Brainstem Brain-Derived Neurotrophic Factor Signaling Is Required for Histone Deacetylase Inhibitor–Induced Pain Relief

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ABSTRACT

Our previous study demonstrated that persistent pain can epigenetically suppress the transcription of Gad2 [encoding glutamic acid decarboxylase 65 (GAD65)] and consequently impair the inhibitory function of GABAergic synapses in central pain-modulating neurons. This contributes to the development of persistent pain sensitization. Histone deacetylase (HDAC) inhibitors increased GAD65 activity considerably, restored GABA synaptic function, and rendered sensitized pain behavior less pronounced. However, the molecular mechanisms by which HDAC regulates GABAergic transmission through GAD65 under pain conditions are unknown. This work showed that HDAC inhibitor–induced increases in colocalization of GAD65 and synaptic protein synapsin I on the presynaptic axon terminals of the nucleus raphe magnus (NRM) were blocked by a TrkB receptor antagonist K252a [9S,10R,12R]-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-j][1,6]benzodiazoizene-10-carboxylic acid methyl ester], indicating that BDNF-TrkB signaling may be required in GAD65 modulation of GABA synaptic function. At the brain-derived neurotrophic factor (BDNF) promoter, HDAC inhibitors induced significant increases in H3 hyperacetylation, consistent with the increase in BDNF mRNA and total proteins. Although exogenous BDNF facilitated GABA miniature inhibitory postsynaptic currents and GAD65 accumulation in NRM neuronal synapses in normal rats, it failed to do so in animals subjected to persistent inflammation. In addition, blockade of the TrkB receptor with K252a has no effect on miniature inhibitory postsynaptic currents and synaptic GAD65 accumulation under normal conditions. In addition, the analgesic effects of HDAC inhibitors on behavior were blocked by NRM infusion of K252a. These findings suggest that BDNF-TrkB signaling is required for drugs that reverse the epigenetic effects of chronic pain at the gene level, such as HDAC inhibitors.

Introduction

The nucleus raphe magnus (NRM), located in the brainstem, is a crucial supraspinal site for pain modulation maintaining a behavioral state of sensitized pain under chronic pain conditions (Porreca et al., 2002; Fields, 2004). Loss of GABAergic inhibition in pain-signaling pathways has been proposed as a primary mechanism for pain-induced maladaptive responses termed central sensitization, a key process in the development of acute pain to chronic pain (Moore et al., 2002; Knabl et al., 2008; Costigan et al., 2009; Munro et al., 2009). Previous studies have shown that persistent inflammation and neuropathic pain can epigenetically suppress Gad2 [encoding glutamic acid decarboxylase 65 (GAD65)] transcription through histone deacetylase (HDAC)–mediated histone hypoacetylation, resulting in impaired GABA synaptic inhibition in the NRM. HDAC inhibitors can increase GAD65 activity, restore GABA synaptic function, and mitigate sensitized pain behavior (Zhang et al., 2011). It is generally acknowledged that GAD65 is critical to the intensification of synaptic activity and that it acts by reversibly binding to the membrane of synaptic GABA vesicles and thus maintaining the highly compartmentalized nature of intracellular and intercellular GABA homeostasis (Walls et al., 2011). Evidence has shown that GAD65 plays a role in the control of the release of neuronal GABA and analgesia in inflammatory pain (Tian et al., 1999). Specifically, GAD65 delivery in vivo produces analgesia and deficits of GAD65 cause thermal hyperalgesia (Kubo et al., 2009). However, the mechanisms underlying synaptic accumulation of GAD65 under pain conditions are unknown.

