HDAC2 regulates atypical antipsychotic responses through the modulation of mGlu2 promoter activity

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Histone deacetylases (HDACs) compact chromatin structure and repress gene transcription. In schizophrenia, clinical studies demonstrate that HDAC inhibitors are efficacious when given in combination with atypical antipsychotics. However, the molecular mechanism that integrates a better response to antipsychotics with changes in chromatin structure remains unknown. Here we found that chronic atypical antipsychotics downregulated the transcription of metabotropic glutamate 2 receptor (mGlu2)22, also known as Gm2, an effect that was associated with decreased histone acetylation at its promoter in mouse and human frontal cortex. This epigenetic change occurred in concert with a serotonin 5-HT2A receptor–dependent upregulation and increased binding of HDAC2 to the mGlu2 promoter. Virally mediated overexpression of HDAC2 in frontal cortex decreased mGlu2 transcription and its electrophysiological properties, thereby increasing psychosis-like behavior. Conversely, HDAC inhibitors prevented the repressive histone modifications induced at the mGlu2 promoter by atypical antipsychotics, and augmented their therapeutic-like effects. These observations support the view of HDAC2 as a promising new target for schizophrenia treatment.

Schizophrenia is a severe and persistent psychiatric condition that affects almost 1% of the world’s population1,2. In some patients with schizophrenia, typical and atypical antipsychotic drugs produce complete remission of psychotic symptoms. However, about 30% of patients are considered treatment resistant and will continue to experience psychotic and other symptoms despite the optimal use of available antipsychotic medications3,4. Over the last 40 years, a variety of adjunctive treatments have been used to enhance the response to antipsychotic medications5. Among these, preclinical6–8 and clinical9–11 studies suggest that drugs such as valproate, one of whose therapeutic-like effects. These observations support the view of HDAC2 as a promising new target for schizophrenia treatment.

Atypical antipsychotic drugs all have in common a high affinity for the serotonin 5-HT2A receptor (5HT2A), as well as a modest affinity for the dopamine D2 receptor16,17. Hallucinogenic drugs, such as lysergic acid diethylamide (LSD), psilocybin and mescaline, recruit specific 5HT2A-mediated signaling pathways to affect behavior in humans and rodents18,19. These findings are consistent with the implication of the 5HT2A receptor in the neurochemical abnormalities responsible for psychosis. Several lines of evidence also associate schizophrenia with dysfunction of glutamatergic transmission20. Indeed, recent preclinical assays in rodents suggest that drugs that activate the metabotropic glutamate 2 receptor (mGlu2)22 represent potential antipsychotic medications21–23, which is further underscored by some clinical measures24. Our previous findings convincingly demonstrate that chronic treatment with the atypical antipsychotic clozapine induces downregulation of mGlu2 expression in mouse frontal cortex25—a brain region that is important in cognition and perception, and has been implicated more recently in schizophrenia and antipsychotic responses17,19,23. Together with the antipsychotic properties of drugs that bind to and activate the mGlu2 receptor, these studies led us to hypothesize that downregulation of mGlu2 expression might restrain the therapeutic effects of atypical antipsychotic drugs.

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Figure 1  
Decreased acetylation of histone H3 (H3ac) at the mGlu2 promoter after chronic treatment with atypical antipsychotics in mouse frontal cortex. (a–d) Clozapine and risperidone, but not haloperidol, modulate the expression of mGlu2 in mouse frontal cortex. Mice were chronically (21 d) injected with vehicle, 10 mg kg⁻¹ clozapine, 4 mg kg⁻¹ risperidone or 1 mg kg⁻¹ haloperidol, and killed 1 d after the last injection. (a–c) [³H]LY341495 binding in mouse frontal cortex after vehicle or clozapine (a; F₂,₉₅ = 65.34, P < 0.001; n = 4 independent experiments performed in triplicate), risperidone (b; F₂,₁₇₇ = 166.4, P < 0.001; n = 6 independent experiments performed in triplicate) or haloperidol (c; F₂,₁₁₆ = 1.29, P > 0.05; n = 6 independent experiments performed in triplicate). Maximum number of binding sites (Bmax) for [³H]LY341495 obtained from saturation curves is decreased by chronic clozapine (t = 6.32) and risperidone (t = 6.53) but not haloperidol (t = 0.97; P > 0.05; NS, not significant); two-tailed Student’s t-test. ** P < 0.01. (d) Expression of SHT2A (F₃,₂₀ = 5.66, P < 0.01), 5HT2C (F₂,₂₀ = 0.29, P > 0.05), D₂ (F₂,₂₀ = 0.31, P > 0.05), mGlu2 (F₃,₂₀ = 6.62, P < 0.01) and mGlu3 (F₂,₂₀ = 0.13, P > 0.05) mRNA in mouse frontal cortex assayed by quantitative reverse transcription (qRT)-PCR (n = 6 mice per group). * P < 0.05; ** P < 0.01; Bonferroni’s post hoc test of one-way ANOVA. (e–h) Decreased acetylation of histone H3 (H3ac) at the mGlu2 promoter by clozapine (e; t = 2.77) and risperidone (f; t = 5.38), but not by haloperidol (g; t = 1.08) or fluoxetine (h; t = 0.41), in mouse frontal cortex. Decreased acetylation of H3ac at 5HT2A promoter by clozapine (e; t = 2.96). * P < 0.01, two-tailed Student’s t-test corrected for multiple null hypotheses by Holm’s Bonferroni method. Error bars, s.e.m.

RESULTS  
Histone modifications at mGlu2 by chronic antipsychotics  
In schizophrenia patients, antipsychotic drugs are administered chronically (weeks to months of sustained drug treatment)²⁶. We have previously shown that chronic treatment with clozapine downregulates mGlu2 expression in mice²⁵. We found here similar effects with chronic (21-d) clozapine and risperidone, but not haloperidol—a first-generation (or typical) antipsychotic drug—in mouse frontal cortex (Fig. 1a–d; see also Supplementary Fig. 1d for the effect of chronic haloperidol on dopamine D2 receptor binding in striatum and Supplementary Fig. 1h for absence of effect of chronic clozapine on mGlu2 expression in thalamus and striatum). Previous work has demonstrated that activation of cortical mGlu2 modulates the cellular and behavioral responses induced by hallucinogenic and antipsychotic 5HT2A ligands²⁵.²⁵. Recent observations also suggest chromatin remodeling in cortical neurons as a mechanism involved in the molecular responses to chronic treatment with antipsychotic drugs²⁶. The notable regulation of mGlu2 expression by atypical antipsychotic drugs prompted us to investigate the effect of chronic antipsychotic treatments on the epigenetic status of the mGlu2 promoter in mouse and human frontal cortex.

