

ACCELERATED COMMUNICATION

Epigenetic Modulation of mGlu2 Receptors by Histone Deacetylase Inhibitors in the Treatment of Inflammatory Pain

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ABSTRACT

Knowing that expression of metabotropic glutamate 2 (mGlu2) receptors in the dorsal root ganglia is regulated by acetylation mechanisms, we examined the effect of two selective and chemically unrelated histone deacetylase (HDAC) inhibitors, *N*-(2-aminophenyl)-4-[*N*-(pyridine-3-ylmethoxy-carbonyl)amino-methyl]benzamide (MS-275) and suberoylanilide hydroamic acid (SAHA), in a mouse model of persistent inflammatory pain. Although a single subcutaneous injection of MS-275 (3 mg/kg) or SAHA (5–50 mg/kg) was ineffective, a 5-day treatment with either of the two HDAC inhibitors substantially reduced the nociceptive response in the second phase of the formalin test, which reflects the development of central sensitization in the dorsal horn of the spinal cord. Analgesia was abrogated by a single injection of the mGlu2/3 receptor antagonist (α S)- α -amino- α -[(1*S*,2*S*)-2-carboxycyclopropyl]-9*H*-xantine-9-propanoic acid (LY341495; 1 mg/kg,

i.p.), which was inactive per se. Both MS-275 and SAHA up-regulated the expression of mGlu2 receptors in the dorsal root ganglion (DRG) and spinal cord under conditions in which they caused analgesia, without changing the expression of mGlu1a, mGlu4, or mGlu5 receptors. Induction of DRG mGlu2 receptors in response to SAHA was associated with increased acetylation of p65/RelA on lysine 310, a process that enhances the transcriptional activity of p65/RelA at nuclear factor- κ B-regulated genes. Transcription of the mGlu2 receptor gene is known to be activated by p65/RelA in DRG neurons. We conclude that HDAC inhibition produces analgesia by up-regulating mGlu2 receptor expression in the DRG, an effect that results from the amplification of NF- κ B transcriptional activity. These data provide the first evidence that HDAC inhibitors cause analgesia and suggest that HDACs are potential targets for the epigenetic treatment of pain.

Histone deacetylase (HDAC) inhibitors are known to modulate gene expression by increasing acetylation of histone proteins, thus remodeling chromatin structure (Strahl and Allis, 2000). Eighteen HDAC isoenzymes have been identified and divided into four classes based on their homology to yeast deacetylase proteins. Class I HDACs include the HDAC1, -2, -3, and -8 isoforms. These enzymes are ubiquitously expressed and have a predominant nuclear localization. Class II HDACs, which include HDAC4, -5, -6, -7, -9,

and -10, are cytosolic enzymes and have a more restricted tissue pattern of expression. Class III HDACs include sirtuins, whereas HDAC11 is the only member of class IV HDAC and shares properties of both class I and II HDACs (Xu et al., 2007; Yang and Seto, 2007).

In addition to histones, a number of nonhistone proteins, mainly transcription factors, are regulated by acetylation and are specifically targeted by HDACs (Spange et al., 2009). In particular, members of the nuclear factor- κ B (NF- κ B) family of transcription factors are known to be regulated by reversible acetylation (Chen et al., 2001). The NF- κ B/Rel family consists of p50, p52, p65/RelA, c-Rel, and RelB, which

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ABBREVIATIONS: mGlu, metabotropic glutamate; HDAC, histone deacetylase; LY 379268, (1*R*,4*R*,5*S*,6*R*)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid; LY 341495, (α S)- α -amino- α -[(1*S*,2*S*)-2-carboxycyclopropyl]-9*H*-xantine-9-propanoic acid; SAHA, suberoylanilide hydroamic acid; MS-275 3-pyridylmethyl-*N*-[4-[(2-aminophenyl)-carbonyl]-benzyl]-carbamate.

form homo- or heterodimers (Xiao, 2004). The most prevalent combination in mammalian cells is the heterodimer p50/p65. Acetylation of p65/RelA at Lys310 by the histone acetyltransferase p300/CREB-binding protein is required for a full transcriptional activity at NF- κ B target genes. In contrast, deacetylation of Lys310 p65/RelA inhibits the transcription of NF- κ B target genes (Chen et al., 2002; Chen and Greene, 2004). Class I HDACs, particularly HDAC1 and -2, directly interact with p65/RelA and deacetylate Lys310 p65/RelA, thus negatively regulating NF- κ B activity (Ashburner et al., 2001).

