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]benzodiazocine-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 effects of chronic organism at ASPET Journals on January 10, 2021 molpharm.aspetjournals.org Downloaded from
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 allostynia. The antinociceptive effect of an infused drug was measured 10–20 minutes later. Gad2−/− 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 7.4). ADC inhibitor–induced synaptic accumulation of GAD65 in antinociception is explored in rat models of inflammatory pain in the brainstem NRM.

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 200–500-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. 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

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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 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 thus reduce chronic pain. There are many target genes that can undergo persistent pain-induced H3 hyperacetylation, which might be involved in epigenetic mechanisms 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), BNF expression in the NRM was examined. ChIP assays showed that 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; and 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); 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; and CFA-injected rats: TsA, 2.2 ± 0.5, n = 7, P < 0.001; 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% of 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 cotreatment with the TrkB inhibitor K252a ([(9S,10F,12R)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-f,g:3′,2′,1′-kl]pyrrolo[3,4,5]R]-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.

**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, 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 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.

**Fig. 2.** Western blot analysis of the acH3 levels of the BDNF promoter (A) and the levels of BDNF (B) in saline- and CFA-injected rats (n = 6 to 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 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
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, \( \mathcal{P} < 0.01 \)). 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 coinjection of K252a (n = 6 rats; Fig. 5A), indicating the involvement of BDNF/TrkB signaling. However, combined coinjection 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). Consistent with the absence of BDNF effects on GABA mIPSCs and GAD65 expression, repeated daily coinjection of BDNF and TsA also failed to alter the CFA-induced sensitized pain behavior in GAD<sup>−/−</sup> 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 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 \( \mu \)-opioid receptors, Nav1.8 sodium channels, and transient receptor potential channels (Uchida et al., 2010). HDAC inhibitors also reduce inflammatory pain by upregulating spinal 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 postsynaptic 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 mIPSCs and GAD65 expression in the 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, which is more relevant to the GAD65 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 hand, 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 HDAC 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–/– 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 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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