# Anxiolytic effects of polydatin through the blockade of neuroinflammation in a chronic pain mouse model

Shao-Yu Guan[1,*], Kun Zhang[1,*], Xin-Shang Wang[2,*], Le Yang[1],
Bin Feng[3], Dan-Dan Tian[4], Mei-Rong Gao[4], Shui-Bing Liu[2],
An Liu[1], and Ming-Gao Zhao[1] (ORCID)

## Abstract

**Background:** Chronic pain is frequently comorbid with anxiety disorder, thereby complicating its treatment. Polydatin, a component from the root of *Polygonum cuspidatum*, has shown neuroprotection in the central nervous system. However, its effects on pain and anxiety processing have been rarely investigated. In this study, mice were injected with complete Freund's adjuvant (CFA) at the hindpaw to induce pain- and anxiety-like behaviors.
**Results:** Treatment with polydatin (25 mg/kg) alleviated the anxiety-like behaviors but not pain perception in these mice. Polydatin treatment reversed the upregulation of N-methyl-D-aspartic acid receptors and GluA1-containing $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors in the amygdala of CFA-injected mice. Additionally, this treatment reduced the levels of proinflammatory cytokines, namely, tumor necrosis factor-alpha and interleukin-1$\beta$, in the amygdala. Furthermore, activated nuclear factor kappa-B signaling was blocked in the amygdala from CFA-injected mice. By using docking technology, we found potential structural binding between polydatin and I$\kappa$B kinase beta.
**Conclusion:** This study indicates the anxiolytic effects of polydatin by suppressing inflammatory cytokines in the amygdala.

## Background

Patients suffering from chronic pain commonly have emotional comorbidities, including sleep disorder, cognitive impairment, and anxiety. Chronic pain is the first determinant of mood disorders.[1] Comorbidities of anxiety show its significant contribution to pain.[2,3] However, most patients with chronic pain are often administered at specialty pain clinics with opioids as the most effective treatment, but they are commonly already suffering from evident mood disorders.[4] Analgesic abuse shows the urgent need for novel analgesics and anxiolytics.[5]

In the central nervous system (CNS), the amygdala coordinates negative emotional responses to threatening stimuli. The amygdala consists of several anatomically and functionally distinct nuclei, such as the lateral (LA) and basolateral (BLA)[6] nuclei and the central nucleus.[7,8]

[1]Department of Pharmacy, Precision Pharmacy and Drug Development Center, Tangdu Hospital, Fourth Military Medical University, Xi'an, China
[2]Key Laboratory of Gastrointestinal Pharmacology of Chinese Materia Medica of the State Administration of Traditional Chinese Medicine, Department of Pharmacology, School of Pharmacy, Fourth Military Medical University, Xi'an, China
[3]Department of Pharmacy, School of Stomatology, Fourth Military Medical University, Xi'an, China
[4]Department of Acupuncture-Moxibustion-Massage, Shaanxi University of Chinese Medicine, Xianyang, China

*These authors contributed equally to this work.

**Corresponding Authors:**
Ming-Gao Zhao, Department of Pharmacy, Precision Pharmacy and Drug Development Center, Tangdu Hospital, Fourth Military Medical University, Xi'an 710038, China.
Email: minggao@fmmu.edu.cn
An Liu, Department of Pharmacy, Precision Pharmacy and Drug Development Center, Tangdu Hospital, Fourth Military Medical University, Xi'an 710038, China.
Email: anran1222@163.com

Studies on amygdala function have focused on the plasticity of sensory inputs from the thalamus and the cortex to the LA and the BLA.[9,10] The amygdala is believed to switch chronic pain on and off[11] and is involved in major depressive disorder.[12,13] Alterations in excitatory/inhibitory (E/I) neurons and synapses in the amygdala have been prominently linked to anxiety disorders.[14] In addition, inflammation is involved in the onset and development of anxiety in the amygdala.[15]

Polydatin (3,4',5-Trihydroxystilbene-3-$\beta$-D-glucoside), also named piceid, is a monocrystalline compound that was first isolated from the rhizome and root of *Polygonum cuspidatum* (Polygonaceae). This compound is also detected in grapes, peanuts, hop cones, red wines, hop pellets, cocoa-containing products, and chocolate products.[16] These herbs are traditionally used to treat symptoms, such as pain, fever, cough, and hypertension.[17] Nowadays, polydatin has been increasingly comprehensively investigated for its pharmacological actions, such as anti-oxidative, anti-platelet aggregative, anti-inflammatory, and anti-cancer effects, and benefits for neurological diseases.[18] However, the effects of polydatin on analgesia and anti-anxiety have been rarely studied. The present study aims to evaluate the effects of polydatin on chronic inflammatory pain and related anxiety.

## Methods

### Animals

Adult male C57BL/6J mice (6–8 weeks) from the Experimental Animal Center of the Fourth Military Medical University (FMMU) were used in the experiments. Male mice were used to avoid the possible effects of hormone cycles on pain. The animals were housed in groups under standard laboratory conditions (12 h light/12 h dark, temperature 22–26°C, and humidity 55–60%). The water and food were freely accessible. Prior to the procedure, animals were allowed to accommodate to laboratory conditions for at least seven days. All experimental procedures were carried out according to protocols approved by the Animal Ethics Committee of the FMMU.

### Induction of chronic inflammatory pain and drug treatment

To induce chronic inflammatory pain, mice were injected subcutaneously with a single dose of complete Freund's adjuvant (CFA) (50% CFA, 10 $\mu$l) into the plantar surface of right hindpaw.[19] Control mice were injected with the same volume of saline. One week after CFA administration, mice received an intraperitoneal injection (i.p.) of polydatin at a dose of 6.25, 25, or 100 mg/kg once a day for 8 to 10 consecutive days between 9:00 a.m. to 10:00 a.m. Polydatin was dissolved in olive oil to the concentration of 5 mg/ml. Last polydatin administrated was 30 min before behavioral tests. Brain samples were collected immediately after behavioral tests. CFA was purchased from Sigma (St. Louis, MO, USA), and polydatin (purity = 99.9%) was purchased from TargetMol (Shanghai, China).

### Open field test

Open field (OF) test was conducted to assess anxiety-like behaviors as reported previously.[20] OF test apparatus (JL Behv-LAM, Shanghai, China) contains a square arena (30 cm × 30 cm × 30 cm) with plastic walls and floor and was placed inside an isolated chamber with illumination. Half of mice in each group were placed into the central area of the box and allowed to freely explore for 15 min. Movement locus of mouse was videotaped using a camera fixed above the floor and analyzed with a video-tracking system (Jiliang, Shanghai, China). OF test was performed before the elevated plus maze (EPM) test on the same day in the morning.

### EPM test

To further detect anxiety-like behaviors, EPM test was conducted as described in a previous report.[21] The apparatus (RD1208-EP, Shanghai Mobiledatum Corporation, China) comprised two open arms (25 cm × 8 cm × 0.5 cm) and two closed arms (25 cm × 8 cm × 12 cm) that extend from a common central platform (8 cm × 8 cm). The apparatus was elevated to a height of 50 cm above the floor. Mice were allowed to habituate to the testing room for one day before the test. For each test, individual animals were placed in the center square, facing an open arm, and allowed to explore freely for 5 min. Mice were videotaped using a camera fixed above the maze and analyzed with a video-tracking system. The number of entries and time spent in each arm were recorded. The anxious degree was evaluated by the number of entries and the time spent in open arms.[22] EPM test was performed after OF test on the same day in the morning.

