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Brainstem BDNF signaling is required for histone deacetylase inhibitor-induced pain relief

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Running title: Brainstem BDNF signaling and HDAC inhibitors in pain relief

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Abbreviations:

GAD65, glutamic acid decarboxylase 65; HDAC, histone deacetylase; CFA, complete Freund's adjuvant; BDNF, brain-derived neurotrophic factor; TsA, trichostatin A; SAHA, suberoylanilide hydroxamic acid CHIP, chromatin immunoprecipitation; PAG, periaqueductal gray, NRM, nucleus raphe mangus; RVM, rostral ventromedial medulla; mIPSC, miniature inhibitory postsynaptic current.

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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. The current work showed that HDAC-inhibitor-induced increases in co-localization 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, 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 (mIPSCs) 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 TrkB receptor with K252a has no effect on mIPSCs 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 BDNF-TrkB signaling is required for drugs that reverse the epigenetic effects of chronic pain at the gene level, such as HDAC inhibitors.

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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 so 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

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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. 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 (Guo et al., 2006). 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, e.g., vesicular GABA transporter (vGAT), 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, 9 to 14 days old and adult rats weighing 200–300 g were used (Charles

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River, Wilmington, MA, U.S.). To induce hyperalgesia of inflammatory pain, complete Freund's adjuvant (CFA, 20 μl, suspended in a 1:1 oil/saline emulsion, Sigma-Aldrich) or saline was injected into the plantar surface of one hindpaw of a rat under brief halothane anesthesia and then the animal was returned to home cages. Pain thresholds were measured every 5 min 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 min later. *Gad2*$^{-/-}$ mice were obtained from Jackson Laboratories. 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 and 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 (4ºC) physiological 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 (35ºC) physiological saline containing the following (in mM): 126 NaCl, 2.5 KCl, 1.2 NaH2PO4, 1.2 MgCl2, 2.4 CaCl2, 11 glucose, and 25 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 (in mM): 126 KCl, 10 NaCl, 1 MgCl2, 11 EGTA, 10 HEPES, 2 ATP, and 0.25

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GTP, pH adjusted to 7.3 with KOH; osmolarity, 280–290 mOsM. An AxoPatch-700B amplifier and AxoGraph software (Molecular Devices) 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. Miniature GABA inhibitory postsynaptic 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-7-nitroquinoxaline-2,3-dione (10 μM) and tetrodotoxin (1 μM), and a sliding mIPSC template custom, 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-polyacrylamide gel electrophoresis. 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 min, 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 (TBS) overnight at room temperature. Membranes were blocked for 2 h at RT in blocking solution and incubated in a polyclonal rabbit BDNF antibody (1: 200, Santa Cruz Biotechnology) and β-tubulin antibodies (1:1000, Santa Cruz Biotechnology) with agitation overnight at 4 °C. Membranes were then incubated in a 1: 10000 dilution of antibody to mouse Ig HRP (Calbiochem) in blocking solution for 1 h at RT. The bands were detected using enhanced chemiluminescence

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(GE Healthcare).

Immunohistochemistry

Frozen coronal sections (20 μm thick) containing the NRM were cut on a cryostat and blocked in PBS containing 0.1% Triton X-100 (Fisher) plus 5% normal donkey serum (Jackson ImmunoResearch Laboratories) for 1 h at RT, 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) and GAD65 (1:1000, Millipore). Sections were then rinsed in PBS and incubated for 1 h at RT with a mixture of FITC-(1:1000, Jackson ImmunoResearch Laboratories) and Cy3-conjuaged secondary antibodies (1:1000, Jackson ImmunoResearch Laboratories). After an additional PBS rinse, sections were mounted on slides using ProLong Gold antifade reagent with DAPI (Invitrogen). 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, and 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 (ChIP) assays

NRM tissues were harvested and immediately cross-linked in 1% formaldehyde for 15–20 min. 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 200–500 bp fragments and diluted 10-fold in ChIP dilution buffer.

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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/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 columns. 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 PCR (Applied Biosystems) was used to measure the amount of acetylated, histone-associated DNA. The following primers (Invitrogen) were used: *Bdnf1,* 5'-TGATCATCACTCACGACCACG-3' and 5'- CAGCCTCTCTGAGCCAGTTACG-3'; *Bdnf2,* 5'-CCGTCTTGTATTCCATCCTTTG-3' and 5'-CCCAACTCCACCACTATCCTC-3'; *Bdnf3*, 5'-GTGAGAACCTGGGGCAAATC-3' and 5'-ACGGAAAAGAGGGAGGGAAA-3' *Bdnf4,* 5'-GGCTTCTGTGTGCGTGAATTTGC-3' and 5'-AAAGTGGGTGGGAGTCCACGAG-3'; *Aprt*, 5'-TGCTGTTCAGGTGCGGTCAC-3' and 5'-AGATCCCCGAGGCTGCCTAC-3'.