We have shown that metabotropic glutamate 2 (mGlu2) receptors are transcriptionally regulated by the NF- κ B pathway, and hyperacetylation of p65/RelA by the analgesic drug L-acetylcarnitine enhances the expression of mGlu2 receptors in cultured dorsal root ganglia (DRG) neurons (Chiechio et al., 2006). These receptors negatively regulate neurotransmitter release from primary afferent fibers in the dorsal horn of the spinal cord (Gerber et al., 2000), and their activation causes analgesia in models of inflammatory and neuropathic pain (Simmons et al., 2002; Yang and Gereau, 2002, 2003; Jones et al., 2005). The analgesic action of L-acetylcarnitine in rats results from an up-regulation of mGlu2 receptors in the DRG and the dorsal horn of the spinal cord (Chiechio et al., 2002), suggesting that the epigenetic control of mGlu2 receptor expression may be targeted by novel analgesic drugs.

Here we examined whether HDAC inhibition in mice affects mGlu2 receptor expression and modulates pain sensitivity in the formalin model of persistent inflammatory pain. We used two selective HDAC inhibitors, the benzamide derivative MS-275 and the hydroxamate derivative suberoylanilide hydroxamic acid (SAHA). These two inhibitors show different selectivity among HDAC isotypes. MS-275 selectively inhibits a subset of class I HDAC isotypes, showing high affinity for HDAC1 and HDAC2, lower affinity for HDAC3, and no affinity for HDAC8 (Khan et al., 2008). In contrast, SAHA inhibits both class I and class II HDACs (Blanchard and Chipoy, 2005).

Materials and Methods

Animals. Male CD1 mice aged between 8 and 9 weeks were used in the present study. All procedures were approved by the institutional animal care and use committee. Animals were maintained on a 12-h light/dark cycle and allowed free access to food and water. For behavioral experiments, mice were acclimated to the experimental room, and tests were performed blind to the pharmacological treatment of the animals.

Drugs. MS-275 (Sigma) and SAHA (Calbiochem) were dissolved in 5% DMSO and stored at -20°C . MS-275 (3 mg/kg s.c.) or SAHA (5–50 mg/kg s.c.) were injected once 24 h or 30 min before behavioral tests or repeatedly for 5 consecutive days with the last injection 24 h before behavioral tests.

Formalin Test. Formalin (5%, 10 μl ; Sigma, St. Louis, MO) was injected into the plantar surface of the right hind paw, and the total time spent licking the injected paw was monitored for 1 h and recorded every 5 min.

Rotarod Test. Motor performance was assessed on an accelerating Rotarod treadmill (Ugo Basile, Comerio, Italy) as described previously (Malmberg et al., 2003).

Immunoblotting. Tissues from the lumbar segment of the spinal cord or DRG from L4–L5 were removed and homogenized. Ten micrograms of total protein were separated by 10% SDS-

polyacrylamide gel electrophoresis and electrophoretically transferred onto protein-sensitive nitrocellulose membranes (Criterion blotter; Bio-Rad Laboratories, Hercules, CA). The membranes were blocked in Odyssey blocker (LI-COR Biosciences, Lincoln, NE) for 1 h, and the following primary antibodies were used: rabbit anti-HDAC1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) rabbit anti-HDAC2 (1:1000; Santa Cruz Biotechnology), mouse anti p65/Rel-A (1:200; Santa Cruz Biotechnology), rabbit anti-acetyl-Lys310-p65/Rel-A (2 $\mu\text{g}/\text{ml}$; Abcam Inc., Cambridge, MA) mouse anti-mGlu2 (1 $\mu\text{g}/\text{ml}$; Abcam), and mouse anti-actin (1:2000; Sigma). Primary antibodies were incubated overnight at 4°C . The following secondary antibodies were incubated for 1 h at room temperature: goat anti-rabbit antibody labeled with IRD800CW (LI-COR) and goat anti-mouse antibody labeled with Alexa Fluor 680 (Invitrogen, Carlsbad, CA). Proteins were detected with the Odyssey Infrared Fluorescence Imaging System (LI-COR).

Transient Transfection. mGlu2 and mGlu3 were cotransfected with green fluorescence protein (GFP) with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. In brief, 4 μg of DNA of each mGlu, 1 μg of GFP, and 10 μl of Lipofectamine solution were first each diluted in 250 μl of Dulbecco's modified Eagle's medium in separate tubes. Each solution was incubated for 5 min before they were combined to allow complexes to form. After a 20-min incubation period, a 500- μl solution of DNA/Lipofectamine complexes was added to each 35-mm plate of HEK cells. Transfection was verified by the expression of GFP. Cells were 90 to 95% confluent at the time of transfection.

