalgesia in mice and cultured astroglia

To explore the antinociceptive mechanism of TET, PGE2 production was measured by EIA in the sera and brain tissues of LPS-induced hyperalgesic mice and in the supernatants of cultured astroglia. In vitro, LPS significantly increased PGE2 levels, which were markedly suppressed by TET pretreatment in dose-dependent manners (Fig 2A, 2B). To exclude the pathophysiological conditions in vivo that may potentially affect the intrinsic reactions, we cultured astroglia cells to verify the mechanism in vitro. The toxicity of TET on astroglia was evaluated by the MTT assay, and we confirmed that TET concentrations from 1×10^{-6} mol/l to 1×10^{-5} mol/l did not significantly repress cell viability (Fig 2C). Therefore, these concentrations of TET were used in further experiments. Treatment of astroglia with 1 μg/ml LPS significantly increased PGE2 levels. Similarly, TET co-treatment attenuated PGE2 levels in a concentration-dependent manner. The repressive peak was at the TET concentration of 1×10^{-6} mol/l (Fig 2D). These results suggest that the antinociceptive effect of TET on LPS-induced hyperalgesia in mice may be partially mediated through downregulation of the PGE2 signaling pathway.

3.4 TET suppressed COX-2, but not COX-1, levels

Following its release from membrane phospholipids by cytosolic or secretory phospholipases, arachidonic acid is converted to PGE2 by COX-1 and COX-2[25]. We then investigated the expression of COX-1 and COX-2 at the mRNA and protein levels. As shown in Figure 3A, brain tissues from LPS-stimulated mice exhibit four-fold increases in COX-2 protein levels, and TET pretreatment decreases these levels of COX-2. No changes in COX-1 were observed in the presence or absence of LPS or TET. This indicates that TET can selectively suppress COX-2 expression. Similar trends in the mRNA levels of COX-1 and COX-2 were observed (Fig 3B and 3C). Thus, the mechanisms of action in vivo and in vitro appear to be the similar processes.

3.5 TET decreased COX-2 expression through IKKβ, which further inhibited the NF-κB pathway

The NF-κB signaling pathway is consensually involved in LPS-induced cell activation and inflammation[26,27]. To investigate whether NF-κB activity is also regulated by TET, the expression of various components of the NF-κB pathway, including P65, pP65, IκBα, and pIκBα, were assessed by western blotting in cultured astroglia. As shown in Figure 4A, P65 expression is not significantly changed in the presence of LPS or TET. However, pP65 was notably up-regulated after LPS stimulus and gradually reversed by TET pretreatment. Meanwhile, pIκBα levels increased dramatically after LPS treatment and decreased with TET pretreatment. No changes in IκBα levels were observed. These results suggest that TET can inactivate the NF-κB signaling pathway through the inhibition of LPS-induced increases in IκBα phosphorylation, thus preventing the degradation of IκBα and retaining NF-κB in the cytoplasm(Fig 4A).

The phosphorylation of IκBα is catalyzed by the IKK complex, which is comprised of the IκKα, IκKβ, and IκKγ subunits. Among these, IκKα and IκKβ serve as the catalytic subunits to phosphorylate IκBα for degradation via ubiquitination[28]. Hence, we investigated whether IκKα and IκKβ are the upstream targets for TET in the NF-κB pathway. Western blotting showed that LPS increased the phosphorylation of IκKβ without affecting IκKα, which was consistent with previous reports[29,30,31]. Similarly, TET pretreatment effectively inhibited IκKβ phosphorylation without affecting IκKα (Fig 4B), which suggests that TET represses IκBα activity by inhibiting IκKβ.

To verify whether TET specifically targets IκKβ, cells were transfected with IκKα or IκKβ siRNA (si) to knock down their respective gene expression levels. As shown in Figure 5A, cells transfected with siIκKβ exhibit decreases in the protein expression of phosphorylated IκKβ, and pP65. LPS stimulus partially rescued these trends, although not to levels of those seen in LPS-treated control siRNA-transfected cells. SiIκKβ transfection also did not affect IκKα phosphorylation, and phosphorylated IκKβ did not functionally affect pIκKα in the case of LPS challenge, as shown by other groups[32,33,34]. On the other hand, transfection of cells with siIκKα only decreased the expression of pIκKα without affecting the levels of pIκKβ or pP65. Collectively, these
results show that IKKα and IKKβ played distinct roles in the pathway of LPS-induced hyperalgesia. Moreover, IKKα may not directly participate in the LPS- and TET-signaling cascade. TET treatment in cells with IKKβ knockdown had no effect on the levels of pIKKβ and pP65. This was partially due to the low levels of IKKβ that already existed in these cells with IKKβ knockdown.

Next, to further determine whether the pathway of “LPS/TET—IKKβ—NF-kB—COX-2/PGE2” practically take effect in astroglia, we investigated the COX-2 generation under the control siRNA, siIKKα, siIKKβ knockdown with or without TET treatment (Fig 5B). Results showed both siIKKβ and TET could decrease COX-2 generation, but not siIKKα, which more solidly supported the conclusion of TET specifically inhibited IKKβ phosphorylation and subsequently downregulate COX-2/PGE2 levels.