We quantified several post-translational histone modifications at the promoter region of the mGlu2 gene in mouse frontal cortex using a series of chromatin immunoprecipitation (ChIP) assays. Histone H3 acetylation, which correlates with transcriptional activation¹⁴, was strongly decreased at the mGlu2 promoter by chronic clozapine and risperidone, but not haloperidol (Fig. 1e–g). Earlier studies have also implicated monoaminergic and glutamatergic receptor genes, such as SHT2A (Htr2a), 5HT2C (Htr2c), dopamine D2 (Drd2) and mGlu3 (Gmr3) in psychosis and antipsychotic responses²⁷–³¹. We found that only clozapine, and neither risperidone nor haloperidol, decreased histone H3 acetylation at the 5HT2A promoter in mouse frontal cortex (Fig. 1e–g; see also Fig. 1d and Supplementary Fig. 1h for decreased 5HT2A mRNA expression and Supplementary Fig. 1a–c for decreased density of 5HT2A binding sites). There were no significant changes at the 5HT2C, D2 and mGlu3 promoters. We obtained similar results with histone H4 acetylation (Supplementary Fig. 2a). Histone H3 methylation at lysine 4 (H3K4me1, H3K4me2 and/or H3K4me3), another marker of gene activation, was not affected by any of the tested chronic antipsychotic treatments at these promoters (Supplementary Fig. 2a).

We next examined whether histone modifications known to correlate with transcriptional repression were altered by chronic treatment with antipsychotics. Chronic clozapine or risperidone, but not haloperidol, elicited an increase in histone H3 trimethylation at lysine 27 (H3K27me3), a repressive histone modification marker, at the mGlu2 promoter, with no apparent changes at the 5HT2A, 5HT2C, D2 and mGlu3 promoters (Supplementary Fig. 2a). None of the tested chronic treatments increased histone H3 trimethylation at lysine 9 (H3K9me3), another histone modification that correlates with transcriptional repression, at these promoters (Supplementary Fig. 2a). In addition, we did not detect the...
Supplementary Table 1

| Gene   | Control | Schizophrenia |
|--------|---------|---------------|
| mGlu2  | 0.78    | 1.18          |
| mGlu3  | 1.01    | 1.23          |
| mGlu4  | 0.95    | 1.08          |

Supplementary Figure 2C

Fig. 2C

Increased HDAC2 expression in mouse frontal cortex of subjects with untreated schizophrenia and matched controls.

Supplementary Figure 4A

Fig. 4A

Increased HDAC2 expression in mouse frontal cortex of subjects with untreated schizophrenia and matched controls.

**5-HT2A-dependent modulation of HDAC2 promoter activity**

Next we explored the mechanism through which chronic antipsychotics upregulated HDAC2 expression in mouse frontal cortex. The high affinity of clozapine for both the 5HT2A and the dopamine D2 receptor16,17 prompted us to consider whether signaling pathways downstream of these receptors may modulate HDAC2 promoter activity. Using promoter−reporter gene constructs in human embryonic kidney cell line HEK293 cells, we examined the impact of 5HT2A− and/or D2−dependent signaling on the transcriptional activity of HDAC2 in tissue culture. Activation of the 5HT2A receptor by the neurotransmitter serotonin resulted in inhibition of HDAC2 promoter activity (Fig. 3I), and, notably, this effect was reversed by clozapine in a concentration−dependent manner (Fig. 3J). Neither dopamine nor clozapine affected the promoter activity of HDAC2 in cells expressing the D2 receptor (Fig. 3K).

These data demonstrate that, in vitro in HEK293 cells, clozapine and serotonin differentially modulate HDAC2 promoter activity in a 5HT2A−dependent manner.

However, the overall signal transduction amplification systems present in HEK293 cells and in mouse frontal cortex and the levels of receptor expressed per cell may differ34, which could consequently affect to different extents the promoter activity of the HDAC2 gene. We therefore assessed whether the ability of clozapine to enhance the transcriptional activity of HDAC2 in mouse frontal cortex was 5HT2A−
dependent. Notably, we found that the upregulation of Hdc2 expression by chronic clozapine observed in wild-type (5HT2A+/+) mice (Fig. 3a,c,e) was abolished in 5HT2A null mutant (5HT2A−/−) mice (Fig. 3b,d,e). No other Hdc tested showed regulation by chronic clozapine in 5HT2A−/− mice (Fig. 3b,d,e). Together with the experiments in tissue culture (Fig. 3i,j,k), this work provides mechanistic insight into how chronic atypical antipsychotics lead to elevations in HDAC2 expression in the frontal cortex.

Clozapine upregulates HDAC2 binding to mGlu2 promoter

The highly specific effect of chronic atypical antipsychotics on HDAC2 expression in frontal cortex, together with the repressive histone modifications induced in promoter region of the mGlu2 gene (Figs. 1 and 2), led us to examine the role of HDAC2 in regulating the transcriptional activity of the mGlu2 promoter. We first tested the association of HDAC1, HDAC2 and HDAC4 with the promoter regions of 5HT2A, 5HT2C, mGlu2, and mGlu3 genes, including the β-actin (Actb) promoter as internal control. HDAC2 was more enriched than HDAC1 or HDAC4 at the mGlu2 and mGlu3 promoters, whereas we detected no binding at promoter regions of the 5HT2A and 5HT2C genes (Fig. 4a,b).