Immunohistochemistry. Intact mice were used for immunohistochemistry. Mice were deeply anesthetized and perfused intracardially with 4% paraformaldehyde in phosphate-buffered saline, pH 7.4. The lumbar segment of the spinal cord was removed and cut into 30- μm transverse sections on a microtome. Sections were washed in Tris-buffered saline (TBS; 100 mM Tris, and 0.9% NaCl), and then incubated in TBS containing 3% normal goat serum for 1 h. Sections were then incubated overnight with anti-HDAC1 (1:200) or anti-HDAC2 (1:500). After three washes in TBS, sections were incubated

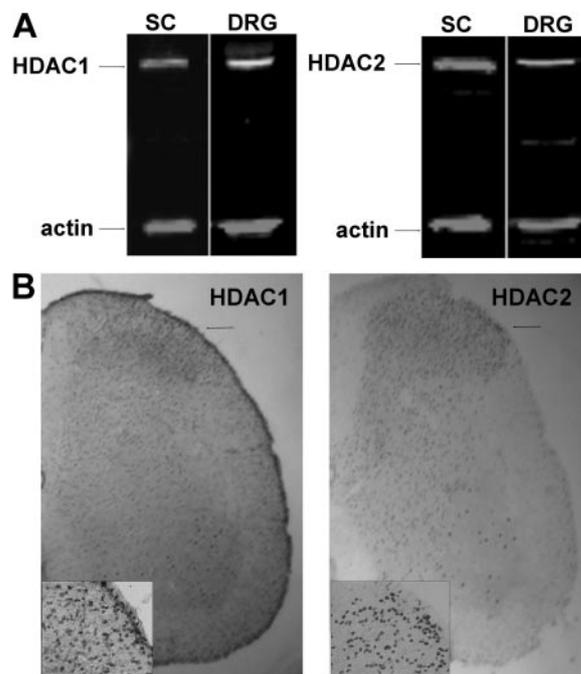


Fig. 1. Expression of HDAC1 and HDAC2 in the mouse spinal cord and DRG. A, immunoblot analysis showing the presence of HDAC1 and -2 in adult mouse DRG and spinal cord. B, immunostaining of HDAC1 and HDAC2 in the lumbar segment of the spinal cord of adult CD1 mice. Note that both HDAC1 and -2 are expressed in the dorsal horn (insets).

for 1 h with a biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories, Burlingame, CA) and then with extravidin (Sigma). Immunoreactivity was visualized using 3,3'-diaminobenzidine (Vector Laboratories).

For mGlu2 immunostaining and histological analysis, mice were deeply anesthetized and L4–L5 DRGs were fixed in Carnoy, and embedded in paraffin. Ten micrometer deparaffinized sections were soaked in 3% hydrogen peroxide to block endogenous peroxidase activity, incubated overnight with the monoclonal mouse anti-mGlu2 antibody (5 ng/ml; Abcam), and then for 1 h with secondary biotinylated anti-mouse antibodies (1:200; Vector Laboratories). The immunoreaction was carried out with 3,3-diaminobenzidine tetrachloride (ABC Elite kit; Vector Laboratories). Nuclear staining was performed with Meyer emallume.

Results

We focused on HDAC1 and -2, which have been shown to deacetylate p65/NF- κ B (Ashburner et al., 2001). Immunoblot

analysis showed the presence of both HDAC isotypes in the mouse DRG (Fig. 1A). Both isotypes were also expressed in superficial laminae of the dorsal horn of the spinal cord, as shown by immunohistochemistry (Fig. 1B). Mice received single or repeated injections of either MS-275 (3 mg/kg s.c.) or SAHA (5 or 50 mg/kg s.c.). At these doses, neither MS-275 nor SAHA impaired motor coordination in the Rotarod test (not shown), although mice treated with MS-275 showed a slight increase in spontaneous locomotor activity. For the assessment of analgesia, mice were subjected to the formalin test 30 min or 24 h after a single injection, or 24 h after a 5-day administration of HDAC inhibitors or vehicle (5% DMSO). In the formalin test, the first phase (immediately after formalin injection in the hind paw) reflects acute pain, whereas the second phase (approximately 20–45 min after formalin injection) reflects the development of chronic pain and involves mechanisms of central sensitization in the dor-

