nature medicine

Epigenetic suppression of GAD65 expression mediates persistent pain

Zhi Zhang, You-Qing Cai, Fang Zou, Bihua Bie & Zhizhong Z Pan

Chronic pain is a common neurological disease involving lasting, multifaceted maladaptations ranging from gene modulation to synaptic dysfunction and emotional disorders. Sustained pathological stimuli in many diseases alter the output activities of certain genes through epigenetic modifications, but it is unclear how epigenetic mechanisms operate in the development of chronic pain. We show here that in the rat brainstem nucleus raphe magnus, which is important for central mechanisms of chronic pain, persistent inflammatory and neuropathic pain epigenetically suppresses *Gad2* (encoding glutamic acid decarboxylase 65 (GAD65)) transcription through histone deacetylase (HDAC)-mediated histone hypoacetylation, resulting in impaired γ-aminobutyric acid (GABA) synaptic inhibition. *Gad2* knockout mice showed sensitized pain behavior and impaired GABA synaptic function in their brainstem neurons. In wild-type but not *Gad2* knockout mice, HDAC inhibitors strongly increased GAD65 activity, restored GABA synaptic function and relieved sensitized pain behavior. These findings suggest GAD65 and HDACs as potential therapeutic targets in an epigenetic approach to the treatment of chronic pain.

Environmental factors and pathological conditions can result in the alteration of transcription of many genes through modifications of chromatin structure, including DNA methylation and histone acetylation, resulting in stable phenotypes[1,2]. Chromatin remodeling dynamically modulates, either positively or negatively, the transcriptional activity of target genes[3]. Histone acetylation increases gene transcription by decondensing chromatin structure, which allows increased accessibility of transcriptional machinery to DNA for transcriptional activation[4]. Epigenetic mechanisms are implicated in adaptive responses to many neurological disorders in which persistent neurochemical stimuli are present[5,6]. For example, histone acetylation crucially regulates synaptic plasticity and memory formation[7], and drugs of abuse alter chromatin structure through histone acetylation and phosphorylation, leading to maladaptive changes that cause drug addiction[8–10].

Chronic pain is a neurological disease caused by nerve injury and persistent tissue inflammation under various pathological conditions such as cancer and neurodegenerative diseases[11]. Distinct from acute pain, chronic pain could induce long-term synaptic and cellular maladaptive changes, involve dynamic memory processes and cause characteristic emotional disorders including depression, stress and anxiety[11–14]. The molecular mechanisms underlying chronic pain development remain poorly understood. The characteristics of chronic pain are strongly suggestive of epigenetic modulations. Evidence is emerging in animal pain models that show antinociceptive effects of HDAC inhibitors[15,16] and epigenetic regulation of C-fiber dysfunction in hypoesthesia[17]. However, how epigenetic mechanisms operate and what the target genes are in chronic pain development are largely unknown. In this study, we explored persistent pain-induced histone modifications in mouse and rat models of inflammatory and neuropathic pain. Whereas spinal adaptive mechanisms are important in chronic pain, our study focused on the brainstem nucleus raphe magnus (NRM), a crucial supraspinal site for maintenance of pain hypersensitivity in behavioral states of chronic pain[18,19].

RESULTS

Inflammatory pain increases global histone acetylation

We first examined global histone acetylation levels in rats with persistent inflammatory pain induced by complete Freund's adjuvant (CFA)[20]. CFA induced persistent pain sensitization (hyperalgesia) (**Fig. 1a**). Sampling NRM tissues at different time points (4 h, 12 h, 1 d, 3 d and 6 d after CFA injection), we found that global histone H3 acetylation was unchanged until 1 d after injection, from which point it showed a continued increase for the following 6 d (**Fig. 1b**,**c**). Total H3 protein levels were unchanged during this period. In tissues taken at 3 d after injection (representing persistent pain), both histone H3 and H4 acetylation levels were increased but the total H4 protein level was not (**Fig. 1d**–**f**). We obtained similar results by ELISA for H3 acetylation at 3 d after injection (171.4% ± 34.1% increase (mean ± s.e.m.), $n = 7$, $P < 0.05$). These results suggest that persistent pain (lasting for >1 d), but not acute pain (lasting for hours), involves global histone hyperacetylation in the NRM.

Persistent pain decreases GABAergic synaptic function

Chronic pain is presumably caused partly by sustained activation of descending pain-facilitatory pathways from NRM[18]. This neuronal hyperactivation could result from loss of inhibitory GABA functions in the NRM. In NRM neurons from CFA-injected rats, we found that the slope of the input-output curve for GABAergic inhibitory postsynaptic currents (IPSCs) was similar to that in controls at 4 h after

Department of Anesthesiology and Pain Medicine, University of Texas MD Anderson Cancer Center, Houston, Texas, USA. Correspondence should be addressed to Z.Z.P. (zzpan@mdanderson.org).

Received 9 May; accepted 12 July; published online 9 October 2011; doi:10.1038/nm.2442

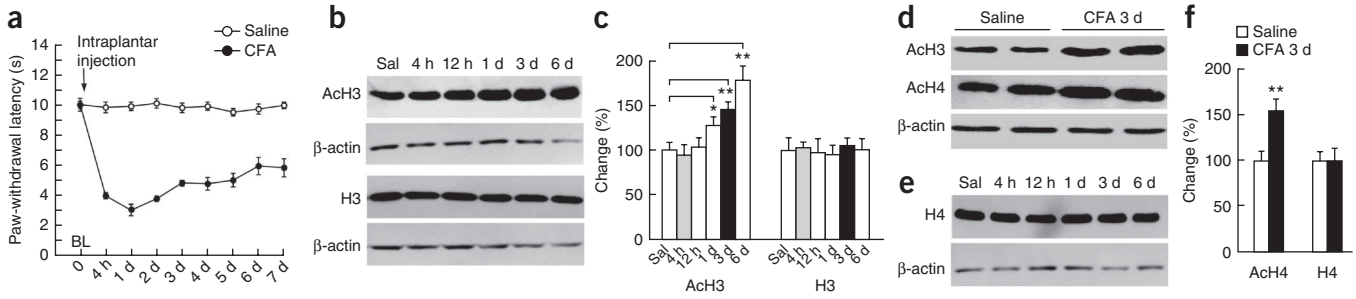

Figure 1 Persistent inflammatory pain induces time-dependent hyperacetylation of histones H3 and H4. (**a**) Time course for the development of persistent pain sensitization induced by CFA and for saline controls, as measured by the paw-withdrawal test ($n = 6$ rats in each group). (**b**,**c**) Western blots (**b**) and summarized data (**c**) ($n = 5$–9 rats for each group) of global acetylated histone H3 (AcH3) and total H3 proteins, normalized to β-actin, in tissues of rat NRM taken at various time points after CFA injection. (**d**) Western blots of AcH3 and acetylated histone H4 (AcH4) 3 d after CFA injection. (**e**,**f**) Western blots (**e**) and summarized results (**f**) ($n = 7$ rats for each group) of AcH4 and total H4 after CFA injection. Data are expressed as mean ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$. BL, baseline; sal, saline.

injection (acute pain) but decreased at 3 d after injection (persistent pain) (**Fig. 2a**,**b**). We saw no difference in the IPSC slopes from hippocampal neurons (**Fig. 2c**).

For the synaptic site of this decrease, we found that the paired-pulse ratio, which is inversely related to presynaptic neurotransmitter release[21,22], was unchanged at 4 h but increased at 3 d after injection (**Fig. 2d**–**f**), and miniature IPSC (mIPSC) frequency, but not amplitude, was reduced at 3 d but not at 4 h after injection (**Fig. 2g**–**j**), indicating decreased presynaptic GABA release. Thus, persistent pain decreases presynaptic function of GABA synapses in NRM neurons.