### Von Frey test

Another half of mice were placed in individual plastic boxes on a metal mesh floor and allowed to adjust to the environment for 20 min. Via Dixon's up-down paradigm, the mechanical sensitivity was determined based on the responsiveness of hindpaw to the point of bending of Von Frey filaments. Von Frey filaments with different bending forces (0.008–2 g) were applied on the middle of dorsum of hindpaw in an ascending order. Positive responses included licking, biting, and sharp

withdrawal of the hindpaw.[23] There was a 3-minute interval between the stimuli. The result was tabulated, and the threshold of 50% withdrawal was analyzed as pain threshold.

## Hot plate test

To assess the thermal hyperalgesia in animals, a commercially available plantar analgesia instrument (BME410A, Institute of Biological Medicine, Academy of Medical Science, China) was employed. Animals were placed in individual plastic boxes and allowed to accommodate the environment for 20 min. Thermal hyperalgesia was assessed by measuring the latency of paw withdrawal (PWL) in response to a radiant heat source.[24] The heat source was turned off automatically when the mice lifted the foot. The time from radiant heat application to withdrawal of the hindpaw was defined as the PWL. In order to prevent tissue damage caused by heat, the heat source would be cut off automatically at 40 s even if the mice did not lift the hindpaw. The experiment was repeated for five times with 5-minute interval each. Hot plate test was performed after Von Frey test on the same day in the morning.

## Western blot analysis

Half of mice in each group were administered with polydatin, and 30 min later, the mice were anesthetized with 4% isoflurane and then decapitated, the brains were extracted, and the amygdala were dissected under the anatomical microscope on Day 16 (Figure 1(a)). Western blot analysis was performed as previously described.[25] Amygdala sample was dissociated with sonication in radioimmunoprecipitation assay (RIPA) lysis buffer containing phosphatase inhibitor and protease inhibitor. Protein level of the samples was quantified by quantified by bicinchonininc acid (BCA) Protein Assay Kit. Equal amounts of proteins (40 $\mu$g) were dispersed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and electrotransferred to PVDF membranes (Millipore, Massachusetts, the USA), which were respectively probed with antibodies after 5% nonfat milk (BD Difco, the USA) incubation for 1.5 h. The antibodies used in the analysis were as follows: anti-$\beta$-actin antibody (1:50000; cat. A5316, Sigma, USA), anti-Iba-1 (1:1000; cat. NB-100-1028ss, Novus Biologicals, USA), anti-PSD95 (1:2000; cat. ab2723, Abcam, UK), anti-GluN2B (1:1000; cat. ab65783, Abcam), anti-synaptophysin (1:1000; cat. ab8049, Abcam), anti-p-GluN2B-S1303 (1:1000; cat. ab81271, Abcam), anti-GluA1 (1:1000; cat. ab31232, Abcam), anti-GluN2A (1:1000; cat. ab1555, Millipore), anti-p-GluA1-S845; 1:1000; cat. ab5849, Millipore), anti-p-GluA1-S831 (1:1000; cat. ab5847, Millipore), anti-nuclear factor kappa-B (NF-$\kappa$B) p65 (1:750; cat.

AF0874, Affinity Biosciences, USA), anti-glial fibrillary acidic protein (GFAP) (1:1000; cat. 3670, Cell Signaling Technology, USA), anti-p-GluN2B-T1472 (1:1000; cat. 4208, Cell Signaling Technology), and anti-p-I$\kappa$B$\alpha$ (1:1000; cat. 9246, Cell Signaling Technology). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit/anti-mouse IgG for the primary antibodies, Affinity Biosciences). All of the chemicals and reagents were commercially available with standard biochemical quality. Densitometric analysis of Western blot was conducted using a Tanon (Shanghai, China) and quantified using Image J software (NIH, Bethesda, MD, USA) according to the instructions. For data analysis, band intensity of each blot was calculated as ratio relative to the $\beta$-actin. The intensity ratio of control group was set as 100%, and the intensity ratios of other treatment groups were expressed as percentage to the control group.

## Enzyme-linked immunosorbent assay

Samples of amygdala were dissociated with sonication in RIPA lysis buffer. The supernatant was collected for commercially available enzyme-linked immunosorbent assay (ELISA) kit to quantify tumor necrosis factor-alpha (TNF-$\alpha$) and interleukin (IL)-1$\beta$ levels in the amygdala of the mice (cat.#JL10484; cat.# JL18442, J&L Biological Co. Ltd. Shanghai, China) according to the manufacturer's instructions. Absorbance (optical density) was measured at 450 nm (BioTek, USA). Concentrations were obtained by interpolation from standard curves.

## In silico docking study of compound polydatin with I$\kappa$B kinase beta

The processes of ligand preparation and optimization were conducted by means of the Prepare Ligands module, a protocol of Discovery Studio 3.5 (Accelrys Inc., San Diego, CA, USA). The prepared ligands were converted to the SD file format. I$\kappa$B kinase beta (IKK$\beta$) crystal structure in Protein Data Bank (PDB) format was downloaded from the RCSB website (http://www.pdb.org). Before the docking procedure, water molecules were removed from the complexes. Hydrogen atoms were added by application of CHARMm force field and the Momany–Rone partial charge as default settings in Discovery Studio 3.5. The ligand-binding site was centered by PHE219 of B chain with 10 Å radius. Docking analyses of compound polydatin with IKK$\beta$ protein in the presence of crystal ligand was performed by means of the CDOCKER module. The number of generated poses was set to 100 for each ligand, and default settings were selected for other parameters.
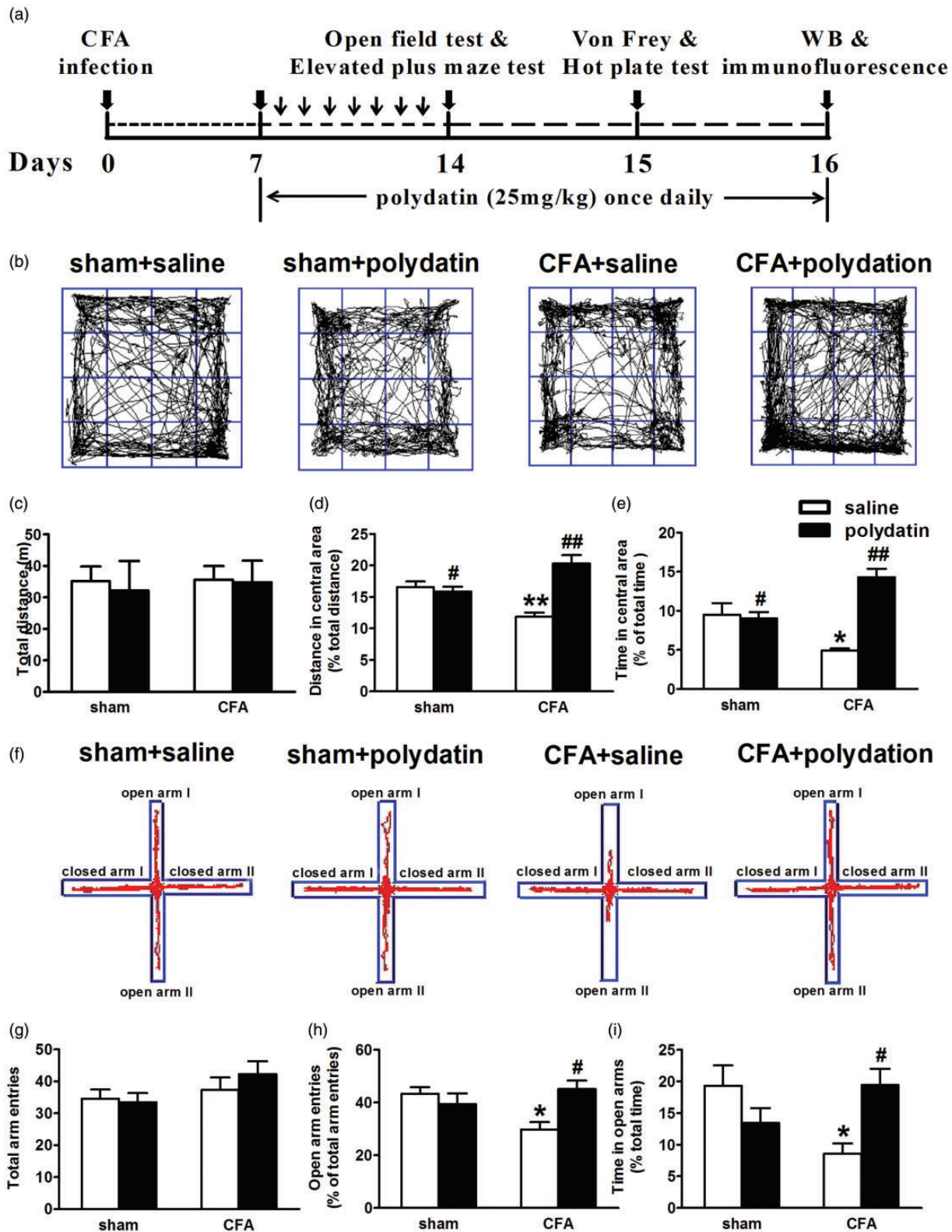
**Figure 1.** Polydatin relieved anxiety-like behaviors in mice injected with CFA. (a) Schedule displayed the experimental procedure. (b) Representative traces in OF test during a period of 15 min. Behavioral tests were performed on Day 14. (c–e) In OF test, administration with polydatin (25 mg/kg) for eight days increased the distance (d) and time spent in the central area (e) but had no effect on the total locomotor distance (c). (f) Representative traces in EPM test during a period of 5 min. (g–i) Polydatin treatment reversed the frequency into open arms (h) and the time spent in open arms (i). However, total arm entries had no change among four groups (g). Data are presented as means ± SEM ($n = 7$ in each group). *$p < 0.05$, **$p < 0.01$ vs. control group; #$p < 0.05$, ###$p < 0.01$ vs. CFA group. CFA: complete Freund's adjuvant; WB: Western blot.