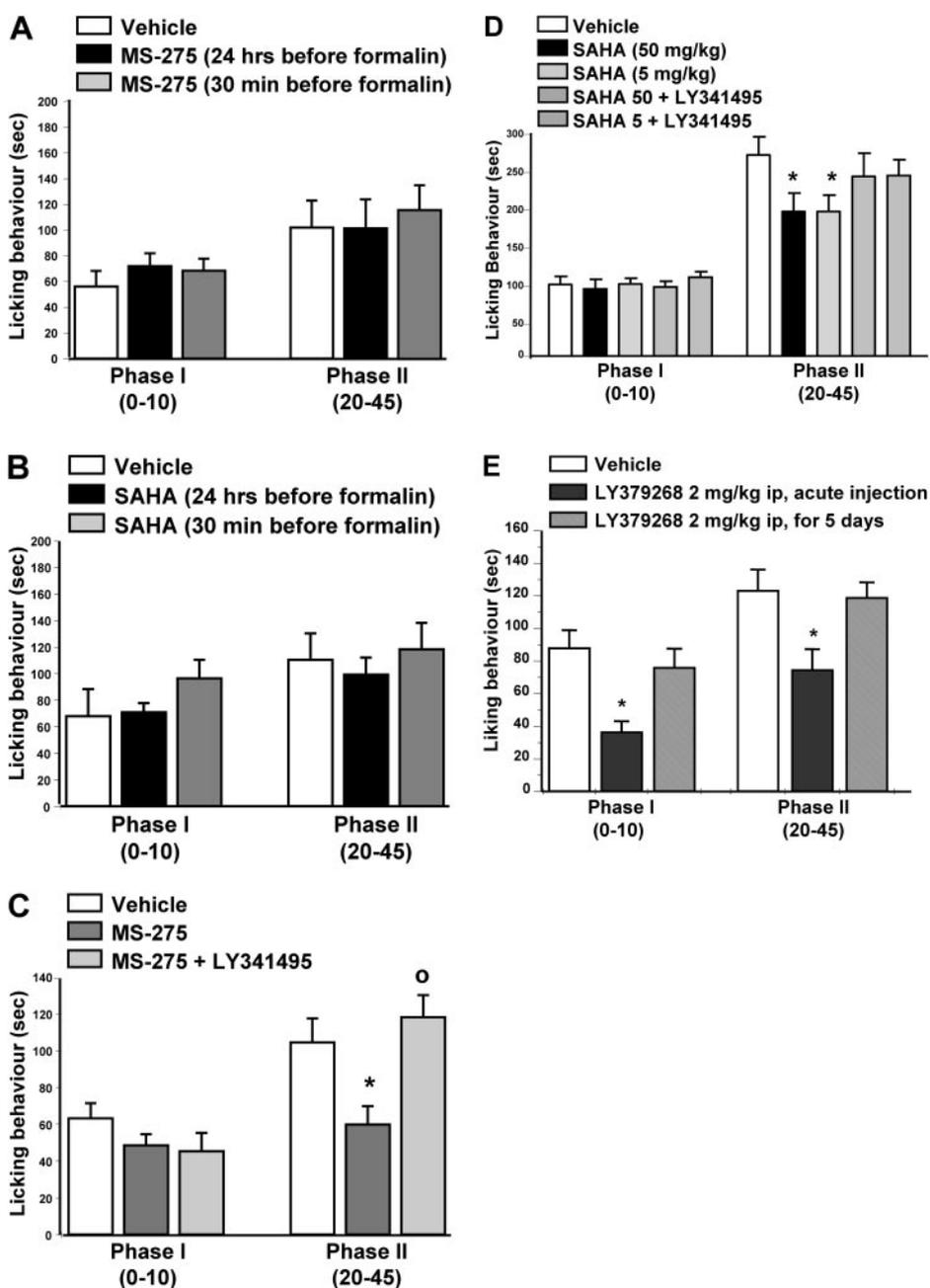


Fig. 2. Repeated systemic injections of MS-275 or SAHA produces analgesia in the second phase of the formalin test in mice. A single injection of MS-275 (3 mg/kg s.c.) (A) or SAHA (50 mg/kg s.c.) (B) either 30 min or 24 h before the injection of formalin in the hind paw did not affect nociceptive behavior. In contrast, a 5-day treatment with MS-275 (3 mg/kg s.c.) (C) or SAHA (5 or 50 mg/kg s.c.) (D) attenuated nociceptive behavior in the second phase of the formalin test. Analgesia was prevented by a single injection of LY341495 (1 mg/kg i.p., administered 30 min before formalin injection) (C and D). Data are means \pm S.E.M. of 12 to 16 mice. *, $p < 0.05$ (one-way ANOVA + Fisher's PLSD) versus the corresponding group treated with vehicle. o, $p < 0.05$ (one-way ANOVA + Fisher's PLSD) versus the corresponding group treated with MS-275. E, for comparison, the effect of LY 379268 (2 mg/kg i.p.; administered as a single pulse 30 min before formalin or injected daily for 5 days) in the formalin test in mice is shown. Data are means \pm S.E.M. of 8 to 12 mice. *, $p < 0.05$ (one-way ANOVA + Fisher's PLSD) versus the corresponding group treated with vehicle.