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 (house-keeping mRNA) as negative control. Differences in signals were calculated as follows: Δ Ct = (Nexp – Nave) x Ctave (Nexp, normalized Ct value of the target or Cttarget/Ctinput; Nave, mean N value for control; and Ctave, mean Ct value for control).

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Quantitative RT-PCR

RNA was extracted 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 ΔCt. The following primers were used: *Bdnf*, forward 5’-GAGGGCTCCTGCTTCTCAA-3’, reverse 5’-GCCTTCATGCAACCGAAGT-3’; *Gapdh*, forward 5’-AGGTCGGTGTGAACGGATTTG-3’, reverse 5’-TGTAGACCATGTAGTTGAGGTCA-3’.

Microinjection

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) was implanted into the brain of an anesthetized rat, aiming the NRM (anteroposterior, –10 from the Bregma; lateral, 0; dorsoventral, –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^{-1}. 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

ANOVA (one-way and two-way) and *post hoc* analysis were used to statistically analyze experimental data between treatment groups with multiple comparisons. Simple comparisons

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of data between two groups were made with the unpaired Students' t test. Behavioral data with multiple measurements were statistically analyzed by two-way ANOVA for repeated measures with the Bonferronic method for *post hoc* tests. Data are presented as mean ± SEM and $p < 0.05$ was considered statistically significant. All statistical analyses were performed with the Prism software version 5.04 (GraphPad Software). Drugs were purchased from Sigma-Aldrich or Tocris Bioscience (Ellisville, MO).

Results

BDNF/TrkB signaling is required for HDAC inhibitor-induced GABA synaptic release

Our previous study showed that persistent pain markedly decreases histone H3 acetylation at *GAD65* promoter and the expression levels of GAD65 mRNA and proteins, resulting in impaired GABA synaptic function in NRM neurons. HDAC inhibitors can reverse the reduction in *GAD65* gene activity, rescue the loss of GABA synaptic inhibition, and so reduce chronic pain. There are many target genes that may underlie persistent pain-induced H3 hyperacetylation, which might be involved in epigenetic mechanism of chronic pain. (Zhang et al., 2011). Because BDNF-TrkB signaling is significantly changed in the induction and maintenance of persistent inflammatory pain (Guo et al., 2006, Wang et al., 2009), BNDF expression in the NRM was here examined. CHIP assays showed microinjection of HDAC inhibitors TsA (16.5 mM, 1 µl for 5 days) or SAHA (100 µM, 1 µl for 5 days) into rat NRM significantly increased H3 acetylation at the *BDNF* promoter regions (Fig. 1A; saline-injected rats, TsA, 2.8 ± 1.0, n = 6, $P < 0.05$; SAHA, 3.2 ± 0.9, n = 6, $P < 0.05$; CFA-injected rats, TsA, 1.8 ± 0.5, n = 7, $P < 0.05$; SAHA, 2.2 ± 0.7, n = 6, $P < 0.05$; normalization to vehicle control);

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a similar increase was observed in the BDNF mRNA (saline-injected rats, TsA, 3.4 ± 1.2, n = 6, $P < 0.05$; SAHA, 2.9 ± 1.0, n = 5, $P < 0.05$; CFA-injected rats, TsA, 2.2 ± 0.5, n = 7, $P < 0.01$; SAHA, 2.4 ± 0.8, n = 5, $P < 0.05$; normalization to vehicle control) (Fig. 1B) and protein levels (saline-injected rats, TsA, 162.2 ± 12.9% of control, n = 6, $P < 0.05$; SAHA, 168.9 ±13.7% of control, n = 5, $P < 0.05$; CFA-injected rats, TsA, 135.3 ± 10.3% control, n = 8, $P < 0.05$; SAHA, 147.9 ± 12.8% of control, n = 5, $P < 0.05$) (Fig. 1C). These results suggest that HDAC inhibitors may promote *BDNF* gene activity through H3 hyperacetylation.