We next evaluated the effect of chronic clozapine on HDAC2 binding to the mGlu2 and mGlu3 promoters in mouse frontal cortex. Notably, chronic clozapine significantly increased HDAC2 binding to the mGlu2, and not to the mGlu3, promoter (Fig. 4c). This did not occur in 5HT2A−/− mice, which further supports 5HT2A-dependent signaling as a modulator of expression and epigenetic function of HDAC2 in 5HT2A+/+ mice, and Supplementary Fig. 2d for absence of effect of chronic atypical antipsychotics on mGlu2 expression in 5HT2A−/− mice, and Supplementary Fig. 2d for absence of effect of chronic atypical antipsychotics on the repressive histone modifications induced at the mGlu2 promoter in 5HT2A−/− mice). Chronic clozapine did not affect binding of HDAC4 to the promoters of mGlu2, mGlu3 and β-actin genes (Fig. 4e).

We further investigated in cell culture the functional significance of this finding. We first tested whether HDAC2 is associated with the mGlu2 promoter in HEK293 cells. ChIP analysis revealed that HDAC2 bound to the mGlu2 promoter, as it was efficiently immunoprecipitated by antibody to Flag in cells overexpressing Flag-tagged HDAC2 (Fig. 4f). Next we determined by promoter reporter assay the effect of HDAC2 on mGlu2 promoter activity. Overexpression of HDAC2 (but not of the deacetylase activity-deficient mutant HDAC2-H141A) impaired mGlu2 promoter function in a concentration-dependent manner, which demonstrated that HDAC2 negatively regulated mGlu2 expression in cultured cells (Fig. 4g; see also Supplementary Fig. 5a for immunoblotting experiments showing overexpression of Flag-HDAC2...
and Flag-HDAC2-H141A in HEK293 cells). These findings provide evidence that HDAC2 is critical for regulating transcriptional activation of the mGlu2 promoter. Taken together, our findings indicate that chronic atypical antipsychotic drugs upregulate HDAC2 promoter activity and expression through an epigenetic mechanism that involves 5HT2A-dependent signaling, which leads to a higher binding of HDAC2 to and negative regulation of the mGlu2 promoter in mouse frontal cortex.

**HDAC2 overexpression induces schizophrenia-like behavior**

To further establish a causal link between changes in HDAC2 expression and transcriptional repression of the mGlu2 promoter, we overexpressed HDAC2 in mouse frontal cortex to examine whether this manipulation would regulate mGlu2 expression and its behavioral function. Mice received intra–frontal cortex injections of bicistronic expression and transcriptional repression of the mGlu2 promoter, and total DNA as a control (input). Representative of three independent experiments. (g) Luciferase activity 24 h after transfection of HEK293 cells transfected with mouse mGlu2 promoter (−241 to +97)–luc2P luciferase plasmid and Flag-HDAC2 plasmid and lysates immunoprecipitated with anti-Flag or control IgG 48 h later. The plasmid-derived mGlu2 promoter and a luc2P fragment were amplified by PCR using immunoprecipitated DNA (ChIP) and total DNA as a control (input). Representative of three independent experiments. (g) Luciferase activity 24 h after transfection of HEK293 cells transfected with mouse mGlu2 promoter–luciferase construct in combination with 1, 2 and 4 μg of Flag-HDAC2 construct (F3.8 = 12.11, P < 0.01, one-way ANOVA, n = 6 independent experiments performed in triplicate) or in combination with the deacetylase activity–deficient mutant construct Flag-HDAC2-H141A (F3.8 = 2.36, P > 0.05, one-way ANOVA, n = 3 independent experiments performed in triplicate). *P < 0.05; **P < 0.01; ***P < 0.001; Bonferroni’s post hoc test. Error bars, s.e.m.

Figure 4 HDAC2 binds to the mGlu2 promoter and represses its function. (a) Binding of HDAC2 to the mGlu2 and mGlu3 promoters in mouse frontal cortex. Association of the 5HT2A (F(3,44) = 3.83, P < 0.05; n = 15 mice per group), mGlu3 (F(3,44) = 9.83, P < 0.001; n = 12 mice per group) and β-actin (F(3,44) = 2.39, P < 0.05, n = 12 mice per group) promoters was measured by quantitative PCR (one-way ANOVA). (b) HDAC2 binding to the mGlu2 promoter in mouse frontal cortex (F(2,42) = 3.71, P < 0.05; two-way ANOVA, n = 8 mice per group). Association of HDAC1 (t = 1.03, P > 0.05) and HDAC4 (t = 0.24, P > 0.05) with the mGlu2 promoter was also measured (n = 8 mice per group). P > 0.05; Holm-corrected two-tailed Student’s t-test. (c–e) Binding of HDAC2 to the mGlu2, but not mGlu3 promoter is increased by chronic clozapine treatment (5HT2A+/+, n = 8; 5HT2A−/−, n = 6 mice per group). P < 0.016, t = 3.50; Holm-corrected two-tailed Student’s t-test. (f) ChIP assay for binding of HDAC2 to the mGlu2 promoter in HEK293 cells. Cells were transfected with mouse mGlu2 promoter (−241 to +97)–luc2P luciferase plasmid and Flag-HDAC2 plasmid and lysates immunoprecipitated with anti-Flag or control IgG 48 h later. The plasmid-derived mGlu2 promoter and a luc2P fragment were amplified by PCR using immunoprecipitated DNA (ChIP) and total DNA as a control (input). Representative of three independent experiments. (g) Luciferase activity 24 h after transfection of HEK293 cells transfected with mouse mGlu2 promoter–luciferase construct in combination with 1, 2 and 4 μg of Flag-HDAC2 construct (F3.8 = 12.11, P < 0.01, one-way ANOVA, n = 6 independent experiments performed in triplicate) or in combination with the deacetylase activity–deficient mutant construct Flag-HDAC2-H141A (F3.8 = 2.36, P > 0.05, one-way ANOVA, n = 3 independent experiments performed in triplicate). *P < 0.05; **P < 0.01; ***P < 0.001; Bonferroni’s post hoc test. Error bars, s.e.m.
mice expressing GFP (Fig. 5e). As activation of the mGlu2 receptor abolishes the head-twitch response induced by hallucinogens25,36, these findings correlate with the lower expression of mGlu2 in mice overexpressing HDAC2 in frontal cortex.