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, has many trophic effects on structural modifications and functional plasticity of GABA synapses in the mammalian brain (Palizvan et al., 2004; Ohba et al., 2005; Betley et al., 2009). It plays a well documented pronociceptive
role in inflammatory and neuropathic pain responses, acting at the brainstem descending pain pathways, including the periaqueductal gray (PAG), rostral ventromedial medulla (RVM), and spinal cord (Pezet and McMahon, 2006; Merighi et al., 2008). High levels of BDNF mRNAs and proteins have been observed within the PAG and RVM (Conner et al., 1997; King et al., 1999). A study by Guo et al. (2006) showed that BDNF in the RVM may have originated from BDNF-containing neurons in the PAG and that BDNF activation of TrkB signaling in the RVM induces descending pain facilitation, suggesting that the signaling cascade of BDNF-TrkB receptors in the RVM circuitry plays a critical role in the development of persistent pain after inflammation. BDNF-TrkB signaling has been shown to regulate the lipid-dependent machinery that directs proteins to synaptic terminals and to promote the stable association of GAD65 with synaptic vesicle proteins such as the vesicular GABA transporter, or the local translation of GAD65 (Jin et al., 2003; Betley et al., 2009). Whether this evidence is relevant to the mechanism by which BDNF-TrkB signaling promotes synaptic accumulation of GAD65 and supraspinal GABAergic inhibition under pain conditions is unclear. In this study, the role of BDNF-TrkB signaling in HDAC inhibitor–induced synaptic accumulation of GAD65 in antinociception is explored in rat models of inflammatory pain in the brainstem NRM.

Materials and Methods

Animals and Pain Models. Male Wistar rats aged 9–14 days and adult rats weighing 200–300 g were used (Charles River Laboratories, Wilmington, MA). To induce hyperalgesia of inflammatory pain, complete Freund's adjuvant (CFA) (20 μl, suspended in a 1:1 oil/saline emulsion; Sigma-Aldrich, St. Louis, MO) or saline was injected into the plantar surface of one hindpaw of a rat under brief halothane anesthesia and the animal was then returned to its home cage. Pain thresholds were measured every 5 minutes or daily by the paw-withdrawal test on a freely moving animal with von Frey filaments for mechanical allodynia. The antinociceptive effect of an infused drug was measured 10–20 minutes later. Gad2 gene–null mice were obtained from Jackson Laboratories (Bar Harbor, ME). All procedures involving the use of animals conformed to the guidelines of the Institutional Animal Use and Care Committee of University of Science and Technology of China as well as the National Institutes of Health Guide for the Care and Use of Laboratory animals.

Brain Slice Preparations and Whole-Cell Recordings. Whole-cell voltage-clamp recordings of NRM neurons were visualized in slice preparations with general methods described previously (Zhang and Pan, 2010; Zhang et al., 2011). The rat brain was cut in a vibratome in cold (4°C) physiologic saline to produce brainstem slices (200-μm thick) containing the NRM for whole-cell recording as described previously (Bie et al., 2005). A single slice was submerged in a shallow recording chamber and perfused with preheated (35°C) physiologic saline containing the following: 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH2PO4, 1.2 mM MgCl2, 2.4 mM CaCl2, 11 mM glucose, and 25 mM NaHCO3 saturated with 95% O2 and 5% CO2 (pH 7.2–7.4). Visualized whole-cell voltage-clamp recordings were obtained from identified NRM neurons with a glass pipette (resistance, 3–5 MΩ) filled with a solution containing the following: 126 mM KCl, 10 mM NaCl, 1 mM MgCl2, 11 mM EGTA, 10 mM HEPES, 2 mM ATP, and 0.25 mM GTP, pH adjusted to 7.3 with KOH (osmolarity, 280–290 mOsm). An AxoPatch-700B amplifier and AxoGraph software (Molecular Devices, Sunnyvale, CA) were used for data acquisition and online and offline data analyses. A seal resistance of 2 GΩ or above and an access resistance of 15 MΩ or below were considered acceptable. Series resistance was optimally compensated and access resistance was monitored throughout the experiment. GABA miniature inhibitory post synaptic currents (mIPSCs) were recorded in the presence of N-methyl-d-aspartate–type glutamate receptor antagonists d-(-)-2-amino-5-phosphonopentanoic acid (50 μM), 6-cyano-7nitroquinolxaline-2,3-dione (10 μM), and tetrodotoxin (1 μM). A sliding custom mIPSC template, defined using acquisition software, was used to detect and analyze the frequency and amplitude of mIPSCs.