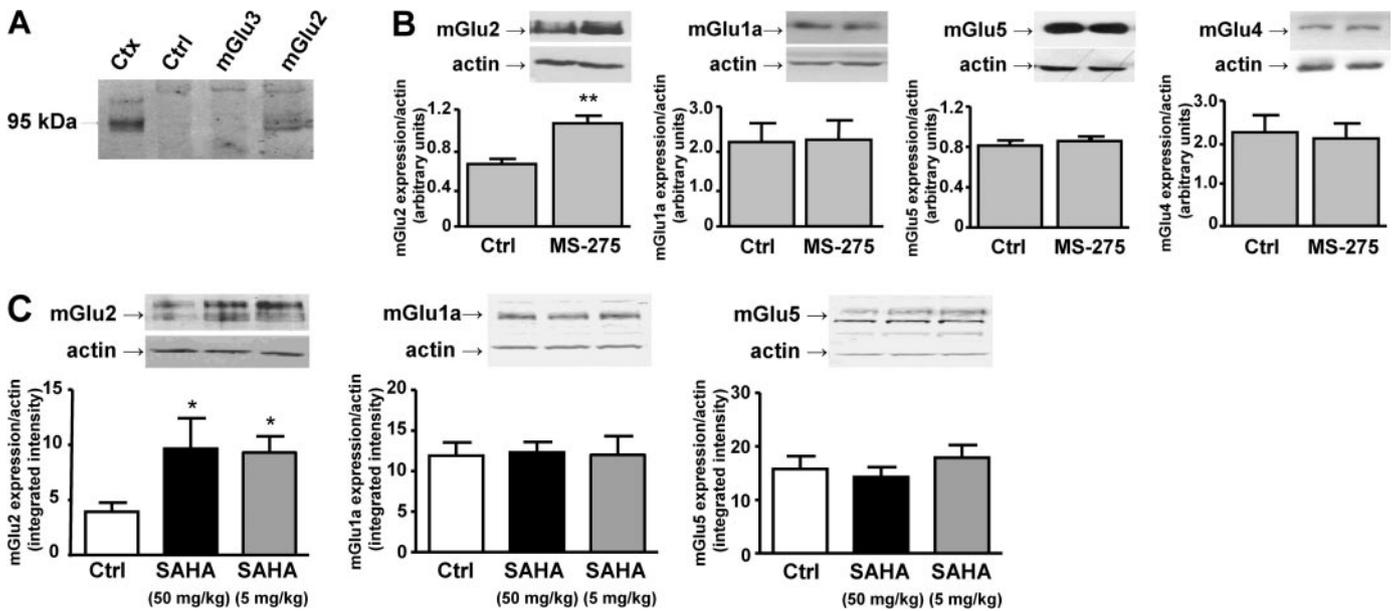


Fig. 3. Repeated injections of HDAC inhibitors increase the expression of mGlu2 receptors in the lumbar segment of the spinal cord. A representative immunoblot showing the specificity of the mGlu2 monoclonal antibody in HEK cells expressing either mGlu2 or mGlu3 receptors is shown in A. The effect of a 5-day treatment with MS-275 (3 mg/kg s.c.) or SAHA (5–50 mg/kg s.c.) on the expression of mGlu2, mGlu1a, mGlu5, and mGlu4 receptors in the lumbar spinal cord is shown in B and C, respectively. **, $p < 0.001$; *, $p < 0.05$ (Student's t test) versus values obtained in animals treated with vehicle for 5 days (Ctrl).