To complement the behavioral findings obtained with hallucinogens, we next investigated the effects of HDAC2 overexpression in frontal cortex on behavioral responses induced by the potent and selective noncompetitive NMDA receptor antagonist MK801 (dizocilpine). MK801 and other noncompetitive NMDA receptor antagonists evoke in healthy humans psychiatric and other symptoms resembling aspects of schizophrenia13. Previous findings demonstrate that activation of mGlu2, and not mGlu3, by the mGlu2 and mGlu3 receptor agonist LY379268 reduces the locomotor hyperactivity induced by MK801 (ref. 22). We found that neither HSV-GFP nor HSV-HDAC2 affected the locomotor activity induced by MK801 (Fig. 5f and Supplementary Fig. 5d). In notable contrast, however, HDAC2 overexpression diminished the ability of LY379268 (8 mg kg−1), the mGlu2 and mGlu3 agonist, to reduce the MK801-dependent locomotor response. Overexpression of HDAC1 or HDAC4 in frontal cortex did not affect the locomotor behavioral effects of MK801 or LY379268 (Fig. 5f and Supplementary Fig. 5d). Spontaneous locomotor activity and time spent in the center of an arena (protocols used in the evaluation of anxiety; see ref. 40) were comparable among mice that received intra–frontal cortex injections of HSV-2 vectors expressing HDAC1, HDAC2, HDAC4, and/or GFP (Supplementary Fig. 5e).

Deficits in the filtering or gating sensory and cognitive information have also been theorized to be core features of schizophrenia. Prepulse inhibition (PPI) is a measure of sensorimotor gating that refers to the reduction in the startle response produced by a low-intensity non-startling stimulus (the prepulse) presented shortly before the startle stimulus41. Patients with schizophrenia exhibit diminished PPI of the acoustic startle reflex42, which represents one operational measure of sensorimotor gating and cognitive impairment. We compared the effects of LY379268 on the impaired PPI response induced by MK801 in mice overexpressing HDAC2 and controls. The MK801-dependent deficit in the PPI test was not affected by virally mediated overexpression of HDAC2 (Fig. 5g). Consistent with previous findings in rodents and healthy human subjects41, LY379268 prevented the PPI deficits evoked by MK801 in HSV-GFP control mice. Notably, the behavioral effects of LY379268 on the MK801-dependent PPI deficits were significantly reduced in mice overexpressing HDAC2 (Fig. 5g). These data suggest that HDAC2 negatively regulates sensorimotor gating of the startle reflex.

Patients with schizophrenia also demonstrate impairments on a variety of working memory tests. Working memory requires the ability to form memory traces over a brief time period in order to manipulate this information to guide a goal-oriented behavior40. In rodents, working memory can be reliably assessed in a T-maze using a discrete-trial delayed spatial alternation task. We found that MK801 impaired choice accuracy in the T-maze delayed alternation
task, an effect that was reversed by LY379268 in HSV-GFP control mice (Fig. 5h and Supplementary Fig. 5g). In contrast, the effect of LY379268 on MK801-induced impairment in the T-maze was reduced in mice overexpressing HDAC2 in frontal cortex, as measured by the percentage of entries into the correct arms (Fig. 5h and Supplementary Fig. 5g; see also Supplementary Fig. 5f for T-maze task in uninfected control mice). Taken together, our findings indicate that increased expression of HDAC2 in mouse frontal cortex results in behaviors that are associated with impaired mGlu2 function.

**SAHA improves antipsychotic-like effects of clozapine**

Recent preclinical and clinical results suggest that drugs that activate the mGlu2 receptor may represent a new approach for the treatment of schizophrenia\(^\text{11-13,21-24}\). Our results thus far indicate that chronic atypical antipsychotics selectively upregulate the expression of HDAC2 in frontal cortex, which precisely parallels the decreased acetylation at the promoter region of the mGlu2 gene in both mouse and human. These findings led us to hypothesize that prevention of this repressive histone modification at the mGlu2 promoter with HDAC inhibitors would augment the therapeutic-like behavioral responses induced in mice by atypical antipsychotic drugs.

to directly examine this possibility, we first investigated the effects of two HDAC inhibitors, suberylanilide hydroxamic acid (SAHA) and MS-275, which are selective inhibitors of class I and II HDACs and have been shown to modulate mGlu2 expression in mammalian CNS\(^\text{43}\). Mice were stereotactically injected in the frontal cortex with a single dose of SAHA, MS-275 or vehicle and were analyzed 24 h later. Both SAHA and MS-275 increased mGlu2 and mGlu3 mRNA expression, whereas they did not affect SHT2A and SHT2C mRNA expression (Supplementary Fig. 4b). ChIP assays showed that histone H3 acetylation was increased at the promoter regions of the mGlu2 and mGlu3 genes by local administration of SAHA or MS-275 in frontal cortex (Supplementary Fig. 4c; see also Supplementary Fig. 4d-e for promoter assay and ChIP experiments with SAHA and MS-275, and another HDAC inhibitor trichostatin A in tissue culture). These data indicate that HDAC inhibitors stimulate mGlu2 promoter activity in vitro as well as in vivo in mouse frontal cortex.

Next, we directly compared the effects of chronic SAHA treatment with that of clozapine on the expression of mGlu2. We tested the effects of SAHA because MS-275 does not effectively penetrate the blood brain barrier when given systemically\(^\text{44}\), and our data validate the ability of chronic intraperitoneal administration of SAHA to increase histone