## Immunofluorescence staining

Immunofluorescence staining was conducted as described previously (45), modified to some extent. Another half of mice in each group were deeply anesthetized with 4% isoflurane. This was followed by perfusion with 0.9% NaCl and then 4% paraformaldehyde (PFA) in 0.1 mM phosphate-buffered saline (PBS) through aorta. Brains were removed and post-fixed in 4% PFA overnight at 4°C. Free-floating coronal sections (20 mm) were obtained using a freezing microtome (CM1950, Leica, Germany). Sections containing the amygdala were washed in 0.1 mM PBS buffer, permeabilized with 0.3% TritonTM in 5% normal goat serum for 1 h. Then, the sections were incubated in primary antibodies (anti-GFAP, 1:400; anti-Iba-1, 1:500) overnight at 4°C in 10% normal goat serum. After washing, sections were incubated with secondary Cy3-conjugated anti-rabbit antibody (1:300, Wuhan Servicebio Technology Co., China) for 2 h at room temperature. Diluted Hoechst 33342 in 0.1 mM PBS (1:1000) was applied to sections after washing for 5 min to stain nuclei. Sections were mounted onto slides using 50% glycerinum. The slides were observed using a confocal laser microscope (FV1000, Olympus, Japan), and images were captured by FV 1000 using standard laser lines and filters.

## Statistical analysis

Data were presented as mean ± SEM. Statistical analysis of multiple groups were performed by two-way analysis of variance followed by least significant difference test or Dunnett's test for post hoc comparisons (SPSS 20.0). In all cases, $p < 0.05$ was considered as statistical significance.

# Results

## Polydatin ameliorates anxiety-like behaviors in CFA-injected mice

Figure 1(a) shows the experimental scheme. At seven days after CFA injection, mice were administered with polydatin (25 mg/kg, i.p.) for 8 to 10 days. OF and EPM tests were used to determine anxiety-like behaviors on Day 14 after 30 min of last polydatin injection (Figure 1). In the OF test, the distance and time traveled in the central area were less in CFA-injected mice than in control mice (Figure 1(b), (d), and (e)). In the EPM test, CFA-injected mice showed fewer entries and less time in open arms than control mice (Figure 1(f), (h), and (i)). Polydatin administration markedly restored the CFA-induced decreased distance and time traveled in the central area and open arm entries. The effects of polydatin in OF and EPM tests were dose dependent

(Supplementary Figure 1). However, the total locomotor distance in the OF test and the arm entries in the EPM test were comparable (Figure 1(c) and (g)), indicating similar locomotor activity between the groups. The data suggest the anxiolytic effects of polydatin in CFA-injected mice.

## Polydatin has no analgesic effects on chronic inflammatory pain

To investigate the mechanism underlying the anxiolytic effects of polydatin, we detected its effects on pain sensory. Von Frey and hot plate tests were performed to determine mechanical allodynia and thermal hyperalgesia on Day 15 (Figure 1(a)). Hindpaw CFA injection reduced the threshold (Figure 2(a)) and PWL (Figure 2(c)) in ipsilateral hindpaw but not in contralateral hindpaw (Figure 2(b) and (d)). Polydatin treatment did not affect the threshold and latency in both sides of hindpaw, even when the dose was increased to 100 mg/kg (i.p.) (Supplementary Figure 2). These data indicate that polydatin had no analgesic effects on mechanical allodynia and thermal hyperalgesia. The anxiolytic effect of polydatin is not related to analgesic effect.

## Polydatin reduces the upregulation of glutamatergic receptors in the amygdala

Amygdala is a critical brain region implicated in the onset and development of anxiety,[26] which is associated with synaptic changes.[27] The alteration of glutamatergic receptors, including N-methyl-D-aspartic acid receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPARs), is associated with anxiety-like behaviors.[28–30] In the current study, GluN2A, GluN2B, p-GluN2B-T1472, p-GluN2B-S1303, PSD95, and synaptophysin levels were markedly increased in the amygdala of CFA-injected mice (Figure 3). Levels of these excitatory synaptic proteins were reversed with polydatin treatment. Polydatin administration alone had no effect on these proteins in control mice (Figure 3(a) to (d)). Similarly, the levels of AMPAR subunitGluA1 and its phosphorylated proteins (p-GluA1-S831 and p-GluA1-S845) were significantly enhanced in the amygdala after CFA injection but could be downregulated by polydatin treatment (Figure 4(a) and (b)). Polydatin treatment alone did not affect the levels of total GluA1and its phosphorylated proteins. These results imply that the anxiolytic effect of polydatin is related to the inhibition of glutamatergic receptors in the amygdala.
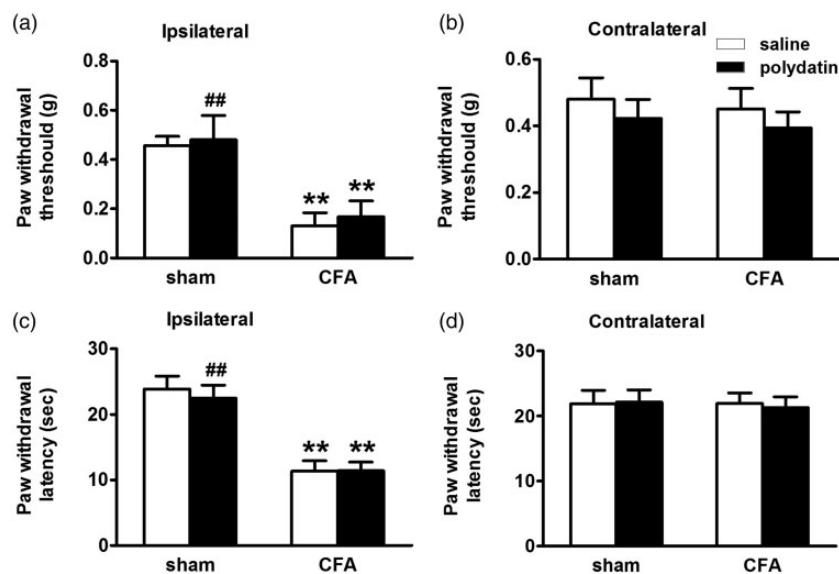
**Figure 2.** Polydatin had no analgesic effects in mice with chronic inflammatory pain. Von Frey and hot plate tests were implemented on Day 15. Polydatin (25 mg/kg) did not diminish CFA-induced mechanical allodynia (a) and thermal hyperalgesia(c) in CFA-injected hind paw (ipsilateral). The basal mechanical allodynia (b) and thermal hyperalgesia (d) in contralateral hind paw were not impacted by CFA and/or polydatin. Data are presented as means $\pm$ SEM ($n = 7$ in each group). **$p < 0.01$ vs. control group; ##$p < 0.01$ vs. CFA group. CFA: complete Freund's adjuvant.