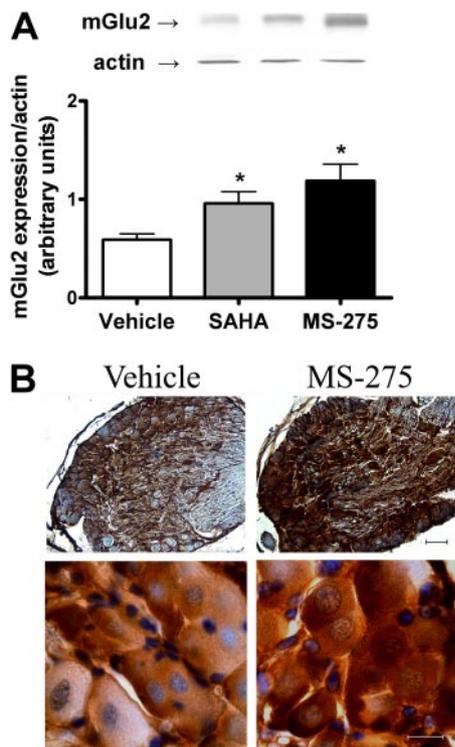


Fig. 4. Repeated injections of SAHA and MS-275 up-regulate the expression of mGlu2 receptors in the DRG. A, the effect of a 5-day treatment with MS-275 (3 mg/kg s.c.) or SAHA (5 mg/kg s.c.) on the expression of mGlu2 in the DRG is shown. Data are the means \pm S.E.M. of 4 animals. *, $p < 0.05$ (Student's t test) versus vehicle group. B, immunohistochemistry of mGlu2 receptors in the DRG of mice treated for 5 days with MS-275 (3 mg/kg s.c.). Low-magnification images of immunostained DRG slices from control (vehicle) and treated (MS-275) animals are shown at top. Images of immunostained DRG neurons are shown at bottom (magnification, 100 \times). Note the up-regulation of mGlu2 receptors in the extranuclear regions of DRG neurons in mice treated with MS-275.

sal horn of the spinal cord (Coderre and Melzack, 1992; Tjølsen et al., 1992). A single injection of MS-275 (3 mg/kg sc) or SAHA (50 mg/kg sc) 30 min or 24 h before formalin did not affect the nociceptive response in either phase of the formalin test (Fig. 2, A and B). When administered daily for 5 days before testing, these doses of either MS-275 or SAHA produced analgesia selectively in the second phase of the formalin test. SAHA was equally effective at the lower dose of 5 mg/kg (Fig. 2, C and D). These data indicate that a prolonged inhibition of HDAC was required for the induction of analgesia. It is noteworthy that analgesia produced by a 5-day treatment with MS275 or SAHA was largely attenuated by a single injection of the preferential mGlu2/3 receptor antagonist LY341495 (1 mg/kg i.p., administered 30 min before the formalin test; i.e., 24 h after the last injection of MS275 or SAHA) (Fig. 2, C and D). LY341495 per se did not affect the nociceptive response after a single injection in control mice treated for 5 days with vehicle (data not shown). We have also tested the analgesic activity of compound LY379268, which behaves as a potent orthosteric agonist of mGlu2 and mGlu3 receptors (for review, see Schoepp et al., 1999). A single injection of LY379268 (2 mg/kg i.p.) reduced nociceptive responses in both the first and second phase of the formalin test. In contrast, a 5-day treatment with LY379268 (2 mg/kg, i.p.; once a day) did not cause analgesia (Fig. 2E).

We then examined whether HDAC inhibitors-induced analgesia could be associated with an increased expression of mGlu2 receptors, a mechanism that is responsible for the analgesic effects of the acetylating agent, L-acetylcarnitine (Chiechio et al., 2002, 2006). For the study of mGlu2 receptor expression, we used a monoclonal antibody that does not cross-react with mGlu3 receptors or other mGlu receptor subtypes (Neki et al., 1996; Gu et al., 2008). We checked the specificity of this antibody using HEK293 cells expressing either mGlu2 or mGlu3 receptors (Fig. 3A). A 5-day treatment with either MS-275 or SAHA substantially enhanced

the expression of mGlu2 receptors in the spinal cord without affecting the expression of mGlu1a, mGlu4, or mGlu5 receptors (Fig. 3, B–H). A similar effect by either MS-275 or SAHA was observed by Western blot analysis in the DRG (Fig. 4A). Immunohistochemistry showed that MS-275 increased membrane and cytoplasmic mGlu2 staining in DRG neuronal cell bodies (Fig. 4B). Finally, we confirmed that HDAC inhibition could hyperacetylate the mGlu2 receptor-regulating transcription factor p65/RelA using SAHA under the same conditions in which we observed analgesia and up-regulation of mGlu2 receptor expression (i.e., 24 h after a 5-day treatment) (Dai et al., 2005). We used an antibody that specifically detects Lys310-acetylated p65/RelA. Acetylation of Lys310 enhances the transcriptional activity of NF- κ B/p65/RelA, increasing the affinity of p65/RelA for the DNA responsive elements and decreasing the affinity for the inhibitory subunit called inhibitor of NF- κ B (Buerki et al., 2008). A 5-day treatment with SAHA (5 or 50 mg/kg) enhanced the levels of Lys310-acetylated p65/RelA without changing the total levels of p65/RelA. Note that SAHA increased the expression of mGlu2 receptors in the same set of experiments (Fig. 5, A and B).