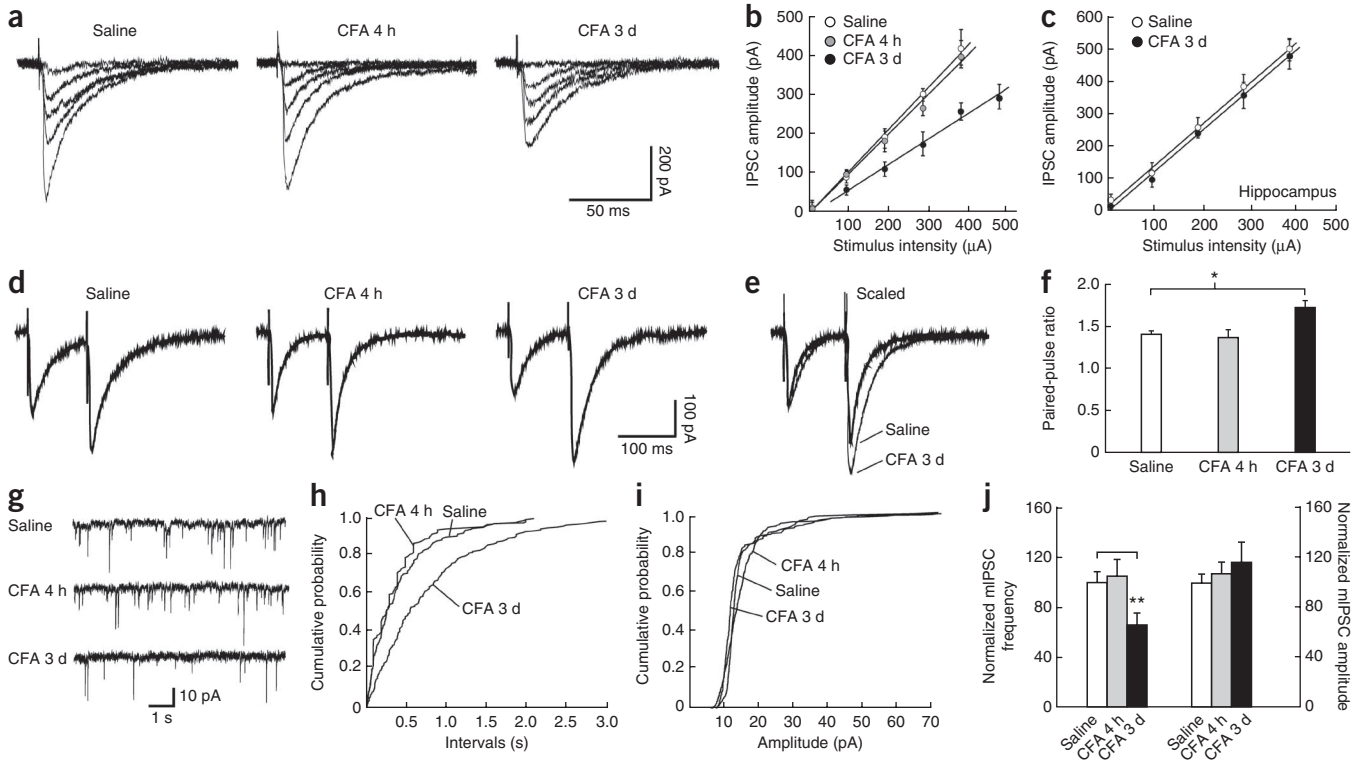

Figure 2 Persistent pain decreases GABAergic synaptic function by inhibiting presynaptic GABA release. (**a**) Representative traces of GABA IPSCs evoked by various stimulation intensities in NRM neurons from a saline-injected rat and a CFA-injected rat at 4 h and 3 d after injection. (**b**) A plot of input-output curves for IPSC amplitudes in neurons from the three treatment groups in **a**. The slopes were as follows: saline, 90.4 ± 11.5 pA 0.1 mA^{-1}, $n = 13$; CFA after 4 h, 88.7 ± 12.9 pA 0.1 mA^{-1}, $n = 26$, $P > 0.05$; and CFA after 3 d, 53.6 ± 10.5 pA 0.1 mA^{-1}, $n = 35$, $P < 0.01$. (**c**) A similar input-output plot of IPSCs from hippocampal CA1 neurons. The slopes were as follows: saline, 98.1 ± 14.8 pA 0.1 mA^{-1}, $n = 15$; and CFA after 3 d, 102.7 ± 16.2 pA 0.1 mA^{-1}, $n = 16$, $P > 0.05$. (**d**) Representative IPSC pairs evoked by two stimuli (100 ms apart) in NRM neurons from the three groups of rats. (**e**) Two representative IPSC pairs from the two indicated groups are superimposed and scaled to the amplitude of the first IPSC. (**f**) Group data of changes in the paired-pulse ratios in the three groups ($n = 15$–35 cells for each group). (**g**) Representative traces of spontaneous miniature IPSCs (mIPSCs) in neurons from the three groups. (**h**–**j**) Distribution plots of mIPSC frequency (**h**) and amplitude (**i**) from neurons of each group and their group data (**j**) ($n = 16$–20 cells). Data are expressed as mean ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$.

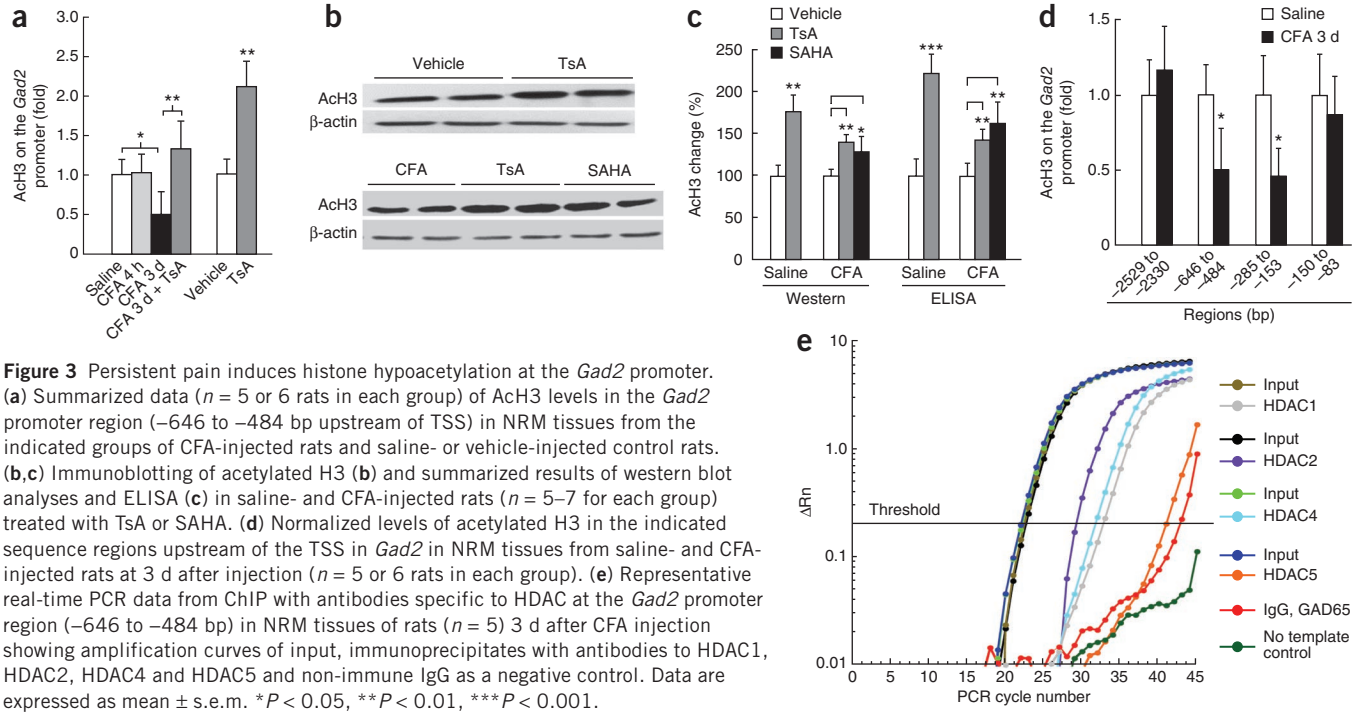

Figure 3 Persistent pain induces histone hypoacetylation at the *Gad2* promoter. (**a**) Summarized data ($n = 5$ or 6 rats in each group) of AcH3 levels in the *Gad2* promoter region (−646 to −484 bp upstream of TSS) in NRM tissues from the indicated groups of CFA-injected rats and saline- or vehicle-injected control rats. (**b**,**c**) Immunoblotting of acetylated H3 (**b**) and summarized results of western blot analyses and ELISA (**c**) in saline- and CFA-injected rats ($n = 5$–7 for each group) treated with TsA or SAHA. (**d**) Normalized levels of acetylated H3 in the indicated sequence regions upstream of the TSS in *Gad2* in NRM tissues from saline- and CFA-injected rats at 3 d after injection ($n = 5$ or 6 rats in each group). (**e**) Representative real-time PCR data from ChIP with antibodies specific to HDAC at the *Gad2* promoter region (−646 to −484 bp) in NRM tissues of rats ($n = 5$) 3 d after CFA injection showing amplification curves of input, immunoprecipitates with antibodies to HDAC1, HDAC2, HDAC4 and HDAC5 and non-immune IgG as a negative control. Data are expressed as mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Persistent pain epigenetically reduces GAD65 expression