## Polydatin attenuates inflammatory response in the amygdala

The inflammatory system has a clear role in the pathophysiology of chronic mental illnesses, such as anxiety disorder.[31] Thus, we determined if the anxiolytic effect of polydatin was connected to inflammatory inhibition. We detected the edema of CFA-injected hindpaw with polydatin treatment, but no alteration by polydatin was found at Day 16 (data not shown). However, CFA injection significantly increased the levels of proinflammatory mediators, namely, TNF-$\alpha$ and IL-1$\beta$, in the amygdala, which were reversed by polydatin treatment (Figure 5(a) and (b)). NF-$\kappa$B p65 and p-I$\kappa$B$\alpha$ levels in the amygdala, which were mitigated by polydatin treatment, were upregulated after CFA injection (Figure 5(c) and (d)).

Astrocytes and microglia are active participants in propagating and regulating neuroinflammation within the brain.[32,33] GFAP and Iba-1 are markers of astrocytes and microglia.[34] The levels of Iba-1 but not GFAP were enhanced in the amygdala after CFA injection, indicating the involvement of microglia in the peripheral pain process. Polydatin administration reduced the levels of Iba-1 (Figure 6(a) and (b)), which was consistent with immunofluorescence staining (Figure 6(c)). The data indicate that polydatin inhibits the neuroinflammation mediated by microglial activation in the amygdala of CFA-injected mice.

## Polydatin has structural interactions with IKK$\beta$

To investigate the underlying mechanism of anti-inflammation, further, we conducted a molecular docking analysis of polydatin. Polydatin was docked to IKK$\beta$ by using the CDOCKER module of Discovery Studio (Accelrys Inc.).The multisubunit protein kinase IKK regulates NF-$\kappa$B activation and contains two possible kinase subunits IKK$\alpha$ and IKK$\beta$.[35] Their functions differentiate from each other. IKK$\beta$ is a potent NF-$\kappa$B activator and plays a key role in the canonical NF-$\kappa$B pathway responsible for immune responses, whereas IKK$\alpha$ is critical in the noncanonical pathway required for developmental processes. Additionally, IKK$\beta$ inhibitors compete with the substrate I$\kappa$B.[36] IKK$\beta$ has unusually high affinity for ATP, the inside binding pocket of which contains an innate crystal ligand.[37] By contrast, conformations of IKK in its active or inactive state would not be differentiated. Therefore, outside of the ATP binding pocket, certain binding site for non-ATP competitive IKK$\beta$ inhibitors exists to change its kinase activity. One of the potential binding sites is remote from the ATP-binding pocket and centers around Phe219, which is displayed as a 12 Å-long and 10 Å-deep channel.[38] In the present study, the OH group on the monosaccharide of polydatin under the binding pocket formed hydrogen bonds with Glu214 and Arg220 on the B chain of polydatin-IKK$\beta$ binding protein (PDB:4KIK). Moreover, the phenyl moiety of polydatin formed Pi-Pi
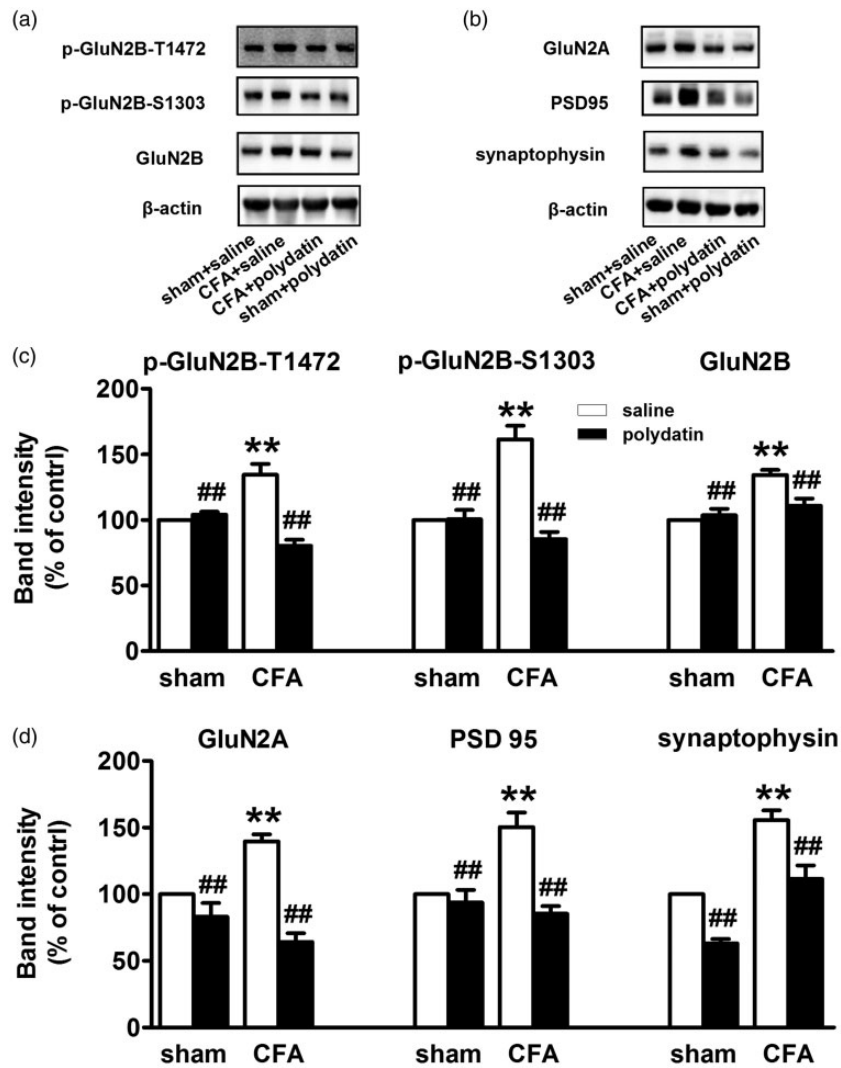
**Figure 3.** Polydatin reduced CFA-induced upregulation of NMDARs in the amygdala. (a and b) Representative Western blot analysis of p-GluN2B-T1472, p-GluN2BS1303, GluN2B, GluN2A, PSD95, and synaptophysin. Polydatin (25 mg/kg) treatment for 10 days reversed the upregulation of p-GluN2B-T1472, p-GluN2BS1303, and total GluN2B (c), and GluN2A, PSD95, and synaptophysin (d). Data are presented as means $\pm$ SEM ($n = 7$ in each group). **$p < 0.01$ vs. control group; ##$p < 0.01$ vs. CFA group. CFA: complete Freund's adjuvant.
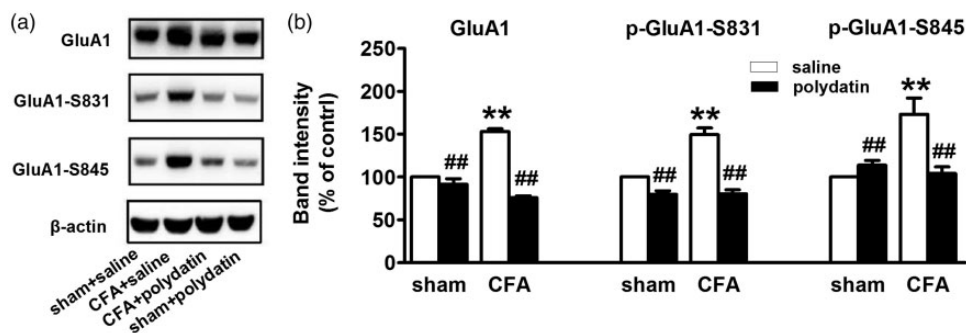