Western Blotting. Total proteins were prepared after tissue lysis and centrifugation for SDS-PAGE. β-tubulin was used as an internal normalizer. Protein concentration was determined before immunoblot analysis and 20 μg was mixed with SDS sample buffer, heated to 100°C for 5 minutes, separated under reducing conditions on a 12% SDS–polyacrylamide gel, and transferred to a nitrocellulose membrane. Nonspecific binding was blocked by incubating the membrane in 3% bovine serum albumin in Tris-buffered saline overnight at room temperature. Membranes were blocked for 2 hours at room temperature in blocking solution and incubated in a polyclonal rabbit BDNF antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and β-tubulin antibodies (1:1000; Santa Cruz Biotechnology) with agitation overnight at 4°C. Membranes were then incubated in a 1:10,000 dilution of antibody to mouse Ig horseradish peroxidase (Calbiochem, San Diego, CA) in blocking solution for 1 hour at room temperature. The bands were detected using enhanced chemiluminescence (GE Healthcare, Wauwatosa, WI).

Immunohistochemistry. Frozen coronal sections (20-μm thick) containing the NRM were cut on a cryostat and blocked in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (Thermo Fisher Scientific, Waltham, MA) plus 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature, as described in previous reports (Zhang et al., 2011). Sections were incubated overnight at 4°C in 1% bovine serum and 0.3% Triton X-100 in PBS with primary antibodies against synapsin I (1:200; Synaptic Systems, Gottingen, Germany) and GAD65 (1:1000; Millipore, Billerica, MA). Sections were then rinsed in PBS and incubated for 1 hour at room temperature with a mixture of fluorescein isothiocyanate– and Cy3-conjugated secondary antibodies (both 1:1000; Jackson ImmunoResearch Laboratories). After an additional PBS rinse, sections were mounted on slides using ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA). A single optimized acquisition exposure time was used for all images acquired from a particular slide. Signals of immunohistochemical staining for GAD65 and synapsin I, as well as their overlap among different experimental groups, were obtained from randomly selected sections and compared manually, with the experimenter blind to treatment groups. Quantitative analysis of microscopic images was conducted on a 400 × 150 μm area within the NRM on four randomly selected sections per rat in each group.

Chromatin Immunoprecipitation Assays. NRM tissues were harvested and immediately crosslinked in 1% formaldehyde for 15–20 minutes. After washes, the NRM tissue was homogenized 10–30 strokes in a cell lysis buffer. The homogenate was centrifuged and the supernatant was removed. The extracted chromatin was sheared by sonication into 200- to 500-bp fragments and diluted 10-fold in chromatin immunoprecipitation (ChIP) dilution buffer. Normal mouse IgG immunoprecipitates with a mouse polyclonal anti-IgG antibody were used as control to normalize appropriate enrichment of signal amplification, and the data were presented after normalization to saline/wild-type (WT) control groups. DNA and histones were dissociated with reverse buffer. Binding buffer was used for DNA precipitation and purification, and elution buffer was used to elute purified DNA from the column. H3 antibodies and all buffers were provided in the ChIP kit.

To quantify the level of histone modification at the gene promoter of interest, quantitative real-time polymerase chain reaction (PCR) (Applied Biosystems, Foster City, CA) was used to measure the amount of acetylated, histone-associated DNA. The following primers