**Figure 6** Chronic treatment with SAHA prevents the repressive histone modifications induced at the mGlu2 promoter by chronic clozapine. (a) \[^3\text{H}\]Ketanserin binding (n = 6 independent experiments performed in triplicate). Effect of clozapine and/or SAHA, F\(_{5,16}\) = 43.59, P < 0.001 (extra sum-of-squares F-test, to determine whether the best-fit values differ between data sets). (b) Maximum number of binding sites (B\(_{\text{max}}\)) obtained from saturation curves in a. F\(_{3,12}\) = 9.55, P < 0.01 (one-way ANOVA). (c) \[^3\text{H}\]LY341495 binding (n = 6 independent experiments performed in triplicate). Effect of clozapine and/or SAHA, F\(_{5,16}\) = 69.34, P < 0.001 (extra sum-of-squares F-test). (d) B\(_{\text{max}}\) obtained from saturation curves in c. F\(_{3,12}\) = 9.55, P < 0.01 (one-way ANOVA). (e) Effect of clozapine and/or SAHA on expression of SHT2A (F\(_{3,28}\) = 8.58, P < 0.001), SHT2C (F\(_{3,20}\) = 0.85, P > 0.05), mGlu2 (F\(_{3,20}\) = 12.71, P < 0.001) and mGlu3 (F\(_{3,20}\) = 5.81, P < 0.01) mRNA in mouse frontal cortex assayed by qRT-PCR (one-way ANOVA, n = 6 mice per group). (f) Effect of clozapine and/or SAHA on association of histone H3 (H3ac) with the SHT2A promoter (H3ac with the SHT2A promoter; F\(_{3,12}\) = 7.40, P < 0.001), SHT2C (F\(_{3,28}\) = 0.53, P > 0.05), mGlu2 (F\(_{3,28}\) = 18.79; P < 0.001) or mGlu3 (F\(_{3,28}\) = 5.81; P < 0.01) promoters, measured by ChIP and quantitative PCR (one-way ANOVA, n = 8 mice per group). (g) Effect of clozapine and/or SAHA on head-twitch behavioral response to DOI (vehicle, n = 6; clozapine, n = 5; SAHA, n = 5; clozapine + SAHA, n = 6 mice per group). F\(_{5,16}\) = 35.74, P < 0.001; one-way ANOVA. (h) Effect of LY379268 (LY379; 1.5 mg kg\(^{-1}\)) and/or SAHA on MK801-stimulated locomotor activity (vehicle, n = 12; LY379, n = 8; SAHA, n = 11; LY379 + SAHA, n = 12 mice per group). F\(_{3,39}\) = 5.30, P < 0.01 (one-way ANOVA). (i) Effect of LY379 (3 mg kg\(^{-1}\)) and/or SAHA on the MKB1-diminished prepulse inhibition (PPI) of startle (vehicle + vehicle, n = 17; vehicle + MK801, n = 24; vehicle + MK801 + LY379, n = 24; SAHA + vehicle, n = 17; SAHA + MK801, n = 17; SAHA + MK801 + LY379, n = 23 mice per group). Effect of SAHA, F\(_{1,115}\) = 4.43, P < 0.05; effect of LY379, F\(_{2,115}\) = 4.75, P < 0.05 (two-way ANOVA). *P < 0.05, **P < 0.01, ***P < 0.001; NS, not significant; Bonferroni’s post hoc test. Error bars, s.e.m.
H3 acetylation in frontal cortex, an effect that was not observed with chronic clozapine (Supplementary Fig. 4f,g). As above (Fig. 1 and Supplementary Fig. 1), chronic clozapine downregulated the expression of 5HT2A and mGlu2, but not 5HT2C and mGlu3, in mouse frontal cortex (Fig. 6a–e). Notably, although chronic SAHA had no effect on 5HT2A (Fig. 6a,b,e), it increased the expression of mGlu2 and mGlu3 and, more importantly, reversed the downregulation of mGlu2 caused by chronic clozapine (Fig. 6c–e). Such transcriptional regulation of the mGlu2 and mGlu3 genes, and the lack of regulation of the 5HT2A gene, was tightly correlated with active binding of acetyl–histone H3 at their respective promoters (Fig. 6f). Together, these data show that chronic SAHA increases the expression of mGlu2 and mGlu3, and that adjunctive SAHA treatment prevents the repressive epigenetic changes induced at the mGlu2 promoter by chronic clozapine.

To examine the therapeutic-like relevance of our findings, we next investigated whether abolition by SAHA of the clozapine-induced histone modifications at the mGlu2 promoter in frontal cortex is associated with alterations in behavioral responses induced by antipsychotic drugs. We treated mice with clozapine, risperidone, haloperidol or vehicle chronically (21 d) or subchronically (2 d) before assessing the head-twitch response to the hallucinogenic drug DOI 1 d after the last injection. The head-twitch response induced by DOI (2 mg/kg) was decreased by chronic, but not subchronic, clozapine or risperidone (Supplementary Fig. 6a,b). Chronic haloperidol did not affect the head-twitch response induced by DOI (Supplementary Fig. 6a). We also observed that chronic SAHA diminished the head-twitch response induced by DOI to a similar extent to that found after chronic clozapine (Fig. 6g). Furthermore, chronic SAHA potentiated this antipsychotic-like effect of chronic clozapine (Fig. 6g), a lasting behavioral effect that was even further accentuated 5 d after the last chronic injection (Supplementary Fig. 6c).

We next compared the effects of chronic SAHA on the behavioral response induced by the psychotomimetic drug MK801. We found that neither a low dose of LY379268 (1.5 mg kg\(^{-1}\)) alone nor chronic SAHA alone influenced the locomotor response induced by MK801 (Fig. 6h and Supplementary Fig. 6c). However, the MK801-dependent locomotor hyperactivity was attenuated in mice that received both chronic SAHA and acute LY379268 (Fig. 6h and Supplementary Fig. 6c; see also Supplementary Fig. 6d for selection of a low dose of LY379268 that by itself does not affect the locomotor activity induced by MK801). We also compared the effects of chronic SAHA on the impaired PPI response induced by MK801. As shown above (Fig. 5f), MK801 induced PPI deficits in control mice, an effect that was reversed by LY379268 (Fig. 6i). Notably, we also found that chronic treatment with SAHA prevented the PPI deficits evoked by MK801, an effect that was similar to that induced by LY379268 (Fig. 6i). Together, these findings demonstrate that chronic treatment with the HDAC inhibitor SAHA enhances the behavioral effects induced by LY379268 using three different mouse models. Recent pathophysiological studies highlight the role of altered brain connectivity and synaptic plasticity in schizophrenia.\(^2\) Our data do not exclude the possibility that HDAC2‐dependent alterations in the expression of other genes, each of which has previously been implicated in cognitive function and synaptic plasticity, also affect the behavioral responses induced by chronic treatment with SAHA (Supplementary Fig. 4h).