TsA and SAHA treatment can significantly augment GABA synaptic function, increasing mIPSC frequency in neurons of CFA-injected and control rats (Zhang et al., 2011). The effects of TsA on GABA mIPSCs were blocked by daily co-treatment with the TrkB inhibitor K252a (80 µg/kg, Fig. 2A, B). Immunohistochemistry was used to analyze the effect of TsA on the co-localization of GAD65 and the synaptic protein synapsin-1 on presynaptic axon terminals. As shown in Fig. 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 co-treatment with K252a. These results suggest that 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

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maturation and inhibitory function of GABA synapses in the brain (Huang et al., 1999). Therefore, the effect of BDNF on GABA IPSCs 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 h, 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 d (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 slice incubated in exogenous BDNF. A previous study showed that GABA mIPSCs, whose frequency, but not amplitude, was significantly reduced in neurons from GAD65$^{-/-}$ mice when compared with those from wild type (WT) mice (72.5 ± 8.1% of WT control, n=26, $P<0.01$). As expected, after incubation of brain slices from GAD65$^{-/-}$ in 50 ng/ml BDNF *in vitro* for 4 h, 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 (Figure 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 co-localization level displayed a ≈2-fold increase (Zhang et al., 2011). This increase was abolished by co-injection of K252a (80 µg/kg in 1 µl,) (Fig. 4C).These

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results indicates 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 co-microinjection 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 a additive or synergic effect on pain relief than TsA alone in CFA-injected mice (n = 5 rats, Fig. 5B). Consistent with the absence of BDNF effects on GABA mIPSCs and GAD65 expression, repeated daily co-microinjection of BDNF and TsA also failed to alter the CFA-induced sensitized pain behavior in $GAD^{-/-}$ mice (n = 5 mice, Fig. 5C).

Discussion

A rat model of inflammatory pain showed that persistent pain epigenetically reduced the expression and output activity of *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). The current 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 of persistent pain.

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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 mu-opioid receptors, Nav1.8 sodium channels, and transient receptor potential channels (Uchida et al., 2010). HDAC inhibitors also reduce inflammatory pain by upregulating spinal metabotropic glutamate 2 receptors (Chiechio et al., 2009).

Current results suggest that chronic pain also alters the expression of *BDNF* gene and 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 IPSCs 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 d. So 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

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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 hyper-excitability. 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 the current 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); the other that responds to HDAC inhibitors, which is more relevant to the GAD65

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action through GABA signaling. Therefore, the upregulated BDNF can be both excitatory and inhibitory. One possibility for excitation is that epigenetically upregulated BDNF induced by persistent pain promotes glutamate AMPA receptor GluA1 subunit trafficking to synaptic terminals, which facilitates pain development(Tao et al., 2014). On the other side, the inhibitory role of BDNF requires HDAC inhibitor-induced GAD65 upregualtion through GABA systems, which is downregulated by persistent inhibitory. Based on the current finding, therefore, 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. Here, we believe that HDAC inhibitor-induced BDNF upregulation predominantly mediates a 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 down regulation. However, based on current knowledge and literature, even though GAD65 expression is upregulated, BDNF is necessary for GAD65 accumulation at the synaptic terminals.

Behavioral experiments were then conducted with site-specific 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 dose not produce a additive or synergic effect on pain relief than TsA alone in the CFA-injected rats. However, combined treatment

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with TsA and BDNF has no effects on pain sensitivity in the CFA-injected $GAD^{-/-}$ mice. This was consistent with the lack of BDNF effects on GABA mIPSCs and GAD65 expression in the CFA-injected $GAD^{-/-}$ mice. In summary, these findings indicates 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 designs: Zhi Zhang

Conducted experiments: Tao, Chen, Zhou and Wang

Performed data analysis: Tao, Chen, Zhou, Wang and Zhang

Wrote or contributed to the writing of the manuscript: Zhi Zhang and Wenjuan Tao

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Footnotes

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Legends of Figures

Fig. 1. Histone deacetylase (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 = 6–7 for each group) after vehicle, TsA 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 4–5 days after injection (*n* = 6–8 rats per group) and pooled data (bottom) normalized to β-tubulin.

Fig.2. BDNF/TrkB signaling is required for histone deacetylase (HDAC)-inhibitor -induced enhancement of GABA synaptic transmission.

(A) Representative current traces of miniature IPSCs (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 co-localization of GAD65 and synapsin I (yellow) in rats with vehicle, TsA or TsA plus K252a treatment (*n* = 5 or 6 rats in each group, and we analyzed and compared 4–6 randomly selected sections from each rats). Scale bars, 50 µm (left three columns) and 2 µm (right column). Summarized data for colocalization of GAD65 and protein synapsin I was normalized to the vehicle group (right). Data are expressed as mean ± SEM. $^*P < 0.05$.