Discussion

LPS, which is a component of the cell wall of gram-negative bacteria, is known to activate a number of cellular signals in various cell types and tissues during inflammation and infection. In addition to its ability to cause endotoxic shock, LPS induces hyperalgesia in mice at lower doses[35]. A single dose of LPS that is administered centrally or peripherally can evoke a hyperalgesic reaction by decreasing mechanical nociceptive thresholds. In this study, we generated a hyperalgesic mouse model, in which BALB/C mice were treated with LPS. The hyperalgesic effect of LPS was verified by the shortened latency time of paw licking using the canonical hot-plate test, as well as the increase in writhing numbers in the acetic acid-induced abdominal constriction test. Because the writhing model is sensitive to the antinociceptive action of non-steroidal anti-inflammatory drugs, including indomethacin[23], and the hot-plate test focuses on the pathophysiological process above the spinal cord level[24], we chose indomethacin and morphine to be the positive controls of the...
Figure 3. Repressive effects of TET on the expression of COX-1 and COX-2 in LPS-induced hyperalgesia. (A) Western blotting of COX-1 and COX-2 in brain tissues and the quantified comparison of relative densities are shown. (B) COX-1 and COX-2 mRNA expression were tested by qRT-PCR in brain tissues. (C) COX-1 and COX-2 mRNA expression were tested by qRT-PCR in cultured astroglia. Values are shown as M±SD, and normalized to the NS groups. *, P<0.05.
doi:10.1371/journal.pone.0094586.g003
writhing and hot-plate tests, respectively. Using these models, tests, and drugs, we were able to investigate the peripheral and central anti-nociceptive effects of TET.

TET dose-dependently reduced the nociceptive responses in the writhing and hot-plate tests in LPS-treated mice, thus suggesting that TET has both central and peripheral anti-nociceptive effects. Because PGE2 is a critical pro-inflammatory and algesic factor, we measured its levels in vivo and in vitro. PGE2 levels were significantly increased and repressed with LPS and TET treatments, respectively, in mouse sera, brain tissues, and cultured astroglia. This suggests that PGE2 plays pivotal roles in LPS-induced hyperalgesia and TET-mediated analgesia. The COXs are key enzymes that regulate the formation of PGE2 from arachidonic acid. LPS increased COX-2 expression in mouse brain tissues and cultured astroglia. No effects on COX-1 were seen. Consistent with the physiology of canonical pain, COX-2 acted as a key regulatory

Figure 4. Regulatory effects of TET on the NF-κB pathway in LPS-induced cultured astroglia. (A) The expression of P65, pP65, IκBα, and pIκBα in the presence or absence of TET and LPS was assessed by western blotting, and the quantified comparisons of relative densities are shown. (B) The protein expression of IKKs in the presence or absence of TET and LPS was assessed by western blotting, and the quantified relative densities. Values are shown as M±SD, and normalized to the NS groups. *, P<0.05.

doi:10.1371/journal.pone.0094586.g004
Figure 5. Signaling targets of LPS and TET. Small RNA-interfering (si) experiments with siIKKα, siIKKβ, and control siRNA were performed to investigate the specific targets of LPS and TET in astroglia. (A) Both LPS and TET initially target the phosphorylation of IKKβ, but not that of IKKα. (B) Both siIKKβ and TET decrease COX-2 generation, but not siIKKα. Values are shown as M±SD, and normalized to the LPS(-)siRNA(-)TET(-) groups. *, P<0.05.

doi:10.1371/journal.pone.0094586.g005
 Knockdown experiments with IKK

TET-induced antinociception. No effects on IKK

by, at least in part, triggering the phosphorylation of IKK

IKK significantly reversed by TET co-treatment, thus implicating the

appropriate central pathway of hyperalgesia. Proportional de-

expression of these inflammatory cytokines, including COX-2 and

IL-1[37]. Therefore, we isolated astrocytes from the brains of

newborn mice and co-treated them with TET and LPS. The phosphorylation of IKKβ, 1k bladder; P65 and COX-2 increased proportionally upon LPS stimulus, and these increases were significantly reversed by TET co-treatment, thus implicating the phosphorylation of IKKβ but not IKKα.

Interestingly, TET specifically targeted IKKβ phosphorylation in LPS-treated astroglia, and eventually depressed NF-κB

activation and COX-2/PGE2 expression. These results allow us to

better understand the mechanisms by which LPS and TET induce hyperalgesia and antinociception, respectively, and show that both effects were elicited via the activation or inhibition of IKKβ phosphorylation and the downregulation of the NF-κB/COX-2/ 
PGE2 pathway.

Although TET appears to mediate analgesia via inhibiting IKKβ phosphorylation, it may also target other components of the pathway that are upstream of IKK. Additionally, the modulation of pain by peripherally derived inflammatory mediators involves factors and effector cells other than PGE2 and astroglia, respectively. The microglia and spinal glia also participate in pain modulation[38,39]. Whether the central modulation of pain involves the actions of the other eicosanoid metabolites, nitric oxide, or pro-inflammatory mediators requires further elucidation.

Therefore, more work needs to be done to reveal the exact mechanisms of hyperalgesia, as well as the main mechanisms behind the analgesic effects of TET.

Author Contributions

Conceived and designed the experiments: YFY JYW. Performed the experiments: HGZ PLL HZL. Analyzed the data: HGZ LZ. Wrote the paper: HGZ.

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