**DISCUSSION**

Chromatin remodeling has been implicated in the neurochemical mechanisms by which antipsychotic drugs achieve their clinical efficacy.\(^8\),\(^47\). Although these findings provide compelling evidence for the involvement of epigenetic processes in the molecular events associated with the treatment of schizophrenia, they do not exclude the possibility of compensatory mechanisms that may emerge in response to chronic antipsychotic drug exposure and ultimately restrain their therapeutic effects. Compensatory mechanisms are seen as negative correlations between processes that are pivotal for system function.\(^48\). Within an individual neuron or individual animal, alterations in one parameter may produce minor changes in the state of the system if there are mechanisms that cause adjustments in another parameter to compensate for the first change. Here we have demonstrated that chronic treatment with atypical antipsychotic drugs markedly decreased the density of 5HT2A binding sites in mouse frontal cortex, which led to repressive histone modifications at the promoter region of the mGlu2 gene. Furthermore, our data reveal that HDAC2 is critical in mediating these 5HT2A‐dependent repressive epigenetic modifications at the mGlu2 promoter in mouse and human frontal cortex. We found that chronic administration of atypical antipsychotic drugs selectively upregulated HDAC2 expression and binding to the mGlu2 promoter, an effect that was associated with the regulation of HDAC2 promoter transcriptional function by activation of the 5HT2A receptor. We then demonstrated that such overexpression of HDAC2 in frontal cortex was sufficient to downregulate mGlu2 expression and its physiological inhibitory effects, which exacerbated schizophrenia-like behavior. Activation of mGlu2 is well known to repress cellular, electrophysiological and behavioral responses that require the 5HT2A receptor.\(^20\). Similarly, drugs that activate the mGlu2 receptor have potential for the treatment of schizophrenia, whereas 5HT2A agonists, such as hallucinogenic compounds, result in the opposite effect. Taken together, these data suggest that the decreased density of 5HT2A binding sites produced by chronic treatment with atypical antipsychotic drugs results in a compensatory mechanism of repressive chromatin structure at the promoter region of the mGlu2 gene, with consequently less inhibitory effects of the mGlu2 receptor on 5HT2A‐regulated pathways and behaviors.

We also observed that adjunctive SAHA abolished the repressive histone modifications induced at the mGlu2 promoter by chronic atypical antipsychotics, which augmented the behavioral effects induced by atypical and glutamate antipsychotics. Overall, our findings support the hypothesis that compensatory epigenetic events at the mGlu2 promoter may be responsible for the high incidence of patients who do not benefit from conventional therapy with atypical antipsychotic drugs, and they provide a biochemical explanation for the clinical association of pharmacological inhibition of HDACs with improved schizophrenia treatment (Supplementary Fig. 7).

Previous studies from our laboratory have shown that 5HT2A and mGlu2 interact through specific transmembrane domains to form a G protein–coupled receptor (GPCR) heterocomplex in mouse and human frontal cortex.\(^23\). The signaling properties of this receptor heterocomplex have been proposed to be necessary for therapeutic-like effects of atypical antipsychotic drugs.\(^23\). We found here that chronic atypical antipsychotic drugs induced 5HT2A‐dependent repressive histone modifications at the mGlu2 promoter in frontal cortex, which is consistent with previous reports describing effects of long‐term treatment with 5HT2A agonists on behavioral responses that require the mGlu2 receptor.\(^36\),\(^37\). Further work will be directed to assessing the relative contributions of these epigenetic factors to the modulation of expression of 5HT2A and mGlu2 as a GPCR heterocomplex that may balance the response to ligand inputs in cortical pyramidal neurons.

Clozapine is the only antipsychotic drug with proven superior efficacy in treatment‐resistant schizophrenia.\(^4\). We found that both clozapine and risperidone, but not haloperidol, decreased the density of 5HT2A binding sites in mouse frontal cortex. Similar findings on
5HT2A receptor binding have been reported in post-mortem human brain from subjects with treated schizophrenia23,49. These results suggest that the 5HT2A receptor is involved in treating the psychotic symptoms of schizophrenia and that downregulation of 5HT2A receptor binding sites by chronic atypical antipsychotic drugs may be one of the mechanisms underlying their therapeutic effects. Notably, only chronic clozapine induced changes in histone acetylation at the 5HT2A promoter that correlated with its transcriptional repression. Although further investigation is needed to understand the mechanisms and consequences of this finding, it is tempting to speculate that these histone modifications at the promoter of the 5HT2A gene may account for the enhanced antipsychotic properties of clozapine. Further work is also needed to determine the molecular mechanisms responsible for downregulation of 5HT2A mRNA expression in sub-cortical regions by chronic treatment with clozapine.

A notable finding of the current study is that chronic treatment with clozapine upregulated HDAC2 in mouse frontal cortex. In concordance with the effects of clozapine in murine models, the expression of HDAC2 was elevated in post-mortem frontal cortex concordance with the effects of clozapine in murine models, the expression of HDAC2 was elevated in post-mortem frontal cortex. In mouse frontal cortex. In mouse frontal cortex. Notably, chronic clozapine upregulated HDAC2 in mouse frontal cortex. In mouse frontal cortex. The upregulation of HDAC2 in frontal cortex of individuals with schizophrenia and that downregulation of 5HT2A receptor binding sites by chronic atypical antipsychotic drugs may be one of the mechanisms underlying their therapeutic effects. Notably, only chronic clozapine induced changes in histone acetylation at the 5HT2A promoter that correlated with its transcriptional repression. Although further investigation is needed to understand the mechanisms and consequences of this finding, it is tempting to speculate that these histone modifications at the promoter of the 5HT2A gene may account for the enhanced antipsychotic properties of clozapine. Further work is also needed to determine the molecular mechanisms responsible for downregulation of 5HT2A mRNA expression in sub-cortical regions by chronic treatment with clozapine.