**Figure 4.** Polydatin reversed CFA-induced upregulation of AMPARs in the amygdala. (a) Representative Western blot analysis of GluA1, p-GluA1-S831, and p-GluA1-S845. CFA injection increased the expressions of GluA1, p-GluA1-S831, and p-GluA1-S845 (b) and polydatin (25 mg/kg) significantly reduced the expression of GluA1, p-GluA1-S831, and p-GluA1-S845 in the amygdala of CFA-injected mice. Data are presented as means $\pm$ SEM ($n = 7$ in each group). **$p < 0.01$ vs. control group; ##$p < 0.01$ vs. CFA group. CFA: complete Freund's adjuvant.
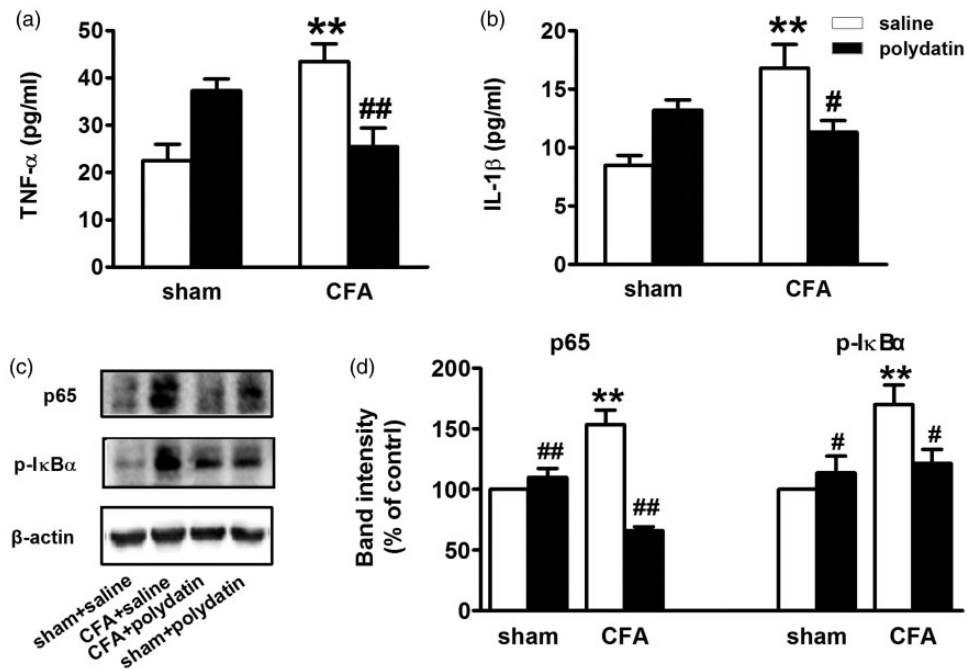
**Figure 5.** Polydatin suppressed CFA-induced production of proinflammatory mediators and NF-$\kappa$B signaling pathway in the amygdala. (a and b) Effects of polydatin against CFA-induced proinflammation by ELISA. Polydatin treatment reduced TNF-α and IL-1$\beta$ levels in the amygdala of CFA-injected mice. (c) Representative bands of Western blot analysis showing the levels of NF-$\kappa$B p65 and p-I$\kappa$Bα. CFA injection evidently increased NF-$\kappa$B p65and p-I$\kappa$Bα (d) in the amygdala, which were reversed by polydatin administration. Data are presented as means ± SEM ($n = 7$ in each group). **$p < 0.01$ vs. control group; #$p < 0.05$, ###$p < 0.01$ vs. CFA group. CFA: complete Freund's adjuvant; TNF-α: tumor necrosis factor-alpha; IL-1$\beta$: interleukin-1$\beta$.

stacked interaction with Phe219 on the B chain, and formative intramolecular hydrogen bonds dramatically improved the stability of the protein–ligand complex (Figure 7). In conclusion, we presume that polydatin acts as a competitor of I$\kappa$B and interacts with IKK$\beta$ underlying the action of the NF-$\kappa$B signaling pathway.

## Discussion

Pain and emotional disorders likely share the same pathobiological pathway, which is noteworthy for treating this comorbidity.[39,40] CFA-injected mouse model is manifested to possess pain and anxiety-like behaviors synchronously. Polydatin, isolated from the rhizome, effectively relieved CFA-induced anxiety-like behaviors in mice. However, this compound did not ameliorate pain-related behaviors in mechanical allodynia and thermal hyperalgesia tests. The present study provides a novel mechanism in brain underlying the anxiolytic effect of polydatin.

In the CNS, the E/I network maintains a finely tuned balance in neural activities, which is vital for central physiological function. Once the balance in E/I signaling is broken, onset patterns of autism, schizophrenia, and seizure arise.[41] Glutamate mediates the majority of excitatory synaptic transmissions in mammalian brains.

Ionotropic and metabotropic glutamate receptors contribute to synaptic transmission, plasticity, and modulation.[42] NMDARs and AMPARs are crucial excitatory postsynaptic receptors, of which the mounting activities indicate neurotransmitter hyperexcitability.[43] Among them, NMDARs typically contain GluN1 and GluN2 subunits. Each type of GluN2, including GluN2A and GluN2B, exerts its function largely by associating with the postsynaptic density protein PSD-95.[44] Moreover, synaptophysin, a major resident of the synaptic vesicle membrane, relates closely with the packaging and storage of synaptic vesicles and release of neurotransmitters.[15,45] In the present study, the upregulation of NMDARs and AMPARs in the amygdala after CFA injection increased the excitatory transmission, which contributed to anxiety-like behaviors in mice. Polydatin treatment reversed the upregulation of NMDARs and AMPARs and restored the E/I balance in the amygdala. This result is consistent with observations from a recent study using ketamine, a noncompetitive antagonist of NMDARs, as a fast antidepressant treatment in rodent models of anxiety/depression.[46]