Discussion

These data demonstrate that two established and unrelated inhibitors of HDAC, MS-275 and SAHA, induce analgesia, and that at least HDAC-1 and -2 are expressed in the DRG and the dorsal horns of the spinal cord. These and other HDAC inhibitors are under clinical development for the treatment of cancer and show in general a good profile of safety and tolerability in humans (Minucci and Pelicci, 2006). We have found that HDAC inhibitors selectively relieve pain

in the second phase of the formalin test, which reflects the development of central sensitization in the dorsal horns of the spinal cord (Coderre, 1992; Tjølsen et al., 1992). This is potentially interesting from a therapeutic standpoint because central sensitization underlies many features of cancer pain and neuropathic pain associated with cancer or the use of anticancer drugs (e.g., vincristine and paclitaxel) (Polomano et al., 2001; Dougherty et al., 2004). It is noteworthy that we have found that MS-275 and SAHA up-regulated the expression of mGlu2 receptors in the DRG and dorsal horn without affecting the expression of other mGlu receptor subtypes typically associated with pain, such as mGlu1a and mGlu5 receptors (Fundytus, 2001; Bleakman et al., 2006). A number of mechanisms might contribute to the analgesic activity of HDAC inhibitors. For example, HDAC inhibitors regulate the expression of 32 δ and κ opioid receptors (Bi et al., 2001; Lin et al., 2008). Under our conditions, however, the analgesic activity of MS-275 and SAHA was largely prevented by the mGlu2/3 receptor antagonist LY341495. We therefore conclude that the increased expression of mGlu2 receptors in DRG neurons is a predominant mechanism of the analgesic activity of HDAC inhibitors in the second phase of the formalin test. Although we cannot exclude a role for mGlu3 because selective mGlu3 receptor antagonists are not yet available, it is noteworthy that expression of mGlu3 receptors in the DRG is not regulated by acetylation mechanisms. L-Acetylcarnitine, which is clinically effective in relieving pain associated with peripheral neuropathies (Chiechio et al., 2007), up-regulates mGlu2 but not mGlu3 receptors in DRG/spinal cord through acetylation of the NF- κ B family member p65/RelA (Chiechio et al., 2002, 2006). Likewise, HDAC inhibitors might produce analgesia by enhancing the