Fig.3. Effects of exogenous BNDF on mIPSC. **(A)** Levels of BDNF proteins as analyzed by Western blotting in NRM tissues from saline- and CFA-injected rats (*n* = 6–7 rats for each

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group). **(B)** Traces of mIPSCs (left) and summarized data of mIPSC frequency (right) in vehicle or BDNF-incubated (50 ng/ml, 4 h) slices from saline- and CFA-injected rats 3 d after injection. **(C)** The effect of exogenous BDNF on NRM mIPSCs from GAD65$^{-/-}$ mouse brain slices. Data are expressed as mean ± SEM. *$P < 0.05$.

Fig. 4. BDNF-TrkB signaling is required for accumulation of synaptic GAD65. (A-C) (A) Immunohistochemical staining for GAD65 (red), the synaptic terminal protein synapsin I (green), and co-localization of GAD65 and synapsin I (yellow) in saline-injected rats with BDNF treatment. **(B** and **C)** Immunohistochemical staining from CFA-injected rats treated with BDNF (B), TsA or TsA plus K252a **(C)**. Summarized data for colocalization of GAD65 and protein synapsin I was normalized (right). n = 5–7 rats per group.

Fig. 5. BDNF/TrkB signaling is required for HDAC inhibitor-induced pain relief. (**A** and **B)** Behavioral changes in pain threshold measured by the paw-withdrawal test with NRM microinjections of vehicle (at arrowheads) (n = 5 rats), TsA (n = 6 rats), TsA plus k252a (n = 6 rats) (**A**), TsA plus BDNF (n = 6 rats) (**B**) in CFA-injected rats**. (C)** The effect of combined treatment of TsA and BDNF on pain threshold in CFA-injected GAD$^{-/-}$ mice (n = 6 mice). Data are expressed as mean ± SEM. * and # $P < 0.05$. ** and ## $P < 0.01$.

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Figure. 1

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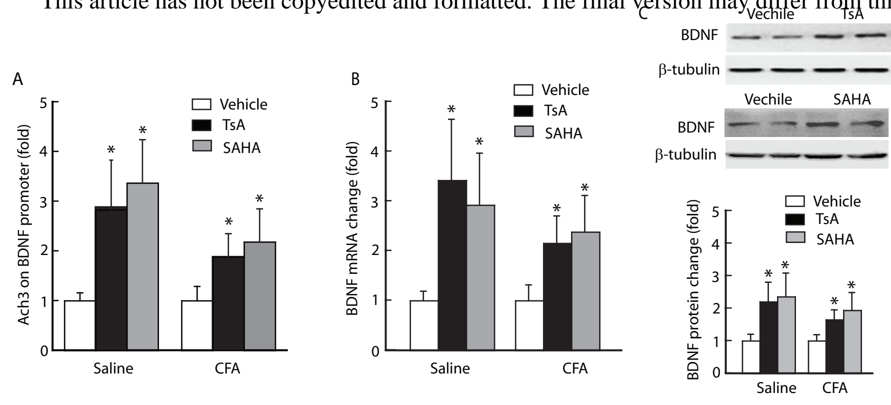

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Figure 2

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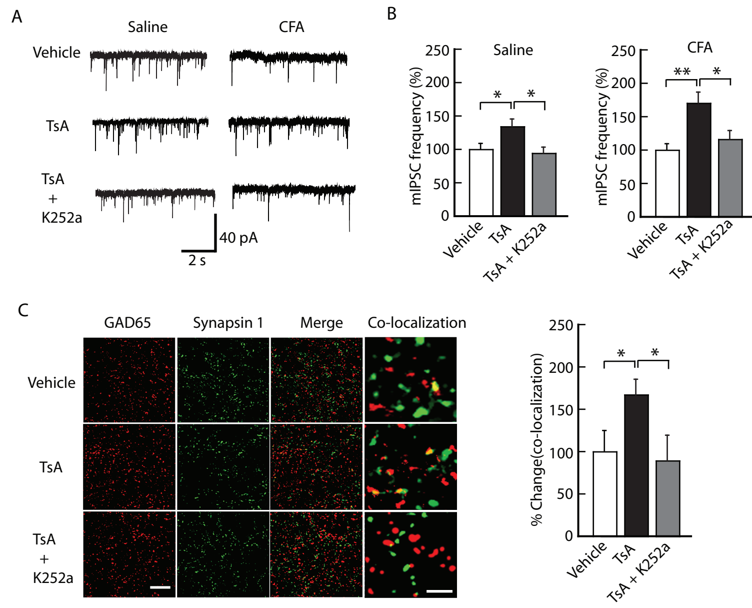