GAD65 is a GABA synthetic enzyme that preferentially synthesizes GABA in the synaptic terminal for vesicle release, whereas GAD67 preferentially synthesizes cytoplasmic GABA[23,24]. We used chromatin immunoprecipitation (ChIP) assays to determine H3 acetylation levels in *Gad2* promoter regions under pain conditions. We found that H3 acetylation in the region of −646 to −484 bp upstream of the transcription start site (TSS) of *Gad2* was reduced in rats at 3 d after CFA injection but not at 4 h afterward (**Fig. 3a**). Systemic treatment with the HDAC inhibitors trichostatin A (TsA) or suberoylanilide hydroxamic acid (SAHA), which increase histone acetylation nonselectively[25], increased the pain-reduced H3 acetylation level in the *Gad2* promoter region and increased global H3 acetylation in the NRM of saline- and CFA-injected rats (**Fig. 3a**–**c**). At 3 d after CFA injection, acetylated H3 was also reduced in the *Gad2* promoter region at −285 to −153 bp upstream of the TSS but not in regions <150 bp or >2 kb upstream (**Fig. 3d**).

We determined the relative levels of HDAC1 and HDAC2 (class I HDACs) and HDAC4 and HDAC5 (activity-dependent class II HDACs)[26,27] present on the *Gad2* promoter in NRM chromatin preparations from rats at 3 d after CFA injection and found chromatin-associated HDAC1, HDAC2 and HDAC4 marks, but not HDAC5 marks, in the *Gad2* promoter (**Fig. 3e**). Additionally, the GAD65 mRNA level was decreased, and so was the GAD65 protein level (**Fig. 4a**–**c**). We saw no change in GAD65 expression 4 h after CFA injection. These reductions in GAD65 transcriptional and translational activities were completely reversed by treatment with TsA (**Fig. 4a**–**c**). TsA also increased GAD65 protein abundance in control rats to a lesser extent (**Fig. 4d**), suggesting a global TsA effect. Persistent pain decreased co-localization of GAD65 and terminal protein synapsin I by 46% ± 10% (mean ± s.e.m.), and the co-localization was increased approximately twofold by TsA in NRM neurons (**Fig. 4e**). These results indicate that CFA-induced persistent hyperalgesia epigenetically suppresses *Gad2* transcription in the NRM and that HDAC-inhibitor–induced global histone hyperacetylation can overwhelm this pain effect, increasing acetylation at the *Gad2* promoter and increasing its transcription.

Next, we examined *Gad2* transcription in rats with spinal nerve ligation (SNL), another rodent model of chronic neuropathic pain that lasts for months[28]. We collected NRM tissues from rats with SNL and sham-operated control rats at 1 d (representing acute pain) and 21 d (representing prolonged pain) after surgery (**Fig. 4f**). We found that the acetylated H3 level in the *Gad2* promoter showed no change at 1 d but was reduced at 21 d after surgery (**Fig. 4g**). Moreover, both GAD65 mRNA and protein levels were decreased at 21 d but not at 1 d after surgery (**Fig. 4g**,**h**). Thus, both prolonged sensitization of neuropathic pain and inflammatory hyperalgesia epigenetically reduce *Gad2* transcription.

Histone hyperacetylation increases GABA synaptic function

As expected from the above results of pain-reduced GABA synaptic function by epigenetic hypoacetylation at *Gad2*, treatment with TsA or SAHA augmented GABA neurotransmission, increasing the mIPSC frequency in neurons from CFA- and saline-injected rats (**Fig. 5a**). This suggests that GAD65 expression is important for GABA neurotransmission and that its epigenetic repression by persistent pain decreases (and its pharmacological augmentation by HDAC inhibitors enhances) GABA synaptic transmission.

Considering the nonselective, acetylation-promoting effects of HDAC inhibitors, we analyzed GABA IPSCs in the NRMs of *Gad2* knockout mice. We found the slope of the IPSC input-output curve to be reduced in neurons from *Gad2*$^{-/-}$ mice when compared with wild-type mice (**Fig. 5b**). This indicates *Gad2*-deletion–induced reduction in GABA synaptic function, consistent with the effects of pain (**Fig. 2b**). Supporting a presynaptic function of GAD65, mIPSC frequency, but not amplitude, was lower in neurons from *Gad2*$^{-/-}$ mice than in those from wild-type mice (**Fig. 5c**,**d**), indicating impaired presynaptic GABA release, also consistent with the effects of pain (**Fig. 2g**–**j**). Furthermore, in *Gad2*$^{-/-}$ mice, both TsA and SAHA failed