- NRM neurons with a glass pipette (resistance, 3–5 MΩ filled with a solution containing the following: 126 mM KCl, 10 mM NaCl, 1 mM MgCl2, 11 mM EGTA, 10 mM HEPES, 2 mM ATP, and 0.25 mM GTP, pH adjusted to 7.3 with KOH (osmolarity, 280–290 mOsm).
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GraphPad Prism software (version 5.04; GraphPad Software, La Jolla, CA). The effects of TsA on GABA mIPSCs were blocked by daily cotreatment with the TrkB inhibitor K252a ([2R,10f,12R]-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-f:3′,2′,1′-ki][pyrrolo[3,4,4][1,6]benzozadiazepine-10-carboxylic acid methyl ester; 80 μg/kg] (Fig. 2, A and B). Immunohistochemistry was used to analyze the effect of TsA on the colocalization of GAD65 and the synaptic protein synapsin-1 on presynaptic axon terminals. As shown in Fig. 1A, vehicle administration significantly reduced the colocalization ratio of GAD65 and synapsin-1 compared with the vehicle group. Treatment with TsA or SAHA significantly increased the colocalization ratio of GAD65 and synapsin-1 compared with the vehicle group. These results suggest that HDAC inhibitors may promote BDNF gene activity through H3 hyperacylation.

**Methods.** Detailed methods of repeated NRM infusions and behavioral pain tests were the same as previously reported (Zhang et al., 2011). A 26-gauge double-guide cannula (Plastics One, Roanoke, VA) was implanted into the brain of an anesthetized rat, aiming the NRM (anteroposterior, 10 from the Bregma; lateral, 0; and dorsalventral, −10.5 from the dura). After recovery from the implantation surgery for >5 days, the rat received an intraplantar injection of CFA or saline. NRM infusions were made through a 33-gauge double injector with an infusion pump at a rate of 0.2 μl/min. Trichostatin A (TsA) or suberoylanilide hydroxamic acid (SAHA) was infused into the NRM once daily for 5 days. As a standard control, TsA infusions into a site 1 mm dorsal to the NRM were without effect (data not shown).

**Data Analysis and Materials.** Analyses of variance (one-way and two-way) and post hoc analysis were used to statistically analyze experimental data between treatment groups with multiple comparisons. Simple comparisons of data between two groups were made with the unpaired t test. Behavioral data with multiple measurements were statistically analyzed by two-way analysis of variance for repeated measures with the Bonferroni method for post hoc tests. Data are presented as means ± S.E.M. P < 0.05 was considered statistically significant. All statistical analyses were performed with GraphPad Prism software (version 5.04; GraphPad Software, La Jolla, CA). Drugs were purchased from Sigma-Aldrich or Tocris Bioscience (Ellisville, MO).

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**Quantitative Real-Time PCR.** RNA was quantified with the RNAqueous-4PCR Kit and reverse transcribed with the RETROscript Kit (Applied Biosystems). cDNA was quantified by real-time PCR and specific cDNA regions of the transcripts were amplified with custom-designed primers (Invitrogen). Fold differences of mRNA levels over controls were calculated using 2ΔCt. The following primers were used: Bdnf, forward 5′-AGGGGCTCCTCTTTGCAAGTG-3′, reverse 5′-GACCGAGGAAATGCTGTCG-3′; Gapdh, forward 5′-AGGTCGGTGTTTATGCAGG-3′, reverse 5′-TGTAAGCATGTAGTTGG-3′; and Aprt, 5′-TGGTCGTTCAAGTGCGGT-3′.

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**DNA Quantification.** Quantitative real-time PCR was performed with a SYBR Green Master Kit (Applied Biosystems) and used to measure the amount of H3-associated DNA with adenine phosphoribosyltransferase (housekeeping mRNA) as negative control. Differences in target or Cttarget over Ctinput, N represent the normalized Ct value of the target or Cttarget/Ctinput, N is the mean N value for the control, and Cttarget is the mean Ct value for the control.

**Results**

**BDNF/TrkB Signaling Is Required for HDAC Inhibitor–Induced GABA Synthetic Release.** Our previous study showed that persistent pain markedly decreases histone H3 acetylation at the GADD51 promoter and the expression levels of GADD51 mRNA and proteins, resulting in impaired GABA synaptic function in NRM neurons. HDAC inhibitors can reverse the reduction in GADD51 gene activity, rescue the loss of GABA synaptic inhibition, and thus reduce chronic pain.