Polydatin did not affect pain-like behaviors, although it had anti-inflammatory effect in the amygdala but not in the CFA-injected site. Since amygdala is critical for pain and anxiety, present study found that polydatin
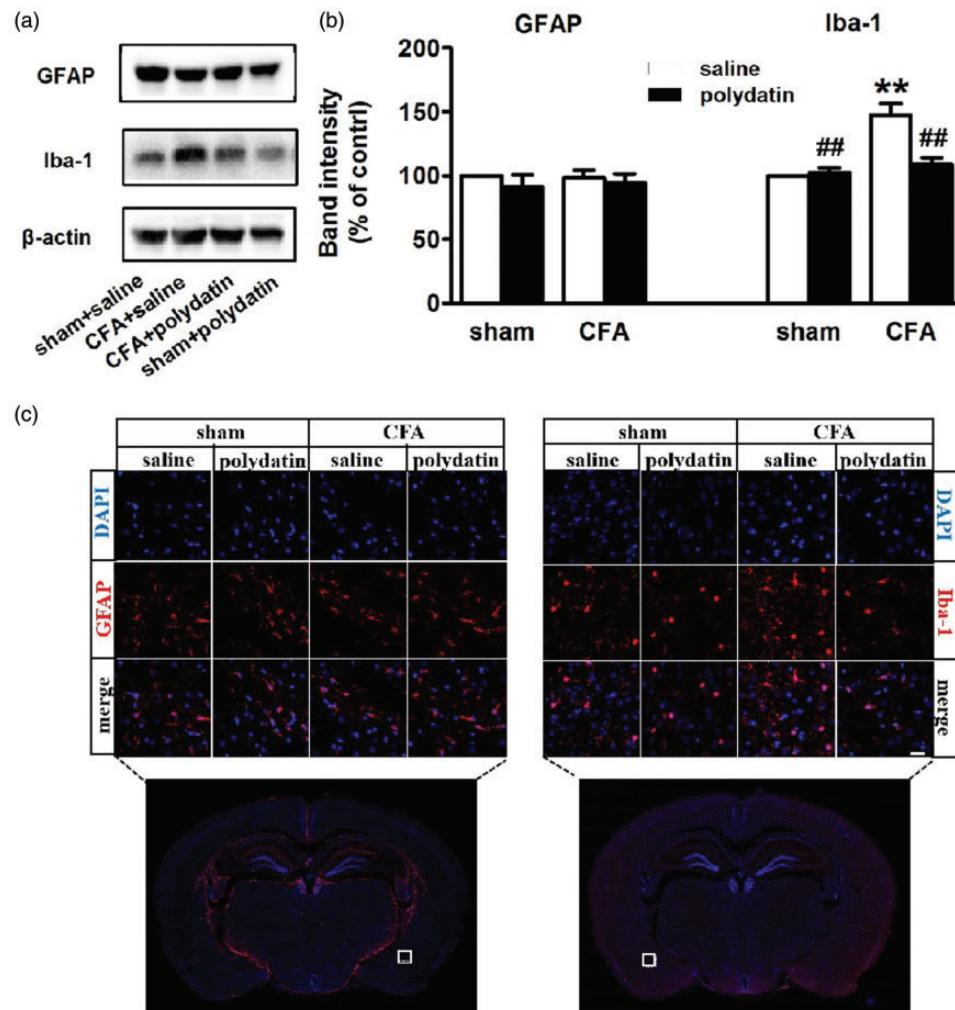
**Figure 6.** Effects of polydatin on CFA-induced microglia activation and GFAP expression. (a) Representative Western blot analysis of GFAP and Iba-1. (b) Polydatin inhibited overexpression of Iba-1 in CFA-treated mice but had no effects on the levels of GFAP among the groups. Data are presented as means $\pm$ SEM ($n = 7$ in each group). $**p < 0.01$ vs. control group; $^{\#\#}p < 0.01$ vs. CFA group. (c) Immunofluorescence staining showed microglia activation by CFA injection in the amygdala by Iba-1 immunoreactivity, which was reversed by polydatin administration. Scale bars: lower 20$\mu$m, upper 1000 $\mu$m. $n = 3$ in each group.
CFA: complete Freund's adjuvant; GFAP: glial fibrillary acidic protein.

selectively reduce anxiety-like behavior but not mechanical hypersensitivity. It raises the possibility that polydatin selectively reduces proinflammatory cytokines in "anxiety, but not pain" related specific area in amygdala. In fact, polydatin selectively reduces proinflammatory cytokines in CFA-injected hindpaw. Furthermore, we did not exclude the anxiolytic effects through modulating function of other brain regions including cingulate cortex, hippocampus, or striatum.

Proinflammatory cytokines, including TNF-$\alpha$, IL-1$\beta$, IL-6, and IL-8, were increased in patients with anxiety, suggesting a key role of inflammation in anxiety.[47] NF-$\kappa$B is a transcript factor involved in the regulation of inflammation and immune response.[48] In its inactive form, NF-$\kappa$B is sequestered in the cytoplasm and bound by members of the I$\kappa$B family, including prototypical I$\kappa$Bs, atypical I$\kappa$Bs, p105, and p100. IKK contains a cytokine-inducible I$\kappa$B kinase activity and controls sequential phosphorylation, ubiquitination, and degradation of the inhibitory subunit I$\kappa$B for NF-$\kappa$B. As a result, the release of NF-$\kappa$B subunit exerts its functions in the nucleus, including the induction of several proinflammatory cytokines and chemokines, which are involved in innate and acquired immune responses.[18,49] The anti-inflammatory and neuroprotective effects of polydatin targeting NF-$\kappa$B signaling have been reported.[50,51] Polydatin is identified as a natural precursor of resveratrol, which is also a stilbene-derived natural compound. Considerable studies reveal the suppressive effect of resveratrol on the NF-$\kappa$B pathway

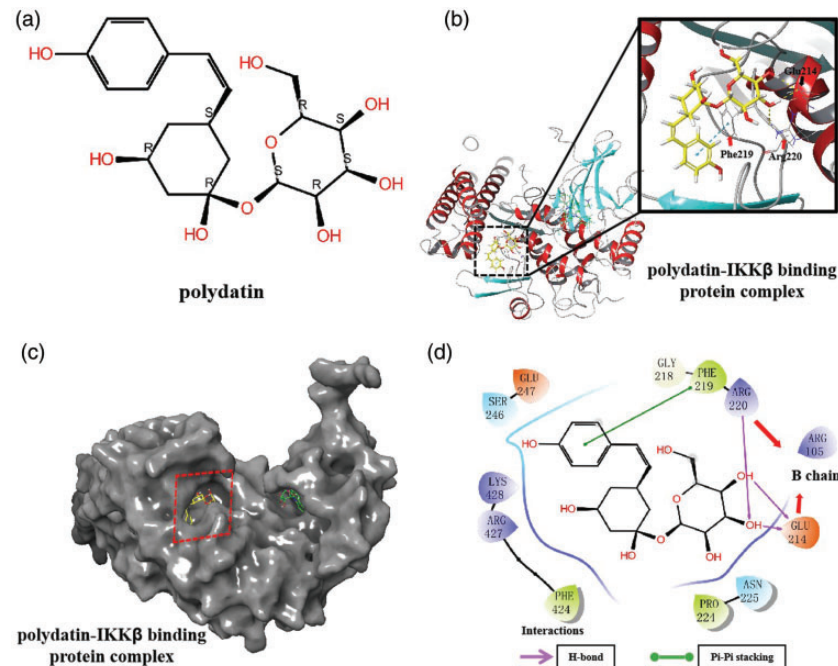**Figure 7.** Characterization of the spatial interactions within the polydatin-IKK$\beta$ binding protein (PDB:4KIK) complex. (a) Chemical structural formula of polydatin. (b and c) Polydatin is shown as yellow sticks inside the kinase activity-binding pocket. IKK$\beta$ innate crystal ligand is shown as green sticks inside the ATP binding site. (d) The phenyl ring forms pi-pi stacking with Phe219 of B chain of PDB:4KIK. The -OH group of polydatin forms hydrogen bond with Glu214 and Arg220 of B chain.
IKK$\beta$: I$\kappa$B kinase beta.

by counteracting the phosphorylation of I$\kappa$B$\alpha$, IKK$\alpha$, and IKK$\beta$.[52,53] In the current study, CFA injection markedly triggered the secretion of TNF-$\alpha$ and IL-1$\beta$ and increased the levels of NF-$\kappa$B p65 and p-I$\kappa$Ba, which were abrogated by polydatin treatment. On the basis of in silico docking analysis, the reverse effect of polydatin on the upregulated expressions of NF-$\kappa$B p65 and p-I$\kappa$B$\alpha$ in CFA-induced mice may be due to the interactive structures of polydatin and IKK$\beta$ in the NF-$\kappa$B pathway.