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Figure 3

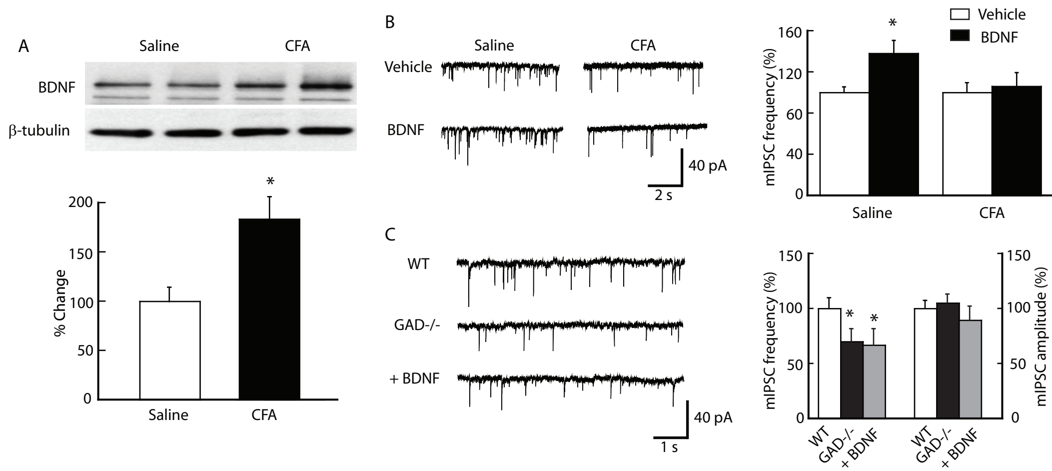

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Figure 4

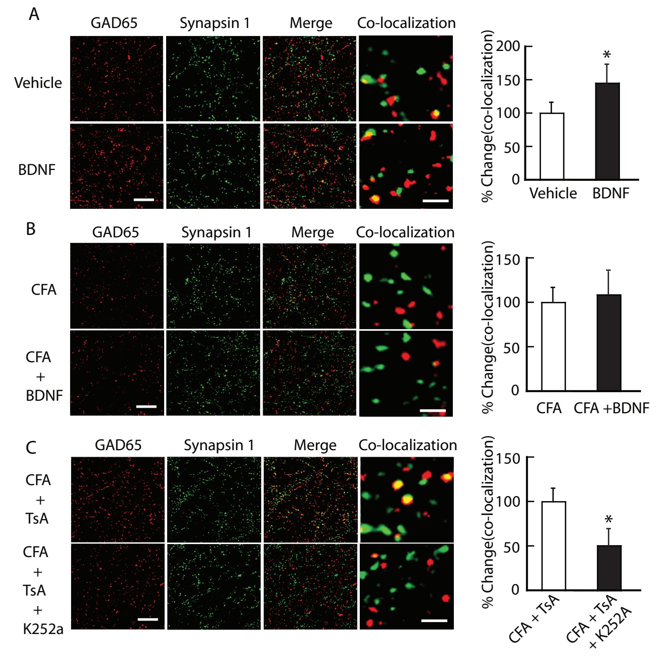

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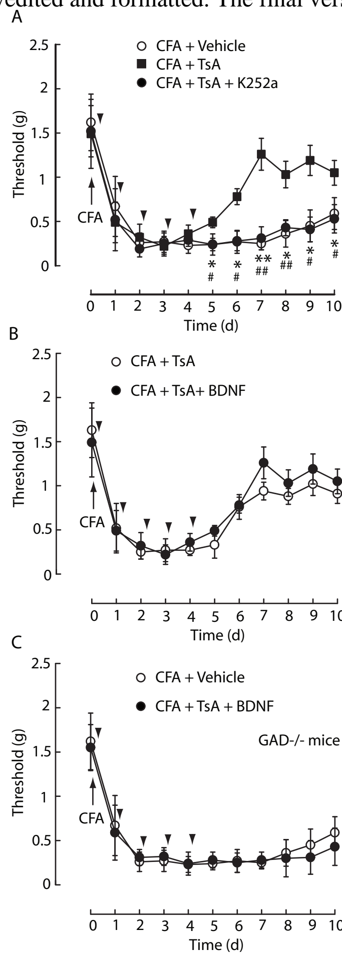
Figure 5



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