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**Brainstem BDNF Signaling and HDAC Inhibitors in Pain Relief**

**Fig. 1.** HDAC inhibitors epigenetically increase BDNF expression. (A and B) Normalized changes in acH3 levels at the BDNF promoter (A) and the levels of BDNF mRNA (B) in saline- and CFA-injected rats (n = 6 to 7 for each group) after vehicle, vehicle, or SAHA treatment in the NRM. (C) Representative Western blot lanes of BDNF proteins (top) in NRM tissues harvested from vehicle- and TsA/SAHA-treated groups 4 to 5 days after injection (n = 6–8 rats per group) and pooled data (bottom) normalized to β-tubulin. *P < 0.05 compared with the vehicle group.
2C, in the NRM tissues, there was an increase in colocalization levels of GAD65 and synapsin-I relative to the vehicle control after TsA treatment. The effect was blocked by daily cotreatment with K252a. These results suggest that the BDNF-TrkB signaling pathway is involved in the effects of TsA-induced histone modifications on GABA synaptic function.

Our recent work showed that persistent pain can epigenetically upregulate BDNF expression through histone modifications, leading to enhancement of excitatory synaptic transmission (Tao et al., 2014). Consistent with this, Western blotting showed a statistically significantly higher level of BDNF protein in the NRM 3 days after CFA injection than that in saline-injected control rats (Fig. 3A; 166.2 ± 19.6%, n = 6, P < 0.05). BDNF promotes the maturation and inhibitory function of GABA synapses in the brain (Huang et al., 1999). Therefore, the effect of BDNF on GABA inhibitory postsynaptic currents in these brainstem neurons is explored here. After incubation of brain slices from saline-injected rats in 50 ng/ml BDNF in vitro for 4 hours, the frequency of GABA mIPSCs was increased (138.5 ± 12.7%, n = 22, P < 0.05), as expected. However, BDNF was no longer effective on mIPSC frequency in neurons from CFA-injected rats at 3 days (Fig. 3B; 106.4 ± 13.5%, n = 19). To confirm the role of BDNF signaling in analgesics using GAD65 upregulation after TsA treatment, mIPSC levels were measured in NRM neurons from GAD65 knockout mice slices incubated in exogenous BDNF. A previous study showed that the

Fig. 2. BDNF/TrkB signaling is required for HDAC inhibitor-induced enhancement of GABA synaptic transmission. (A) Representative current traces of mIPSCs with TsA or TsA plus K252a in an NRM neuron from saline- and CFA-treated rats. (B) Summarized data of the effects of TsA on the frequency of mIPSCs in neurons from the saline group (n = 5 rats) and CFA group (n = 6 rats). (C) Micrographs of immunohistochemical staining for GAD65 (red), the synaptic terminal protein synapsin I (green), and colocalization of GAD65 and synapsin I (yellow) in rats with vehicle, TsA, or TsA plus K252a treatment (n = 5 or 6 rats in each group; 4–6 randomly selected sections from each rat were analyzed and compared). Summarized data for colocalization of GAD65 and protein synapsin I was normalized to the vehicle group (right). Data are expressed as means ± S.E.M. *P < 0.05; **P < 0.01. Bar, 2 μm in (C), right column; 50 μm in (C), other columns.

Fig. 3. Effects of exogenous BNDF on mIPSCs. (A) Levels of BDNF proteins as analyzed by Western blotting in NRM tissues from saline- and CFA-injected rats (n = 6 to 7 rats for each group). (B) Traces of mIPSCs (left) and summarized data of mIPSC frequency (right) in vehicle or BDNF-incubated (50 ng/ml, 4 hours) slices from saline- and CFA-injected rats 3 days after injection. (C) The effect of exogenous BDNF on NRM mIPSCs from GAD65−/− mouse brain slices. Data are expressed as means ± S.E.M. *P < 0.05.
frequency of GABA mIPSCs, but not amplitude, was significantly reduced in neurons from GAD65<sup>−/−</sup> mice compared with those from WT mice (72.5 ± 8.1% of WT control, n = 26, P < 0.01). As expected, after incubation of brain slices from GAD65<sup>−/−</sup> in 50 ng/ml BDNF in vitro for 4 hours, it failed to alter mIPSC frequency (68.7 ± 6.8% of WT control, n = 21) and amplitude (88.9 ± 8.5% of WT control, n = 21) (Fig. 3C). This shows that exogenous BDNF is not sufficient to rescue the reduction of GABA synaptic function induced by persistent pain or GAD65 deficiency.