Microglia is a dynamic immune cell response to brain damage, degeneration, and neuroinflammation, and it produces various neurotoxic and neuroprotective factors. The most neurotoxic factor from activated microglia is glutamate.[54] Astrocytes are activated by inflammatory mediators but contribute to the local inflammatory response by producing proinflammatory cytokines and alleviating neuronal damage through anti-inflammatory factors in the CNS.[32] Under neuro-inflammatory conditions, astrocytes uptake excessive extracellular glutamate by membrane-bound glutamate transporters, thereby playing a critical role in preventing glutamate excitotoxicity.[55] Astrocyte dysfunction results in the decrease of glutamate uptake, loss of neuronal synapses, and increased release of cytokines and inflammatory mediators.[56] Here, we found that CFA injection

markedly activated microglia in the amygdala. This effect was inhibited by polydatin treatment. Astroglios did not occur after CFA injection and/or polydatin treatment, indicating that astrocytes were not involved in the anxiolytic effects of polydatin.

In summary, the present data provide solid evidence for the anxiolytic effects of polydatin in mice with chronic inflammatory pain. The underlying mechanisms are related to its neuroinflammation inhibition in the amygdala.

## Author Contributions

Shao-Yu Guan, Kun Zhang, Xin-Shang Wang, and Shui-Bing Liu performed biological experiments. Bin Feng, Dan-Dan Tian, and Mei-Rong Gao performed behavioral tests. Ming-Gao Zhao and An Liu designed and wrote the article.

## Declaration of Conflicting Interests

## Funding

## ORCID iD

Ming-Gao Zhao (iD) https://orcid.org/0000-0002-7539-3887

## Supplemental Material

Supplemental material for this article is available online.

## References

1. Humo M, Lu H, Yalcin I. The molecular neurobiology of chronic pain-induced depression. *Cell Tissue Res* 2019; 377: 21–43.
2. Lerman SF, Rudich Z, Brill S, Shalev H, Shahar G. Longitudinal associations between depression, anxiety, pain, and pain-related disability in chronic pain patients. *Psychosom Med* 2015; 77: 333–341.
3. Zhuo M. Neural mechanisms underlying anxiety-chronic pain interactions. *Trends Neurosci* 2016; 39: 136–145.
4. Feingold D, Brill S, Goor-Aryeh I, Delayahu Y, Lev-Ran S. Depression and anxiety among chronic pain patients receiving prescription opioids and medical marijuana. *J Affect Disord* 2017; 218: 1–7.
5. van Rijswijk SM, van Beek M, Schoof GM, Schene AH, Steegers M, Schellekens AF. Iatrogenic opioid use disorder, chronic pain and psychiatric comorbidity: a systematic review. *Gen Hosp Psychiatry* 2019; 59: 37–50.
6. Blackburn-Munro G, Blackburn-Munro RE. Chronic pain, chronic stress and depression: coincidence or consequence? *J Neuroendocrinol* 2001; 13: 1009–1023.
7. Rainnie DG, Bergeron R, Sajdyk TJ, Patil M, Gehlert DR, Shekhar A. Corticotrophin releasing factor-induced synaptic plasticity in the amygdala translates stress into emotional disorders. *J Neurosci* 2004; 24: 3471–3479.
8. Tasan RO, Nguyen NK, Weger S, Sartori SB, Singewald N, Heilbronn R, Herzog H, Sperk G. The central and basolateral amygdala are critical sites of neuropeptide Y/Y2 receptor-mediated regulation of anxiety and depression. *J Neurosci* 2010; 30: 6282–6290.
9. Ehrlich I, Humeau Y, Grenier F, Ciocchi S, Herry C, Luthi A. Amygdala inhibitory circuits and the control of fear memory. *Neuron* 2009; 62: 757–771.
10. Wang SH, de Oliveira Alvares L, Nader K. Cellular and systems mechanisms of memory strength as a constraint on auditory fear reconsolidation. *Nat Neurosci* 2009; 12: 905–912.
11. Rouwette T, Vanelderen P, Roubos EW, Kozicz T, Vissers K. The amygdala, a relay station for switching on and off pain. *Eur J Pain* 2012; 16: 782–792.
12. Hamilton JP, Siemer M, Gotlib IH. Amygdala volume in major depressive disorder: a meta-analysis of magnetic resonance imaging studies. *Mol Psychiatry* 2008; 13: 993–1000.
13. Sacher J, Neumann J, Funfstuck T, Soliman A, Villringer A, Schroeter ML. Mapping the depressed brain: a meta-analysis of structural and functional alterations in major depressive disorder. *J Affect Disord* 2012; 140: 142–148.
14. Babaev O, Piletti Chatain C, Krueger-Burg D. Inhibition in the amygdala anxiety circuitry. *Exp Mol Med* 2018; 50: 18.
15. Tao Y, Chen YJ, Shen C, Luo Z, Bates CR, Lee D, Marchetto S, Gao TM, Borg JP, Xiong WC, Mei L. Erbin interacts with TARP gamma-2 for surface expression of AMPA receptors in cortical interneurons. *Nat Neurosci* 2013; 16: 290–299.
16. Du QH, Peng C, Zhang H. Polydatin: a review of pharmacology and pharmacokinetics. *Pharm Biol* 2013; 51: 1347–1354.
17. Liu W, Chen P, Deng J, Lv J, Liu J. Resveratrol and polydatin as modulators of Ca(2+) mobilization in the cardiovascular system. *Ann N Y Acad Sci* 2017; 1403: 82–91.
18. Ravagnan G, De Filippis A, Carteni M, De Maria S, Cozza V, Petrazzuolo M, Tufano MA, Donnarumma G. Polydatin, a natural precursor of resveratrol, induces beta-defensin production and reduces inflammatory response. *Inflammation* 2013; 36: 26–34.
19. Koga K, Descalzi G, Chen T, Ko H-G, Lu J, Li S, Son J, Kim T, Kwak C, Huganir R L, Zhao M-G, Kaang B-K, Collingridge G L and Zhuo M. Coexistence of Two Forms of LTP in ACC Provides a Synaptic Mechanism for the Interactions between Anxiety and Chronic Pain. *Neuron* 2015; 86: 1109. doi: 10.1016/j.neuron.2015.05.016
20. Zhang K, Li YJ, Guo Y, Zheng KY, Yang Q, Yang L, Wang XS, Song Q, Chen T, Zhuo M, Zhao MG. Elevated progranulin contributes to synaptic and learning deficit due to loss of fragile X mental retardation protein. *Brain* 2017; 140: 3215–3232.
21. Feng B, Liu JC, Zhang J, Ozaki K, Guo YY, Yi DH, Li XQ, Zhao MG. Anxiolytic actions of motilin in the basolateral amygdala. *Mol Neurobiol* 2013; 47: 892–902.
22. Zhou SM, Guan SY, Yang L, Yang LK, Wang L, Nie HF, Li X, Zhao MG, Yang Q, Wu H. Cucurbitacin IIa exerts antidepressant-like effects on mice exposed to chronic unpredictable mild stress. *Neuroreport* 2017; 28: 259–267.
23. Fan YF, Guan SY, Luo L, Li YJ, Yang L, Zhou XX, Guo GD, Zhao MG, Yang Q, Liu G. Tetrahydroxystilbene glucoside relieves the chronic inflammatory pain by inhibiting neuronal apoptosis, microglia activation, and GluN2B overexpression in anterior cingulate cortex. *Mol Pain* 2018; 14: 1744806918814367.
24. Sun YY, Li L, Liu XH, Gu N, Dong HL, Xiong L. The spinal notch signaling pathway plays a pivotal role in the development of neuropathic pain. *Mol Brain* 2012; 5: 23.
25. Guo HL, Xiao Y, Tian Z, Li XB, Wang DS, Wang XS, Zhang ZW, Zhao MG, Liu SB. Anxiolytic effects of sesamin in mice with chronic inflammatory pain. *Nutr Neurosci* 2016; 19: 231–236.
26. Felix-Ortiz AC, Beyeler A, Seo C, Leppla CA, Wildes CP, Tye KM. BLA to vHPC inputs modulate anxiety-related behaviors. *Neuron* 2013; 79: 658–664.
27. Riazi K, Galic MA, Kentner AC, Reid AY, Sharkey KA, Pittman QJ. Microglia-dependent alteration of

glutamatergic synaptic transmission and plasticity in the hippocampus during peripheral inflammation. *J Neurosci* 2015; 35: 4942–4952.