The ultimate goal of understanding the pathophysiology of schizophrenia is to develop therapeutic strategies that improve or restore normal brain activity and, ultimately, ameliorate the associated deficits in sensory processing, perception and memory function. The functions of HDAC2 described here have implications for the molecular basis of the limited response to treatment with atypical antipsychotics. We propose that atypical antipsychotic drugs induce a selective upregulation of HDAC2 in frontal cortex of individuals with schizophrenia, which alters the chromatin state of the mGlu2 promoter and thereby limits the therapeutic effects of these agents. Identification of the epigenetic mechanisms through which administration of HDAC inhibitors potentiates the biochemical and behavioral responses to atypical antipsychotics will help in discovering more effective treatments to improve the clinical efficacy of the available antipsychotic medications. Specifically, our findings encourage the development and testing of HDAC2-selective inhibitors for schizophrenia.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.K. and J.G.-M. designed experiments, analyzed data and wrote the manuscript. M.K. performed experiments. J.G.-M. supervised the research. T.H. performed behavior experiments. A.G.-B. performed experiments in post-mortem schizophrenia-affected brain. A.K. assisted with cloning and performed promoter assay experiments. J.L.M. performed radioligand binding experiments. M.H. assisted with ChIP and DNA methylation assays. G.M., A.M.G., J.H., A.U. and J.M.K. assisted with preparation of R.L.N. performed viral packaging. L.S. performed biostatistical analyses. I.F.C. and J.J.M. obtained and classified post-mortem human brain samples. S.A.G. supervised by S.J.R., helped with stereotaxic surgery. P.J.K. and D.M.D. supervised by E.J.N., helped with viral overexpression. A.K.F., supervised by M.-H.H., performed electrophysiological studies. N.T. supervised by J.D.B., helped with prepulse inhibition test. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

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ONLINE METHODS

Materials and drug administration. 1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane (DOI), [5R,10S]-(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d]cyclohepten-5,10-imine hydrogen maleate (dizocilpine, (-)-MK-801), serotonin (5HT), dopamine (DA), and picrotoxin were purchased from Sigma-Aldrich. Clozapine, risperidone, haloperidol, fluoxetine, (1R,4R,5S,6R)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268), and trichostatin A (TSA) were obtained from Tocris Cookson Inc. Suberyolylanilide hydroxamic acid (vorinostat; SAHA) was purchased from Cayman Chemical. N-2-(Aminophenyl)-4-[N-(pyridine-3-ylmethoxy-carbonyl)aminomethyl]benzamide (MS-275) was obtained from Enzo Life Sciences. The injected doses (i.p.) were clozapine, 10 mg/kg; risperidone, 4 mg/kg; haloperidol, 1 mg/kg; fluoxetine, 20 mg/kg; and SAHA, 20 mg/kg; unless otherwise indicated. DOI and LY379268 were dissolved in saline. MK-801, SAHA, risperidone, haloperidol and fluoxetine were injected after suspension in a minimal amount of DMSO and made up to volume with saline. Clozapine was dissolved in DMSO supplemented with a minimal amount of acetic acid and suspended in saline. SAHA and MS-275 were administered locally in frontal cortex after suspension in a minimal amount of DMSO and made up to volume with saline. [3H]Ketanserin and [3H]raclopride were purchased from PerkinElmer Life and Analytical Sciences, Inc. [3H]25-2-amino-2-(1S,2S,2-carboxycylopropan-1-yl)-3-(xanth-9-yl)-propionic acid ([3H]LY341495) was purchased from American Radiolabeled Chemicals, Inc. All other chemicals were obtained from standard sources.

Transfection of HEK293 cells. Human embryonic kidney (HEK293) cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% (v/v) fetal bovine serum at 37 °C in a 5% CO2 humidified atmosphere. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

Plasmid construction. All PCR reactions were performed using PfU Ultra Hotstart DNA polymerase (Stratagene) in a Mastercycler Ep Gradient Auto thermal cycler (Eppendorf). All the constructs were confirmed by DNA sequencing. For the HDAC2 promoter construct, mouse HDAC2 promoter (−481 to +111 bp) was PCR amplified from mouse genomic DNA (Clontech) using the following primers: 5′-TGAAACAGCTGGGAAATATCG-3′ and 5′-GCCAGACAGAAGGGCTGAC-3′. The amplicon was inserted into pCR-blunt vector (Invitrogen), and then sequenced. The product was re-amplified using the primers 5′-GAATTCAACGGGCTGGGAAATATCG-3′ and 5′-CTAGATGAGGACAGGCTGAC-3′, and then digested with XhoI and HindIII and subcloned into the XhoI and HindIII sites of pGL4.11 [luc2P] plasmid (Promega). For the mGlu2 promoter construct, mouse mGlu2 promoter (−241 to +97 bp) was PCR amplified from mouse genomic DNA (Clontech) using the following primers: 5′-CCACCTCTGGTGGCACTTCCG-3′ and 5′-CAGAAGGGCTGACTACCTAC-3′. The amplicon was inserted into pCR-blunt vector (Invitrogen), and then sequenced. The product was re-amplified using the primers 5′-GAATTCAACGGGCTGGGAAATATCG-3′ and 5′-CTAGATGAGGACAGGCTGAC-3′, and then digested with XhoI and HindIII and subcloned into the XhoI and HindIII sites of pGL4.11 [luc2P] plasmid (Promega). For the Hsv-HDAC2 construct, mouse HDAC2 cDNA (gift of Dr. Patrizia Casaccia) along with the Flag tag was PCR amplified using the following primers: 5′-AGTGG atcATGGACTCAAAGGACAGGCA-3′ and 5′-CCGGGCGGCTGAGAGTT-3′. The PCR product was inserted into the BamHI and XhoI sites of pcDNA3.1 (+) plasmid (Invitrogen) and the bicistronic p1005+ HSV plasmid expressing GFP under the control of the CMV promoter5, and then sequenced. The catalytically inactive mutant HDAC2-H141A (ref. 52) was constructed using site-directed mutagenesis according to the manufacturer’s protocol (Stratagene).

Luciferase reporter assay. Luciferase activity was measured with a luminometer (TD-20/20; Turner Biosystems) using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Transfection efficiency was normalized with coexpressed pGL4.75 hRluc/CMV (Promega) (0.02 μg).

Mice. Experiments were performed on adult (8–12 weeks old) male 129S6/SvEJ mice. 5HT2A−/− mice, developed in 129S6/SvEJ strain, have been previously described19,34. For experiments involving 5HT2A+/− mice, 5HT2A−/− littersmates were used as controls. Animals were housed at 12 h light/dark cycle (lights on, 8:00 to 20:00) at 23 °C with food and water ad libitum. For the working memory test (see below), experiments were performed on adult (8–14 weeks) male C57BL/6 mice because 129S6/SvEJ mice are best avoided for this protocol53. The Institutional Animal Use and Care Committee at Mount Sinai School of Medicine approved all experimental procedures. More than ~1,000 mice were used.