**BDNF-TrkB Signaling Is Required for Accumulation of Synaptic GAD65 for Analgesics.** TrkB signaling is required for accumulation of synaptic GAD65 in the NRM (Fig. 2C). In saline-injected rats, exogenous BDNF microinjected into the NRM also significantly increased GAD65 expression in synaptic terminals (Fig. 4A), but this effect was not observed in CFA-injected rats (Fig. 4B). In addition, after TsA (16.5 mM in 1 μl) treatment in vivo in CFA-injected rats, the colocalization level displayed an approximately 2-fold increase (Zhang et al., 2011). This increase was abolished by coinjection of K252a (80 μg/kg in 1 μl) (Fig. 4C). These results indicate that BDNF signaling may be required, but this alone is not sufficient to rescue the impaired GAD65 expression at the synaptic terminals induced by persistent pain.

If BDNF signaling is indeed critical to HDAC inhibitor–induced analgesics by reversing reductions in GAD65 levels induced by persistent inflammation, the blockade of BDNF signaling would interfere with TsA-induced persistent pain relief (Zhang et al., 2011). Repeated local administration of TsA into the NRM produced a significant antinociceptive effect in CFA-injected rats (Zhang et al., 2011). These effects of HDAC inhibitors were abolished by conmicroinjection of K252a (n = 6 rats; Fig. 5A), indicating the involvement of BDNF/TrkB signaling. However, combined microinjection of BDNF (30 pmol in 1 μl) and TsA (16.5 mM in 1 μl) did not produce an additive or synergic effect on pain relief compared with TsA alone in CFA-injected mice (n = 5 rats; Fig. 5B).

**Discussion**

A rat model of inflammatory pain showed that persistent pain epigenetically reduced the expression and output activity of the GAD65 gene, impairing the inhibitory function of GABAergic synapses in central pain-modulating neurons
and contributing to the development of chronic pain (Zhang et al., 2011). This study demonstrates that histone hyperacetylation reversed the pain effect by promoting GAD65 expression and activity in GABA synaptic terminals in a BDNF/TrkB-dependent manner, thereby reducing the intensity of behavior associated with persistent pain.

Chronic pain involves altered expression of many genes, and the mechanisms underlying these changes are unknown (Lacroix-Fralish et al., 2007). Given the multifaceted spinal and supraspinal mechanisms of chronic pain, it is highly likely that genes other than GAD65 are also epigenetic targets of chronic pain through chromatin remodeling (Zhang et al., 2011). In nerve injury–induced loss of touch sensitivity, the C-fiber dysfunction is reported to be mediated by epigenetic upregulation of the neuron-restrictive silencer factor, a transcriptional repressor of genes that contain the neuron-restrictive silencer element sequence, including those encoding μ-opioid receptors, Nav1.8 sodium channels, and transient receptor potential channels (Uchida et al., 2010). HDAC inhibitors also reduce inflammatory pain by upregulating spinal metabolotropic glutamate 2 receptors (Chiechio et al., 2009).

Our results suggest that chronic pain also alters the expression of the BDNF gene and that BDNF-TrkB signaling is important to the epigenetic modulation of chronic pain. Persistent pain can epigenetically upregulate BDNF expression through histone modifications. Previous reports have shown that BDNF promotes the inhibitory function of GABA synapses in the brain (Huang et al., 1999; Palizvan et al., 2004; Ohba et al., 2005; Betley et al., 2009). This raises the question of what the effects of BDNF on GABA inhibitory post synaptic currents may be in these neurons. After incubation of brain slices from vehicle-injected rats in BDNF in vitro, the frequency of GABA mIPSCs was increased, as expected; however, BDNF was no longer effective on mIPSC frequency in neurons from CFA-injected rats at 3 days. Therefore, it appears that epigenetically upregulated BDNF is unlikely to be the direct or sufficient cause for the reduction of GABA synaptic function induced by persistent pain.