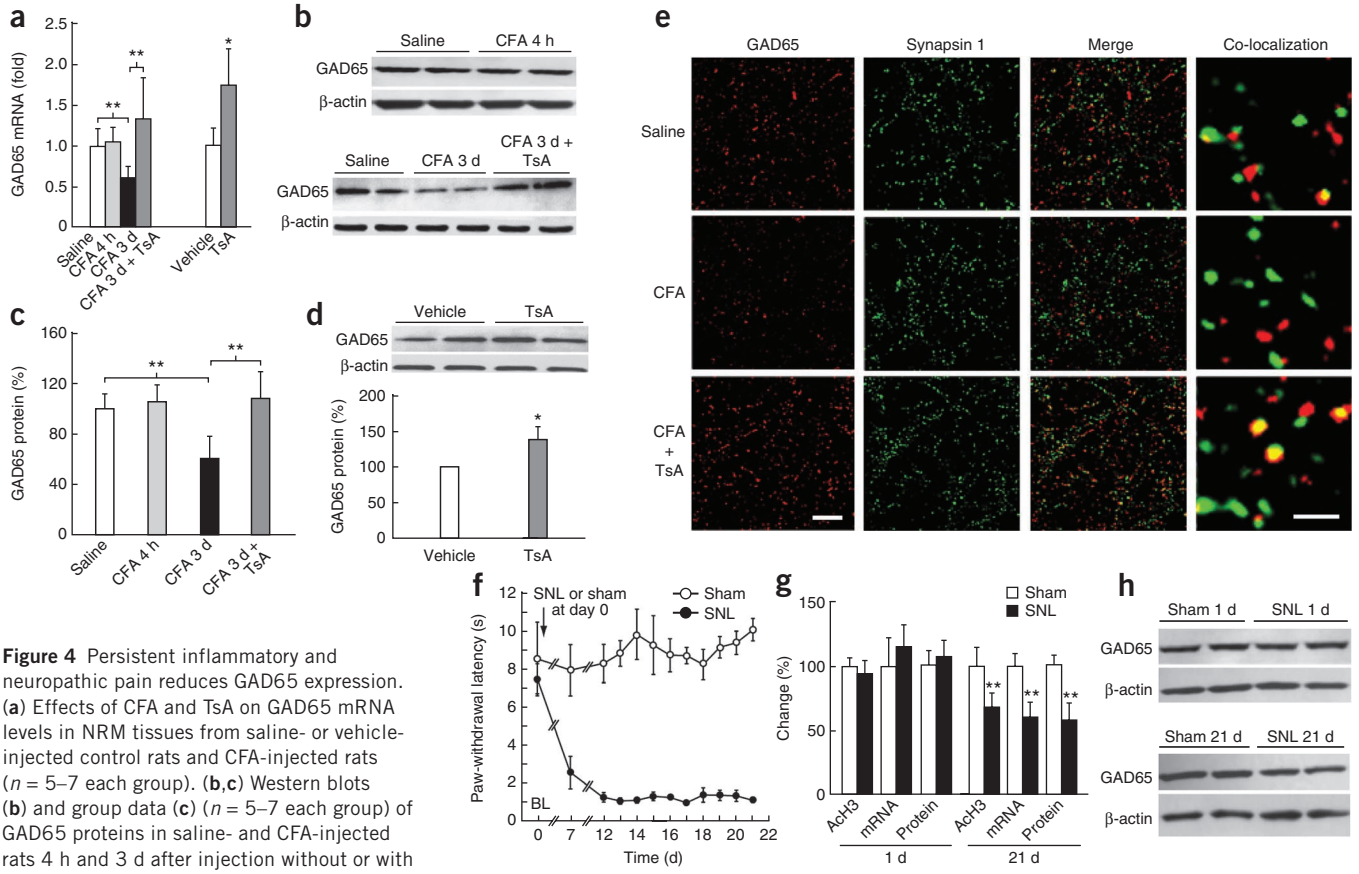

Figure 4 Persistent inflammatory and neuropathic pain reduces GAD65 expression. (**a**) Effects of CFA and TsA on GAD65 mRNA levels in NRM tissues from saline- or vehicle-injected control rats and CFA-injected rats (n = 5–7 each group). (**b**,**c**) Western blots (**b**) and group data (**c**) (n = 5–7 each group) of GAD65 proteins in saline- and CFA-injected rats 4 h and 3 d after injection without or with the TsA treatment *in vivo*. (**d**) Western blots of GAD65 proteins in NRM tissues from normal rats. (**e**) Micrographs of immunohistochemical staining for GAD65 (red), the synaptic terminal protein synapsin I (green), and co-localization of GAD65 and synapsin I (yellow) in saline- (top row) and CFA-injected (bottom two rows) rats without or with the TsA treatment (n = 5 or 6 rats in each group, and we analyzed and compared 4–6 randomly selected sections from each rat). Scale bars, 50 μm (left three columns) or 2 μm (right column). (**f**) Time course of changes in pain threshold in rats after spinal nerve ligation (SNL) (n = 6) or sham surgery (n = 5) performed on day 0 (arrow). (**g**) Normalized changes in levels of acetylated H3 at the *Gad2* promoter (–646 to –484 bp), GAD65 mRNA and protein in NRM tissues from sham-operated and SNL rats at 1 d and 21 d after surgery (n = 5 or 6 rats in each group). (**h**) Representative western blots of GAD65 and β-actin proteins from a sham-operated rat and an SNL rat at 1 d and at 21 d. BL, baseline. Data are expressed as mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$.

to increase mIPSC frequency (**Fig. 5c**,**d**), and the mIPSC amplitude was unaffected. Thus, it seems that HDAC inhibitors enhance GABA neurotransmission (**Fig. 5a**) by promoting histone acetylation at *Gad2*, as they lost this effect in *Gad2*$^{-/-}$ mice.

In examining potential GAD67 roles in pain-reduced presynaptic GABA function despite its predominant cytoplasmic localization, we found that the acetylated H3 level in the three *Gad1* (encoding GAD67) sequence regions we looked at was unchanged at 3 d after CFA injection (**Fig. 5e** and **Supplementary Fig. 1**). The GAD67 mRNA level was also unchanged. However, CFA produced a small but significant decrease in GAD67 protein expression (**Fig. 5e**,**f**). TsA increased GAD67 protein levels in CFA- and saline-injected rats at 3 d after injection (**Supplementary Fig. 2**). Thus, it seems unlikely that CFA has substantial effects on *Gad1* transcription through histone acetylation, but cytosolic GAD67 may have some role in CFA-induced pain sensitization.

Histone hyperacetylation relieves pain

We examined the neuronal excitability of two previously characterized classes of NRM neurons: μ-opioid receptor (MOR)-lacking and MOR-expressing cells[29]. The latter presumably comprises the descending pain-facilitatory system[19,30]. We found that MOR-expressing cells, identified by hyperpolarization with the MOR agonist [D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin (DAMGO) (1 μM) (**Fig. 5g**), had a larger number of depolarization-evoked action potentials in CFA-injected rats 3 d after injection than in control rats (**Fig. 5g**). We did not observe this difference in cells lacking MOR (**Fig. 5h** and **Supplementary Fig. 3**). Thus, the increased excitability of MOR-expressing NRM cells may underlie the cellular mechanism for CFA-induced activation of descending pain facilitation.

To determine whether GAD65-promoting HDAC inhibitors could alleviate pain, we conducted behavioral experiments *in vivo*. TsA repeatedly (once a day for 4 d) infused into the NRM attenuated CFA-induced hyperalgesia in a dose-dependent manner, as did SAHA (**Fig. 6a**–**c**). A single TsA infusion was ineffective (data not shown). Repeated TsA pretreatment before CFA injection failed to alter the effect of CFA at 4 h after injection (**Fig. 6d**), which excludes an effect of TsA on the acute effect of CFA.

Histone-hyperacetylation–induced pain relief requires GAD65

We obtained additional evidence supporting the role of GAD65 in the pain mechanism from behavioral experiments on *Gad2*$^{-/-}$ mice. Compared to wild-type mice, *Gad2*$^{-/-}$ mice had a lower baseline pain threshold, indicating a sensitized basal pain state (basal hyperalgesia) (**Fig. 6e**), consistent with CFA-induced hyperalgesia through epigenetic