28. Lehner M, Wisłowska-Stanek A, Gryz M, Sobolewska A, Turzyńska D, Chmielewska N, Krząścik P, Skórzewska A, Płaźnik A. The co-expression of GluN2B subunits of the NMDA receptors and glucocorticoid receptors after chronic restraint stress in low and high anxiety rats. *Behav Brain Res* 2017; 319: 124–134.

29. Liu SB, Tian Z, Guo YY, Zhang N, Feng B, Zhao MG. Activation of GPR30 attenuates chronic pain-related anxiety in ovariectomized mice. *Psychoneuroendocrinology* 2015; 53: 94–107.

30. Yue J, Wang XS, Guo YY, Zheng KY, Liu HY, Hu LN, Zhao MG, Liu SB. Anxiolytic effect of CPEB1 knockdown on the amygdala of a mouse model of inflammatory pain. *Brain Res Bull* 2018; 137: 156–165.

31. Michopoulos V, Powers A, Gillespie CF, Ressler KJ, Jovanovic T. Inflammation in fear- and anxiety-based disorders: PTSD, GAD, and beyond. *Neuropsychopharmacology* 2017; 42: 254–270.

32. Norden DM, Trojanowski PJ, Villanueva E, Navarro E, Godbout JP. Sequential activation of microglia and astrocyte cytokine expression precedes increased Iba-1 or GFAP immunoreactivity following systemic immune challenge. *Glia* 2016; 64: 300–316.

33. O'Loughlin E, Pakan JMP, Yilmazer-Hanke D, McDermott KW. Acute in utero exposure to lipopolysaccharide induces inflammation in the pre- and postnatal brain and alters the glial cytoarchitecture in the developing amygdala. *J Neuroinflammation* 2017; 14: 212.

34. Meneses CS, Muller HY, Herzberg DE, Uberti B, Werner MP, Bustamante HA. Microglia and astrocyte activation in the spinal cord of lame horses. *Vet Anaesth Analg* 2018; 45: 92–102.

35. Perkins ND. Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol* 2007; 8: 49–62.

36. Burke JR, Pattoli MA, Gregor KR, Brassil PJ, MacMaster JF, McIntyre KW, Yang X, Iotzova VS, Clarke W, Strnad J, Qiu Y, Zusi FC. BMS-345541 is a highly selective inhibitor of I kappa B kinase that binds at an allosteric site of the enzyme and blocks NF-kappa B-dependent transcription in mice. *J Biol Chem* 2003; 278: 1450–1456.

37. Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A, Rao A. IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science* 1997; 278: 860–866.

38. Liu S, Misquitta YR, Olland A, Johnson MA, Kelleher KS, Kriz R, Lin LL, Stahl M, Mosyak L. Crystal structure of a human IkappaB kinase beta asymmetric dimer. *J Biol Chem* 2013; 288: 22758–22767.

39. Doan L, Manders T, Wang J. Neuroplasticity underlying the comorbidity of pain and depression. *Neural Plast* 2015; 2015: 504691.

40. Failo A, Beals-Erickson SE, Venuti P. Coping strategies and emotional well-being in children with disease-related pain. *J Child Health Care* 2018; 22: 84–96.

41. Hartmann J, Kunig G, Riederer P. Involvement of transmitter systems in neuropsychiatric diseases. *Acta Neurol Scand, Suppl* 1993; 146: 18–21.

42. Zhuo M. Cortical kainate receptors and behavioral anxiety. *Mol Brain* 2017; 10: 16.

43. Yasmin F, Saxena K, McEwen BS, Chattarji S. The delayed strengthening of synaptic connectivity in the amygdala depends on NMDA receptor activation during acute stress. *Physiol Rep* 2016; 4: e13002.

44. Zhao C, Du CP, Peng Y, Xu Z, Sun CC, Liu Y, Hou XY. The upregulation of NR2A-containing N-methyl-D-aspartate receptor function by tyrosine phosphorylation of postsynaptic density 95 via facilitating Src/proline-rich tyrosine kinase 2 activation. *Mol Neurobiol* 2015; 51: 500–511.

45. He S, Yang J. Maturation of neurotransmission in the developing rat cochlea: immunohistochemical evidence from differential expression of synaptophysin and synaptobrevin 2. *Eur J Histochem* 2011; 55: e2.

46. Willis MC, LeCuyer KA, Meisenheimer KM, Uhlenbeck OC, Koch TH. An RNA-protein contact determined by 5-bromouridine substitution, photocrosslinking and sequencing. *Nucleic Acids Res* 1994; 22: 4947–4952.

47. Felger JC. Imaging the role of inflammation in mood and anxiety-related disorders. *Curr Neuropharmacol* 2018; 16: 533–558.

48. Hoesel B, Schmid JA. The complexity of NF-kappaB signaling in inflammation and cancer. *Mol Cancer* 2013; 12: 86.

49. Yang L, Chen J, Han X, Zhang E, Huang X, Guo X, Chen Q, Wu W, Zheng G, He D, Zhao Y, Yang Y, He J, Cai Z. Pirh2 mediates the sensitivity of myeloma cells to bortezomib via canonical NF-kappaB signaling pathway. *Protein Cell* 2018; 9: 770–784.

50. Huang B, Liu J, Meng T, Li Y, He D, Ran X, Chen G, Guo W, Kan X, Fu S, Wang W, Liu D. Polydatin prevents lipopolysaccharide (LPS)-induced Parkinson's disease via regulation of the AKT/GSK3beta-Nrf2/NF-kappaB signaling axis. *Front Immunol* 2018; 9: 2527.

51. Ji H, Zhang X, Du Y, Liu H, Li S, Li L. Polydatin modulates inflammation by decreasing NF-kappaB activation and oxidative stress by increasing Gli1, Ptch1, SOD1 expression and ameliorates blood-brain barrier permeability for its neuroprotective effect in pMCAO rat brain. *Brain Res Bull* 2012; 87: 50–59.

52. Ma C, Wang Y, Dong L, Li M, Cai W. Anti-inflammatory effect of resveratrol through the suppression of NF-kappaB and JAK/STAT signaling pathways. *Acta Biochim Biophys Sin (Shanghai)* 2015; 47: 207–213.

53. Zhang L, Li Y, Gu Z, Wang Y, Shi M, Ji Y, Sun J, Xu X, Zhang L, Jiang J, Shi W. Resveratrol inhibits enterovirus 71 replication and pro-inflammatory cytokine secretion in rhabdosarcoma cells through blocking IKKs/NF-kappaB signaling pathway. *PLoS One* 2015; 10: e0116879.

54. Suzumura A. Neuron-microglia interaction in neuroinflammation. *Curr Protein Pept Sci* 2013; 14: 16–20.
55. Block L, Bjorklund U, Westerlund A, Jorneberg P, Biber B, Hansson E. A new concept affecting restoration of inflammation-reactive astrocytes. *Neuroscience* 2013; 250: 536–545.
56. Kaur D, Sharma V, Deshmukh R. Activation of microglia and astrocytes: a roadway to neuroinflammation and Alzheimer's disease. *Inflammopharmacology* 2019; 27: 663–677.