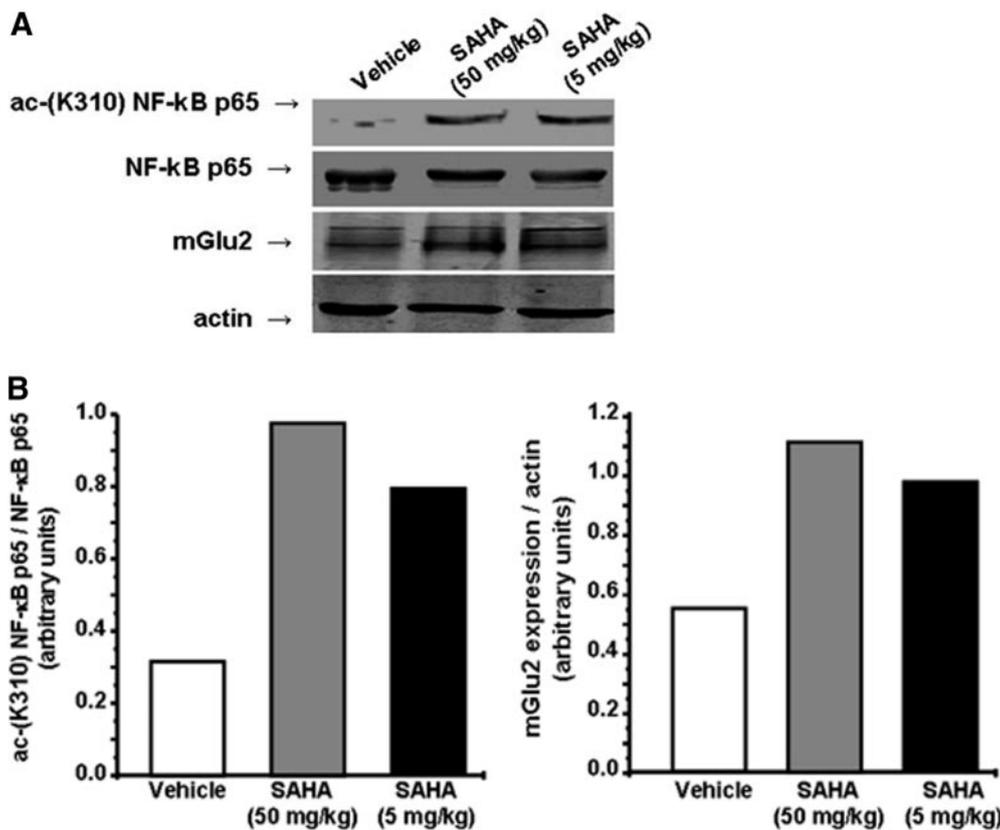


Fig. 5. Repeated injections of SAHA up-regulate the expression of mGlu2 receptors in the DRG and acetylate p65/RelA. A representative immunoblot of acetylated-(Lys310)p65/RelA, total p65/RelA, and mGlu2 receptors in DRG extracts from mice treated for 5 days with vehicle or SAHA (5 or 50 mg/kg s.c.) is shown in A. Because of the low signal of acetylated-(Lys310)p65/RelA, each hybridization signal refers to DRG protein extracts obtained by pooling tissues of 5 different mice per group. Densitometric analysis of acetylated-(K310)p65/RelA normalized by total p65/RelA is shown in B. The experiment has been repeated twice with similar results.

transcriptional activity of p65/RelA, thereby enhancing mGlu2 receptor expression in the DRG. p65/RelA is a member of the NF- κ B family of transcription factors, which also includes c-Rel, RelB, NF- κ B1 (p105/p50), and NF- κ B2 (p100/p5). p50 and p52 form heterodimers with Rel proteins and regulate transcription of NF- κ B-dependent genes (Chen et al., 2001; Kiernan et al., 2003; Hu and Colburn, 2005). Acetylation/deacetylation processes mediated by histone acetyltransferase and HDACs, respectively, affect the transcriptional activity of p65/RelA in a manner that depends on the lysine residue that is acetylated (Xu et al., 2007; Yang and Seto, 2007; Spange et al., 2009). For example, acetylation of Lys310 enhances the transcriptional activity of p65/RelA, whereas acetylation of Lys122 and other lysine residues produces opposite effects (Kiernan et al., 2003; Buerki et al., 2008). The mGlu2 receptor gene (*GRM2*) contains several putative binding sites for the p50-p65/RelA heterodimer, and transcription of the gene is regulated by acetylation (Chiechio et al., 2006). The ability of SAHA to increase acetylation of p65/RelA on lysine 310 and up-regulate mGlu2 receptor expression in the DRG provides a novel putative mechanism for analgesic drugs based on the epigenetic regulation of *GRM2* and perhaps other receptors that are implicated in the pathophysiology of chronic pain. It is noteworthy that the NF- κ B pathway is activated in DRG and spinal cord neurons or astrocytes in response to peripheral inflammation or nerve injury and may support the development of chronic pain through the induction of type-2 cyclooxygenase and other mechanisms (Niederberger and Geisslinger, 2008). At least in our model, the analgesic branch of the NF- κ B pathway seems to be prevalent unless additional mechanisms contribute to the overall analgesic activity of HDAC inhibitors.

Consistent with previous reports (Simmons et al., 2002; Jones et al., 2005), we have found that direct activation of mGlu2/3 receptors with the orthosteric agonist LY379268 also produced analgesia. However, as opposed to HDAC inhibitors, LY379268 (2 mg/kg i.p) reduced nociceptive responses in both the first and second phases of the formalin test; remarkably, the drug was active after a single injection but not after a 5-day treatment. The different analgesic profile of LY379268 and HDAC inhibitors may be explained as follows. The first phase of the formalin test reflects acute nociceptive pain, which is tightly related to the activation of peripheral nociceptors. Both mGlu2 and mGlu3 receptors are localized in peripheral nociceptors (Carlton et al., 2001), and pharmacological activation of both receptors with LY379268 may relieve acute nociceptive pain by hyperpolarizing nociceptors or through other mechanisms. Perhaps mGlu3 receptors may play a predominant role in inhibiting the first phase of the formalin test.

The lack of analgesia in response to repeated administrations of LY379268 is consistent with previous findings (Jones et al., 2005) and is likely to reflect the development of tolerance as a result of receptor desensitization. However, it should be kept in mind that, at least in the mouse cerebral cortex, LY379268 is more efficient in desensitizing mGlu3 than mGlu2 receptors (Iacovelli et al., 2009). Tolerance may not develop in response to HDAC inhibitors because overexpressed mGlu2 receptors are transiently activated by the endogenous glutamate, not continuously activated by a pharmacological agonist. We cannot exclude the idea that sub-threshold doses of LY379268 (which, by definition, do not

produce receptor desensitization) act synergistically with HDAC inhibitors in causing analgesia. Combination studies between mGlu2/3 receptor agonists (or mGlu2 receptor enhancers) and HDAC inhibitors will be particularly interesting because mGlu2/3 receptor agonists are under clinical development for the treatment of schizophrenia and other neuropsychiatric disorders (Patil et al., 2007; Dunayevich et al., 2008).

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