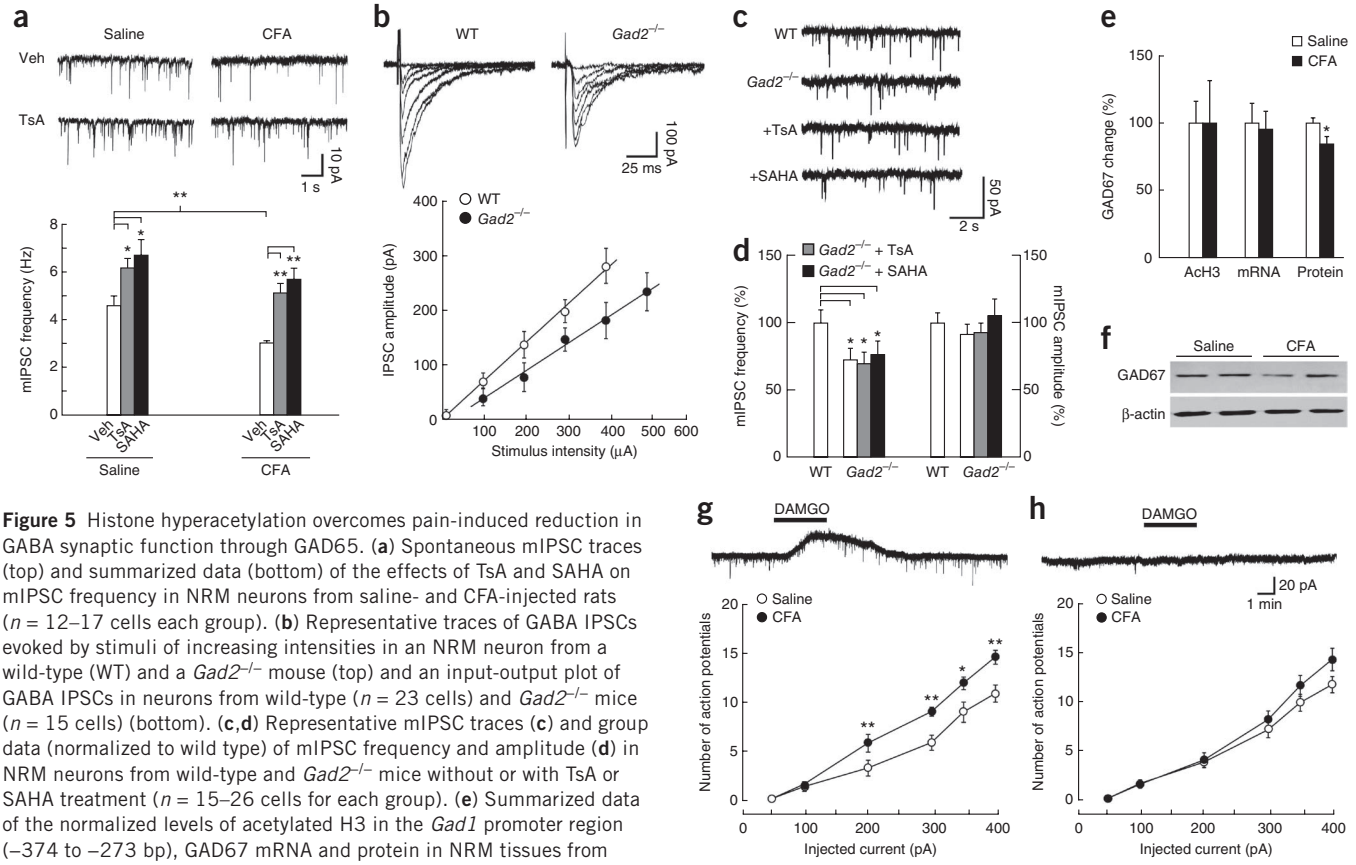

Figure 5 Histone hyperacetylation overcomes pain-induced reduction in GABA synaptic function through GAD65. (**a**) Spontaneous mIPSC traces (top) and summarized data (bottom) of the effects of TsA and SAHA on mIPSC frequency in NRM neurons from saline- and CFA-injected rats (n = 12–17 cells each group). (**b**) Representative traces of GABA IPSCs evoked by stimuli of increasing intensities in an NRM neuron from a wild-type (WT) and a *Gad2*$^{-/-}$ mouse (top) and an input-output plot of GABA IPSCs in neurons from wild-type (n = 23 cells) and *Gad2*$^{-/-}$ mice (n = 15 cells) (bottom). (**c**,**d**) Representative mIPSC traces (**c**) and group data (normalized to wild type) of mIPSC frequency and amplitude (**d**) in NRM neurons from wild-type and *Gad2*$^{-/-}$ mice without or with TsA or SAHA treatment (n = 15–26 cells for each group). (**e**) Summarized data of the normalized levels of acetylated H3 in the *Gad1* promoter region (–374 to –273 bp), GAD67 mRNA and protein in NRM tissues from saline- and CFA-injected rats at 3 d after injection (n = 4–6 rats for each group). (**f**) Representative western blots of GAD67 protein from a saline- or CFA-injected rat. (**g**,**h**) Representative membrane current traces with response to the MOR agonist DAMGO (1 μM) (top) and graphs of evoked action potential firing (bottom) in MOR-expressing NRM neurons (**g**) and in NRM neurons lacking MOR (**h**) from saline- and CFA-injected rats. Data are expressed as mean ± s.e.m. *P < 0.05, **P < 0.01. Veh, vehicle.

inhibition of GAD65 expression. Furthermore, in *Gad2*$^{-/-}$ mice, similar NRM infusions of TsA could no longer ameliorate the sensitized pain behavior (**Fig. 6e**), further supporting the role of GAD65 in histone-hyperacetylation–induced pain relief. To determine how *Gad2*$^{-/-}$ mice might respond to CFA differently, we treated them with CFA and found that, in addition to the basal hyperalgesia seen in *Gad2*$^{-/-}$ mice, CFA induced further pain sensitization to a level similar in amplitude to that in wild-type mice at 1 d after treatment (**Fig. 6f**). This indicates that this acute CFA effect is independent of GAD65 and may be mediated by as-yet unidentified mechanisms. Notably, at 3 d after injection, the amplitude of hyperalgesia remained unchanged in wild-type mice but was partially recovered in *Gad2*$^{-/-}$ mice (**Fig. 6f**). The underlying mechanisms of this effect remain to be investigated.

Next, we reasoned that, if GAD65-suppression–induced loss of GABA synaptic inhibition contributes to pain hypersensitivity, pharmacologically promoting GABA inhibition under those conditions should alleviate hyperalgesia. As predicted, in rats at 3 d after CFA injection, acute NRM infusion of the $GABA_A$ receptor agonist muscimol induced an antinociceptive effect (**Fig. 6g**). Therefore, like histone-hyperacetylation–mediated upregulation of GAD65 transcription and GABA function, pharmacological activation of inhibitory GABA function also can relieve pain.

Depression and proinflammatory cytokines

Chronic pain is often associated with psychophysiological disorders such as depression[31]. To determine the potential effects of depression, we treated SNL rats and sham-operated rats with the antidepressant drug fluoxetine for 21 d, a treatment that reverses depressive behavior in rodents[32]. We found that fluoxetine had no effect on the SNL-reduced expression of NRM GAD65 protein (**Supplementary Fig. 4**), thus probably excluding a general effect of depression on GAD65 expression in the NRM.

CFA is known to release pain-facilitating proinflammatory cytokines, including interleukin 1 (IL-1)[33]. We examined the effect of IL-1β on NRM GAD65 expression and pain threshold. IL-1β infused into the NRM decreased the pain threshold in naive rats acutely for about 4 h (**Fig. 6h**). Co-infusion of IL-1β and the IL-1 receptor antagonist IL-1Ra largely blocked the IL-1β effect. However, unlike CFA-induced hyperalgesia, repeated NRM infusions of IL-1β did not produce lasting hyperalgesia over 3 d, although its acute effect remained (**Fig. 6h**). In NRM tissues from rats treated repeatedly with IL-1β, we found no change in GAD65 protein (**Fig. 6i**,**j**). Furthermore, repeated NRM infusions of IL-1Ra failed to block the CFA-induced reduction in GAD65 (**Fig. 6i**,**j**). These results indicate that, although proinflammatory cytokines are important in chronic pain development[33,34], NRM IL-1 is unlikely to have a major role in CFA-induced modulation of GAD65 expression and related pain mechanisms.

DISCUSSION

In rat and mouse models of chronic pain, we have shown that persistent pain, but not acute pain, epigenetically suppresses the transcription of *Gad2* and consequently causes impaired inhibitory function