The exact role of BDNF in the epigenetic mechanisms of chronic pain relief remains unknown. GAD65 is a presynaptic protein required for GABA synaptic release and maintenance of highly compartmentalized GABA homeostasis on synaptic terminals (Soghomonian and Martin, 1998; Tian et al., 1999; Patel et al., 2006). Interfering with the release of GABA can cause the loss of GABAergic inhibition and consequent neuronal hyperexcitability. It has been proposed that this may be one of the spinal mechanisms underlying chronic pain (Tian et al., 1999). BDNF induces accumulation of synaptic GAD65 during maturation of spinal GABAergic neurons (Betley et al., 2009). Increased GABA mIPSC frequencies were observed after BDNF treatment under control conditions in this study. This is consistent with the hypothesis that BDNF generally promotes the release of GABA (Wardle and Poo, 2003). However, BDNF did not show this effect under persistent pain conditions. This could be attributable to the pain-induced, concurrent loss of GAD65 function with respect to the release of GABA and pain-induced epigenetic upregulation of endogenous BDNF. This loss of GAD65 function occluded the exogenous BDNF effect. Because HDAC inhibitor–induced histone hyperacetylation upregulates the expression of both GAD65 and BDNF and increases mIPSC frequency in a TrkB-dependent manner, it was here presumed that BDNF is required but not sufficient to significantly promote GABA release under chronic pain conditions. Another possibility of the failure for the exogenous BDNF to work could also result from the occlusion effect of already enhanced endogenous BDNF levels in persistent pain states.

It appears that there are two types of upregulation: one induced by persistent pain, which probably causes excitation (pro-pain) rather than inhibition as shown by a recent study (Tao et al., 2014), and the other that responds to HDAC inhibitors. The inhibitory role of BDNF requires HDAC inhibitor–induced GAD65 upregulation through GABA systems, which is downregulated by persistent inhibition. The current findings indicate that, under persistent pain conditions, BDNF predominantly works on excitatory systems because the impaired GAD65 expression at the GABA synaptic terminals. For example, exogenous BDNF has no effect on GABA mIPSCs and GAD65 expression in synaptic terminals under pain conditions. After HADC inhibitor treatment under pain conditions, the upregulated BDNF can be both excitatory and inhibitory. In this study, we believe that HDAC inhibitor–induced BDNF upregulation predominantly mediates an inhibitory effect because BDNF upregulated by persistent pain has already worked on the excitatory system. It is possible that the main action of HDAC inhibitors here is to prevent GAD65 downregulation. However, on the basis of current knowledge and the literature, although GAD65 expression is upregulated, BDNF is necessary for GAD65 accumulation at the synaptic terminals.

Behavioral experiments were then conducted with sitespecific microinjections in rats in vivo. Repeated local administration of HDAC inhibitors into the NRM produced a significant antinociceptive effect in rats that had already been injected with CFA. The effects of HDAC inhibitors were abolished by TrkB receptor blockade, indicating the involvement of TrkB signaling. Combined treatment with TsA and BDNF did not produce an additive or synergic effect on pain relief compared with TsA alone in the CFA-injected rats. However, combined treatment with TsA and BDNF had no effects on pain sensitivity in the CFA-injected GAD deficient mice. This was consistent with the lack of BDNF effects on GABA mIPSCs and GAD65 expression in the CFA-injected GAD deficient mice. In summary, these findings indicate that BDNF/TrkB signaling is critical to HDAC inhibitor–induced antinociception, which works by promoting GAD65 accumulation in GABA synaptic terminals.

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Authorship Contributions
Participated in research design: Zhang.
Conducted experiments: Tao, Chen, Zhou, L. Wang, Y. Wang.
Performed data analysis: Tao, Chen, Zhou, L. Wang, Y. Wang, Zhang.
Wrote or contributed to the writing of the manuscript: Zhang, Tao.

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