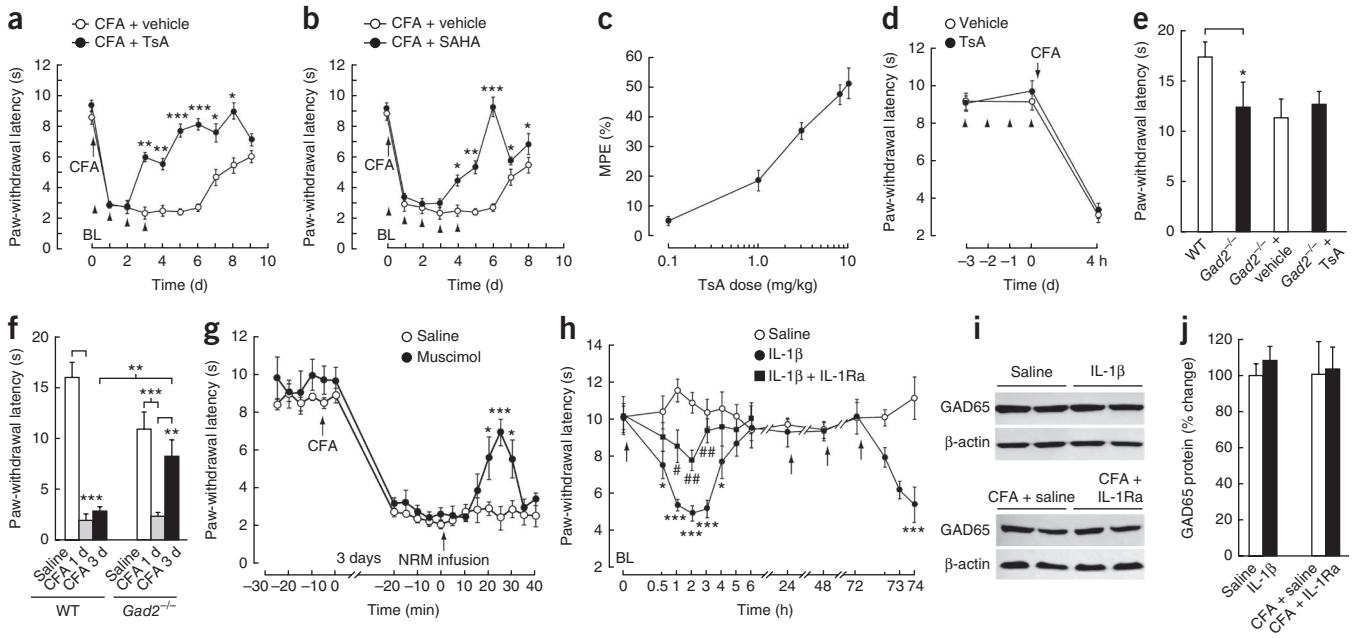

Figure 6 HDAC inhibitors relieve pain through GAD65. (**a**,**b**) Changes in pain threshold in CFA-injected rats with repeated NRM infusions (arrowheads) of vehicle ($n = 5$) or TsA ($n = 8$) (**a**) and vehicle ($n = 5$) or SAHA ($n = 6$) (**b**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the vehicle group. (**c**) Effects of TsA on pain threshold at the indicated doses ($n = 5$ or 6 rats for each data point). (**d**) Effect of TsA pretreatment by NRM infusions (arrowheads) on CFA-induced acute hyperalgesia. (**e**) Averaged baseline pain threshold in wild-type (WT) ($n = 10$) and *Gad2*$^{-/-}$ mice ($n = 7$) and in *Gad2*$^{-/-}$ mice with NRM infusions of vehicle ($n = 7$) or TsA ($n = 7$). $^{*}P < 0.05$. (**f**) Pain threshold changes in saline- and CFA-injected wild-type and *Gad2*$^{-/-}$ mice. $^{**}P < 0.01$, $^{***}P < 0.001$, comparisons of two groups indicated by the bars. (**g**) Effects of single NRM infusion of saline or the $GABA_A$ receptor agonist muscimol (50 ng) on CFA-induced hyperalgesia ($n = 5$ rats in each group). $^{*}P < 0.05$, $^{***}P < 0.001$. (**h**) Pain threshold changes following repeated NRM infusions of saline ($n = 5$) or the proinflammatory cytokine IL-1β (3 μg) ($n = 5$) (arrows) and following single NRM infusion of IL-1β plus IL-1 receptor antagonist (IL-1Ra, 17 μg) ($n = 6$). $^{*}P < 0.05$, $^{***}P < 0.001$ compared to the saline group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared to the IL-1β + IL-1Ra group. (**i**,**j**) Western blots (**i**) and summarized data (**j**) of GAD65 protein normalized to β-actin in NRM tissues from saline-injected rats after repeated NRM infusions of saline or IL-1β and from CFA-injected rats treated with similar 4-d NRM infusions of saline or IL-1Ra (17 μg). BL, baseline; MPE, maximum possible effect. Error bars are s.e.m.

of GABAergic synapses in central pain-modulating neurons, contributing to the development of persistent pain sensitization. These results are supported by observations in *Gad2*$^{-/-}$ mice showing impaired GABA synaptic function in the same neurons and having sensitized pain behavior. In addition, histone hyperacetylation overcomes these molecular and synaptic changes by promoting *Gad2* transcription and GABAergic synaptic function, thereby relieving the sensitized behavior of persistent pain.

Chronic pain involves altered expression of many genes through unknown mechanisms[35]. In drug addiction, histone H3 and H4 acetylation modulates the expression of several genes that regulate transcription, including *Cdk5*, *Fos*, *Creb* and *ΔFosB*[8]. In nerve-injury–induced hypoesthesia, C-fiber dysfunction is reportedly mediated by epigenetic upregulation of the transcriptional repressor neuron-restrictive silencer factor, but pain sensitization does not seem to involve upregulation of this factor[17]. Notably, HDAC inhibitors reduce inflammatory pain by upregulating spinal metabotropic glutamate 2 receptors[16]. The present study identifies *Gad2* as an important target gene of histone modifications induced by persistent pain conditions, providing a potential epigenetic mechanism for the development of chronic pain.

GAD65 is preferentially targeted to presynaptic terminals of central neurons for synthesis of GABA within synaptic vesicles and is required for active GABA synaptic release[23,24,36]. Impaired GABA release would cause loss of GABAergic inhibition, leading to neuronal hyperactivation. For instance, nerve injury induces a loss of GABA inhibition in spinal neurons, and enhancing GABA synaptic inhibition is effective in relieving injury-induced pain[37–39]. Our results from rats and GAD65-deficient mice suggest that the persistent pain induced by inflammation and neuropathy downregulates GAD65 transcription, causing impairment of GABA synaptic inhibition in the NRM and increasing the excitability of presumably pain-facilitating neurons. This is in line with recent reports that *Gad2*$^{-/-}$ mice show sensitized pain behavior[40] and that viral delivery of *Gad2* produces orofacial analgesia[41]. Given the multifaceted mechanisms of chronic pain, it is likely that other genes in addition to *Gad2* also are targets of chronic pain-induced chromatin remodeling. This probably accounts for our observation of increased global histone acetylation by CFA, indicating increased activities of other genes that remain to be investigated. Although the pain-induced changes in GAD65 transcription and GABA synaptic function indicate a likely neuronal locus, further studies are necessary to verify its localization within the nucleus of central neurons for pain-induced histone modification.

GAD67 is a GABA-synthesizing enzyme for cytoplasmic GABA and its tonic release from neurons[23]. Our data show a major role of presynaptic GAD65 in the pain mechanism, but this does not preclude a role for cellular GAD67, particularly in pain-induced adaptive changes in neuronal excitability for sensitized pain behaviors. Although our results do not indicate pain-induced epigenetic modulation of *Gad1* through histone acetylation, GAD67 could participate in pain mechanisms by decreasing tonic inhibition among neurons through reducing the levels of cellular GABA, by reducing synaptic GABA through presynaptic effects or by compensatory changes in response to GAD65 deficiency. The detailed mechanisms by which GAD67 might play a role in pain warrants further study.

How functionally distinct populations of NRM neurons adapt to chronic pain conditions and mediate sensitized pain behaviors in chronic pain remains unclear. Under normal conditions, opioids produce analgesia partly by reducing basal GABA transmission in NRM neurons, thereby activating the descending pain-inhibition system[29,42]. Consistent with this, $GABA_A$ receptor antagonists applied to the NRM induce antinociception, whereas $GABA_A$ receptor agonists produce pain sensitization[43,44]. However, under chronic pain conditions, considerable adaptive changes may have occurred both in GABA input activities on different classes of NRM neurons and in $GABA_A$ receptor properties. Our results indicate that the pain-induced impairment of GABA synaptic inputs may preferentially affect and consequently hyperactivate MOR-expressing neurons. Activation of this neuron class presumably facilitates spinal pain transmission[29,30], contributing to sensitized pain behaviors. A pain-induced decrease in GABA neurotransmission has also been reported recently in amygdala neurons from a rat model of arthritic pain[45]. This GABA-impairment–induced pain sensitization is further supported by our behavioral results that enhancing GABA inhibition by activating NRM $GABA_A$ receptors produces an antinociceptive effect. Detailed molecular and cellular adaptations in GABA and glutamate synapses under chronic pain conditions are subjects of ongoing research.

Proinflammatory cytokines, released into the peripheral and central circulation by immune cells and glia in response to tissue inflammation and trauma, cause augmented pain[33,34], but the underlying cellular and molecular mechanisms of this process remain unclear, particularly in chronic pain states. Proinflammatory cytokines may contribute to the development of chronic pain by sustained release from their sources and by their sensitized signaling mechanisms in nociceptors and central neurons to augment pain responses after healing. Our observations of both the relatively acute hyperalgesic effect of IL-1β and the inability of repeated IL-1β administration in the NRM to change GAD65 expression indicate that this proinflammatory cytokine, at least in the NRM, is not substantially involved in the GAD65-mediated pain mechanisms that mediate prolonged pain behaviors induced by SNL and CFA.

A current common clinical problem is the transition from analgesic-responsive acute pain to chronic pain, of which some types are poorly responsive to currently available analgesics and often lead to long-term neuropsychiatric disorders such as depression, stress and drug addiction[10,12,46,47]. Although multiple forms of neuronal plasticity have been identified for the pathogenesis of chronic pain[13], the mechanisms underlying this crucial transition from acute pain to chronic pain remain poorly understood. Our findings of chromatin modifications emerging only days after pain development indicate that the epigenetic mechanism of *Gad2* modulation might underlie the persistent phase of these pain conditions, and this, together with changes in the expression of other genes, could be an important initial step in the transition that leads to the development of chronic pain and associated disorders. In this regard, drugs such as HDAC inhibitors that overcome the effects of persistent pain on the output activities of *Gad2* and other target genes may serve as a new promising class of analgesics[48], as they could collectively block the upstream cause of pain-induced alterations that lead to multiple system malfunctions and clinical symptoms during chronic pain development.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

This work was supported by US National Institutes of Health National Institute on Drug Abuse grants DA023069 and DA027541. We thank L. Gao of Baylor College of Medicine for his generous gifts of GAD67 primers.

AUTHOR CONTRIBUTIONS

Z.Z. designed the studies, performed most of the experiments and wrote a draft manuscript. Y.-Q.C., F.Z. and B.B. conducted some of the molecular and behavioral experiments. Z.Z.P. was involved in the overall designs of the project and individual experiments, the data analyses and the writing of the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/naturemedicine/.
Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Kouzarides, T. Chromatin modifications and their function. *Cell* **128**, 693–705 (2007).
2. MacDonald, J.L. & Roskams, A.J. Epigenetic regulation of nervous system development by DNA methylation and histone deacetylation. *Prog. Neurobiol.* **88**, 170–183 (2009).
3. Jenuwein, T. & Allis, C.D. Translating the histone code. *Science* **293**, 1074–1080 (2001).
4. Strahl, B.D. & Allis, C.D. The language of covalent histone modifications. *Nature* **403**, 41–45 (2000).
5. Urdinguio, R.G., Sanchez-Mut, J.V. & Esteller, M. Epigenetic mechanisms in neurological diseases: genes, syndromes, and therapies. *Lancet Neurol.* **8**, 1056–1072 (2009).
6. Chuang, D.M., Leng, Y., Marinova, Z., Kim, H.J. & Chiu, C.T. Multiple roles of HDAC inhibition in neurodegenerative conditions. *Trends Neurosci.* **32**, 591–601 (2009).
7. Guan, J.S. *et al.* HDAC2 negatively regulates memory formation and synaptic plasticity. *Nature* **459**, 55–60 (2009).
8. Tsankova, N., Renthal, W., Kumar, A. & Nestler, E.J. Epigenetic regulation in psychiatric disorders. *Nat. Rev. Neurosci.* **8**, 355–367 (2007).
9. Grayson, D.R., Kundakovic, M. & Sharma, R.P. Is there a future for histone deacetylase inhibitors in the pharmacotherapy of psychiatric disorders? *Mol. Pharmacol.* **77**, 126–135 (2010).
10. Reichling, D.B. & Levine, J.D. Critical role of nociceptor plasticity in chronic pain. *Trends Neurosci.* **32**, 611–618 (2009).
11. Campbell, J.N. & Meyer, R.A. Mechanisms of neuropathic pain. *Neuron* **52**, 77–92 (2006).
12. Scascighini, L. & Sprott, H. Chronic nonmalignant pain: a challenge for patients and clinicians. *Nat. Clin. Pract. Rheumatol.* **4**, 74–81 (2008).
13. Costigan, M., Scholz, J. & Woolf, C.J. Neuropathic pain: a maladaptive response of the nervous system to damage. *Annu. Rev. Neurosci.* **32**, 1–32 (2009).
14. Milligan, E.D. & Watkins, L.R. Pathological and protective roles of glia in chronic pain. *Nat. Rev. Neurosci.* **10**, 23–36 (2009).
15. Bai, G., Wei, D., Zou, S., Ren, K. & Dubner, R. Inhibition of class II histone deacetylases in the spinal cord attenuates inflammatory hyperalgesia. *Mol. Pain* **6**, 51 (2010).
16. Chiechio, S. *et al.* Epigenetic modulation of mGlu2 receptors by histone deacetylase inhibitors in the treatment of inflammatory pain. *Mol. Pharmacol.* **75**, 1014–1020 (2009).
17. Uchida, H., Ma, L. & Ueda, H. Epigenetic gene silencing underlies C-fiber dysfunctions in neuropathic pain. *J. Neurosci.* **30**, 4806–4814 (2010).
18. Porreca, F., Ossipov, M.H. & Gebhart, G.F. Chronic pain and medullary descending facilitation. *Trends Neurosci.* **25**, 319–325 (2002).
19. Fields, H. State-dependent opioid control of pain. *Nat. Rev. Neurosci.* **5**, 565–575 (2004).
20. Zhang, L. & Hammond, D.L. Cellular basis for opioid potentiation in the rostral ventromedial medulla of rats with persistent inflammatory nociception. *Pain* **149**, 107–116 (2010).
21. Zhang, Z. & Pan, Z.Z. Synaptic mechanism for functional synergism between delta- and mu-opioid receptors. *J. Neurosci.* **30**, 4735–4745 (2010).
22. Zucker, R.S. & Regehr, W.G. Short-term synaptic plasticity. *Annu. Rev. Physiol.* **64**, 355–405 (2002).
23. Soghomonian, J.J. & Martin, D.L. Two isoforms of glutamate decarboxylase: why? *Trends Pharmacol. Sci.* **19**, 500–505 (1998).
24. Tian, N. *et al.* The role of the synthetic enzyme GAD65 in the control of neuronal γ-aminobutyric acid release. *Proc. Natl. Acad. Sci. USA* **96**, 12911–12916 (1999).
25. Finnin, M.S. *et al.* Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature* **401**, 188–193 (1999).
26. Chawla, S., Vanhoutte, P., Arnold, F.J., Huang, C.L. & Bading, H. Neuronal activity-dependent nucleocytoplasmic shuttling of HDAC4 and HDAC5. *J. Neurochem.* **85**, 151–159 (2003).

27. Kurdistani, S.K. & Grunstein, M. Histone acetylation and deacetylation in yeast. *Nat. Rev. Mol. Cell Biol.* **4**, 276–284 (2003).
28. Kim, S.H. & Chung, J.M. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* **50**, 355–363 (1992).
29. Pan, Z.Z., Tershner, S.A. & Fields, H.L. Cellular mechanism for anti-analgesic action of agonists of the κ-opioid receptor. *Nature* **389**, 382–385 (1997).
30. Pan, Z.Z. Mu-opposing actions of the κ-opioid receptor. *Trends Pharmacol. Sci.* **19**, 94–98 (1998).
31. Blackburn-Munro, G. & Blackburn-Munro, R.E. Chronic pain, chronic stress and depression: coincidence or consequence? *J. Neuroendocrinol.* **13**, 1009–1023 (2001).
32. Duric, V. *et al.* A negative regulator of MAP kinase causes depressive behavior. *Nat. Med.* **16**, 1328–1332 (2010).
33. Watkins, L.R. & Maier, S.F. The pain of being sick: implications of immune-to-brain communication for understanding pain. *Annu. Rev. Psychol.* **51**, 29–57 (2000).
34. Watkins, L.R. & Maier, S.F. Beyond neurons: evidence that immune and glial cells contribute to pathological pain states. *Physiol. Rev.* **82**, 981–1011 (2002).
35. Lacroix-Fralish, M.L., Ledoux, J.B. & Mogil, J.S. The Pain Genes Database: an interactive web browser of pain-related transgenic knockout studies. *Pain* **131**, 3.e1–3.e4 (2007).
36. Patel, A.B., de Graaf, R.A., Martin, D.L., Battaglioli, G. & Behar, K.L. Evidence that GAD65 mediates increased GABA synthesis during intense neuronal activity *in vivo*. *J. Neurochem.* **97**, 385–396 (2006).
37. Moore, K.A. *et al.* Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. *J. Neurosci.* **22**, 6724–6731 (2002).
38. Munro, G., Ahring, P.K. & Mirza, N.R. Developing analgesics by enhancing spinal inhibition after injury: GABAA receptor subtypes as novel targets. *Trends Pharmacol. Sci.* **30**, 453–459 (2009).
39. Knabl, J. *et al.* Reversal of pathological pain through specific spinal GABAA receptor subtypes. *Nature* **451**, 330–334 (2008).
40. Kubo, K. *et al.* Thermal hyperalgesia via supraspinal mechanisms in mice lacking glutamate decarboxylase 65. *J. Pharmacol. Exp. Ther.* **331**, 162–169 (2009).
41. Vit, J.P. *et al.* Adenovector *GAD2* gene delivery into the rat trigeminal ganglion produces orofacial analgesia. *Mol. Pain* **5**, 42 (2009).
42. Pan, Z.Z., Williams, J.T. & Osborne, P.B. Opioid actions on single nucleus raphe magnus neurons from rat and guinea pig *in vitro*. *J. Physiol. (Lond.)* **427**, 519–532 (1990).
43. Gilbert, A.K. & Franklin, K.B. GABAergic modulation of descending inhibitory systems from the rostral ventromedial medulla (RVM). Dose-response analysis of nociception and neurological deficits. *Pain* **90**, 25–36 (2001).
44. Heinricher, M.M. & Kaplan, H.J. GABA-mediated inhibition in rostral ventromedial medulla: role in nociceptive modulation in the lightly anesthetized rat. *Pain* **47**, 105–113 (1991).
45. Ren, W. & Neugebauer, V. Pain-related increase of excitatory transmission and decrease of inhibitory transmission in the central nucleus of the amygdala are mediated by mGluR1. *Mol. Pain* **6**, 93 (2010).
46. Apkarian, A.V., Baliki, M.N. & Geha, P.Y. Towards a theory of chronic pain. *Prog. Neurobiol.* **87**, 81–97 (2009).
47. Woolf, C.J. & Hashmi, M. Use and abuse of opioid analgesics: potential methods to prevent and deter non-medical consumption of prescription opioids. *Curr. Opin. Investig. Drugs* **5**, 61–66 (2004).
48. Doehring, A., Geisslinger, G. & Lotsch, J. Epigenetics in pain and analgesia: an imminent research field. *Eur. J. Pain* **15**, 11–16 (2010).

ONLINE METHODS

Animals. Male Wistar rats, 9–14 d old or weighing 200–300 g, were used. *Gad2*$^{-/-}$ mice were obtained from Jackson Laboratories. All procedures involving the use of animals conformed to the guidelines by the University of Texas MD Anderson Cancer Center Animal Care and Use Committee.

Pain models. We injected CFA (40 µl) into a hindpaw of the rats to induce hyperalgesia of inflammatory pain[20]. We induced mechanical allodynia of neuropathic pain by ligating the left L5 and L6 spinal nerves as previously described[28]. We conducted pain tests as previously described[21]. We injected TsA (4 mg per kg body weight, in alcohol) or SAHA (40 mg per kg body weight, in DMSO) intraperitoneally once daily for 4 d. We harvested NRM tissues 4 h after the last injection.

Histone proteins extraction and western blotting. We modified the protocol for histone protein extraction from a previous report[49]. After tissue homogenization and lysate centrifugation, we separated the acid supernatant and nuclear pellet and added acetone to the pellet. We mixed thirty micrograms of protein with sodium dodecyl sulfate sample buffer. We transferred samples to a nitrocellulose membrane and incubated in solutions containing antibodies specific for histone or acetylated histone (1 in 1,000; Histone H3 (cat. 9715), histone H4 (cat. 2592), acetyl-histone H3 (Lys9/Lys14) (cat. 9677), acetyl-histone H4 (Lys12) (cat. 2591), Cell Signaling Technology) and β-actin (1 in 1,000; β-actin (ACTBD11B7), cat. sc-81178, Santa Cruz Biotechnology). We performed global histone H3 acetylation assays according to the directions of the EpiQuik Global Histone H3 Acetylation Assay Kit (Epigentek Group). We performed western blotting as previously described[21].

ChIP assays. We modified ChIP assays from the protocol of the EpiQuik Tissue Acetyl-Histone H3 ChIP Kit (Epigentek Group Inc). NRM tissues were collected, crosslinked and frozen. After tissue homogenization and centrifugation, we sheared the extracted chromatin by sonication into 200–500 bp fragments. After dilution, we transferred chromatin samples and 'input' DNA to each well for protein and DNA immunoprecipitation. We added DNA release buffer containing proteinase K, followed by the addition of reverse buffer to dissociate the DNA and histones. After reversing DNA crosslinks, we used binding buffer for DNA precipitation and purification. We used elution buffer to elute purified DNA from the columns. We used antibodies to acetyl histone H3 targeting Lys9 and Lys14, acetyl histone H4 targeting Lys12 (Cell Signaling Technology), HDAC1, HDAC2, HDAC4 and HDAC5 (HDAC1 (cat. 2062), HDAC2 (cat. 2540), HDAC4 (cat. 2072), HDAC5 (cat. 2082), Cell Signaling Technology).

Quantification of DNA. We performed quantitative real-time PCR (Applied Biosystems) as previously described[50]. On the basis of the consensus sequence of cAMP-response element (CRE) for potential binding sites of the transcription factor CREB in the *Gad2* and *Gad1* promoter regions, we designed primers to amplify representative promoter regions encompassing the CRE sequence from immediately upstream to >2 kb upstream of the TSS. We calculated fold differences by the ΔC_t method. We used the following primers (Invitrogen): *Gad2*, 5′-GCCCTGACTCGAACACTCAC-3′ and 5′-ACACAGGGACAGGAAACGTG-3′ (−150 to −83 bp); 5′-CTTCCTCC CTCTTTGGTTCCTT-3′ and 5′-ACCAGGGAGACCTTGACAATCT-3′ (−285 to −153 bp); 5′-ATAAGCAGCAGCCAAGGTCAC-3′ and 5′-CGCTGGAGT CTATCACTGAGGA-3′ (−646 to −484 bp); and 5′-TCTGCTGCCTCCTTTG TGAA-3′ and 5′-CTCCCCACTTCGGATACAGG-3′ (−2,529 to −2,330 bp). *Gad1*, 5′-TTGCGCCTCTAGACTTGAGAGT-3′ and 5′-TCTCGGAGACAG AAGGGAAAC-3′ (−212 to −66 bp); 5′-TGATCTTTTCCCTGCTGTCA-3′ and 5′-TCCCATGAGTAATCCAGAACG-3′ (−374 to −273 bp); and 5′-AAGAG ACAGGCCTGGGATAAAC-3′ and 5′-GGTCTGTCTGAGTGATGGGAAG-3′ (−2,841 to −2,704 bp). *cgd3_140* (β-tubulin) 5′-TAGAACCTTCCTGCGGTC GT-3′ and 5′-TTTTCTTCTGGGCTGGTCTC-3′ were used as controls.

Quantitative RT-PCR. We extracted RNA with the RNAqueous-4PCR Kit (Applied Biosystems), and performed reverse transcription with the RETROscript Kit (Applied Biosystems). We quantified complementary DNA by real-time PCR[50]. We used the following primers (Invitrogen): *Gad2*, 5′-GCCCAGGCTCATCGCATTCACGTC-3′ and 5′-CCTCCACCCC AAGCAGCATCCACA-3′; *Gad1*, 5′-GGTTTCTTGCAAAGGACCAA-3′ and 5′-CACCAGGGTCACTGTTTTCA-3′; and *Gapdh*, 5′-AACGACCCCTTCA TTGAC-3′ and 5′-TCCACGACATACTCAGCAC-3′.

Recordings. We performed visualized whole-cell recordings of NRM neurons in slice preparations as previously described[21]. We used neonatal rats in recording experiments because of limited cell visibility and quality in the NRM slices from older rats. Both similarities and differences have to be recognized between neonates and adults in the inflammatory responses of NRM neurons for data interpretation[20].

Immunohistochemistry. We conducted immunohistochemistry as previously described[50]. We used antibodies to synapsin I (1 in 200; (cat. 106001), Synaptic Systems) and GAD65 (1 in 1,000; (cat. AB5082), Millipore) and Cy3-conjuaged secondary antibodies (1 in 1,000; Jackson ImmunoResearch Laboratories). We obtained immunohistochemical staining for GAD65 and synapsin I and their overlap (n = 5 or 6 rats for each experimental group) from randomly selected sections (n = 4–6 sections from each rat) and quantitatively compared manually, with the experimenter blind to the treatment groups.

Microinjection. We performed NRM infusions and behavioral pain tests as previously described[21,51]. We infused TsA (16.5 mM in 1 µl) or SAHA (100 µM in 1 µl) into the NRM once daily for 4 d, as in the systemic treatment. As a standard control, TsA infusions into a site 1 mm dorsal to the NRM were without effect (data not shown).

Statistical analyses and materials. We used analysis of variance (one way and two way) and *post hoc* analyses to analyze data groups with multiple comparisons. We made simple statistical comparisons using Student's t tests. We purchased drugs from Sigma or Tocris Cookson, except SAHA, which was purchased from Cayman Chemical.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

49. Roozendaal, B. *et al.* Membrane-associated glucocorticoid activity is necessary for modulation of long-term memory via chromatin modification. *J. Neurosci.* **30**, 5037–5046 (2010).
50. Ma, J., Zhang, Y., Kalyuzhny, A.E. & Pan, Z.Z. Emergence of functional delta-opioid receptors induced by long-term treatment with morphine. *Mol. Pharmacol.* **69**, 1137–1145 (2006).
51. Ma, J. & Pan, Z.Z. Contribution of brainstem GABA(A) synaptic transmission to morphine analgesic tolerance. *Pain* **122**, 163–173 (2006).

doi:10.1038/nm.2442