Mouse brain samples. The day of the experiment, mice were killed by cervical dislocation, and bilateral frontal cortex (bregma 1.90 to 1.40 mm) was dissected and frozen at −80 °C, or immediately processed for RNA extraction, chromatin immunoprecipitation and/or biochemical assays. The coordinates were taken according to a published atlas of the 129S6/Sv mouse strain54.

Post-mortem human brain tissue samples. Human brains were obtained at autopsies performed in the Basque Institute of Legal Medicine, Bilbao, Spain, in compliance with policies of research and ethical boards at Mount Sinai School of Medicine and the University of the Basque Country for post-mortem brain studies. Deaths were subjected to retrospective searching for previous medical diagnosis and treatment using examiner’s information and records of hospitals and mental health centers. After searching of ante-mortem information was fulfilled, 21 subjects who had met criteria of schizophrenia according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV)55 were selected. A toxicological screening for antipsychotics, other drugs and ethanol was performed on blood, urine, liver and gastric contents samples. All subjects who were drug-free before death (as revealed by the absence of prescriptions in medical histories) also gave negative results in the toxicological screening. The toxicological assays were performed at the National Institute of Toxicology, Madrid, Spain, using a variety of standard procedures including radioimmunoassay, enzymatic immunoassay, high-performance liquid chromatography and gas chromatography-mass spectrometry. Controls for the present study were chosen among the collected brains on the basis, whenever possible, of the following cumulative criteria: (1) negative medical information on the presence of neuropsychiatric disorders or drug abuse; (2) appropriate gender, age, post-mortem delay (time between death and autopsy), and freezing storage time to match each subject in the schizophrenia group; (3) sudden and unexpected death (motor vehicle accidents); and (4) toxicological screening for psychotropic drugs with negative results except for ethanol. Specimens of prefrontal cortex (Brodmann’s area 9) were dissected at autopsy (0.5–1 g tissue) on an ice-cooled surface and immediately stored at −80 °C until use. The definitive pairs of antipsychotic-untreated subjects with schizophrenia and respective matched controls, and the definitive pairs of atypical antipsychotic-treated subjects with schizophrenia and respective matched controls are shown in Supplementary Table 1. Pairs of samples from subjects with schizophrenia and matched controls were processed simultaneously and under the same experimental conditions. Tissue pH values56 were within a relatively narrow range (control subjects: 6.33 ± 0.05; subjects with schizophrenia: 6.23 ± 0.07). All brain samples were assayed for RNA integrity number (RIN)59 using the Agilent 2100 Bioanalyzer (Applied Biosystems) (see Supplementary Table 1).

Quantitative real-time PCR. Quantitative real-time PCR (qRT-PCR) assays were carried out in quadruplicate as previously described with minor modifications19,25,34. See Supplementary Table 2 for primer pair sequences.
Chromatin immunoprecipitation assay in mouse frontal cortex. Chromatin immunoprecipitation (ChIP) experiments were performed using the EZ-Magna ChIP Kit (Millipore) according to the manufacturer's instructions. Primary antibodies were added to diluted lysates, and incubated at 4 °C for 12 h with 20 μl of fully suspended protein A or G magnetic beads (Millipore). The following primary antibodies were used: acetyl-histone H3 (Millipore 06-599, 1:200), acetyl-histone H4 (Millipore 06-866, 1:200), mono/di/tri-methyl-histone H3 (Lys4) (Millipore 05-791, 1:300), tri-methyl-histone H3 (Lys9) (Millipore 07-442, 1:250), tri-methyl-histone H3 (Lys27) (Millipore 07-449, 1:100), HDAC1 (Abcam 31263, 1:200), HDAC2 (Abcam 12169, 1:200), HDAC4 (Abcam 1437, 1:200) and Flag (Sigma F7245, 1:200). Input and immunoprecipitated DNAs were subjected to quantitative real-time PCR (see Supplementary Table 2 for primer pair sequences).

Immunoprecipitation of native chromatin in post-mortem human brain. Post-mortem tissue samples (50 mg) were homogenized with douncing buffer (4 mM HEPES, pH 7.6, supplemented with 1 mM EDTA, 0.01% ascorbic acid, and 5 mM MgCl2) and histone H1 (Santa Cruz sc-10806, 1:200). Incubation with the secondary antibody (1:5,000) coupled to peroxidase (Amersham Biosciences NA931V/AD and NA934V/AC) was performed at room temperature for 90 min, followed by repeated washing with TBST. Immunoreactive proteins were visualized with enhanced chemiluminescence (Thermo Scientific) according to the manufacturer's instructions. Western blot experiments in mouse brain samples were quantified by densitometry using the NIH Image 1.62 software. Nuclear-cytoplasmic fractionation was performed using NE-PER extraction kit (Thermo Scientific), following manufacturer's instructions (see Supplementary Fig. 4g). Western blot experiments in post-mortem human brain samples were analyzed with the Odyssey infrared imaging system (LI-COR Biosciences). The membranes were incubated with secondary antibodies (Alexa 680 Fluor goat anti-mouse, Invitrogen A21057, 1:1,500; Alexa 680 Fluor goat anti-rabbit, Invitrogen A21076, 1:1,500; Alexa 680 Fluor donkey anti-goat, Invitrogen A21084, 1:2,500; IRDye 800 donkey anti-mouse, Rockland Immunocytologicals 610-732-002, 1:10,000; IRDye 800 goat anti-rabbit, Rockland Immunocytologicals 611-312-002, 1:10,000). The membranes were then scanned on an Odyssey infrared imaging system, and images were acquired and analyzed according to manufacturer's instructions.

Viral mediated gene transfer. HDAC1, HDAC2 and HDAC4 cDNAs were subcloned into a published bicistronic HSV-GFP virus vector (see) and viral particles were then packaged as described previously. HSV-HDAC1, HSV-HDAC2, HSV-HDAC4, or control HSV-GFP was injected into the frontal cortex by stereotaxic surgery according to standard methods. Mice were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg) during the surgery. The virus was delivered bilaterally with a Hamilton syringe at a rate of 0.1 μl/min for a total volume of 0.5 μl on each side. The following coordinates were used: +1.6 mm rostral-caudal, −2.4 mm dorsal-ventral, +2.6 mm medial-lateral from bregma (relative to dura) with a 10° lateral angle. The coordinates were taken according to a published atlas of the 129/Sv mouse strain. The Nissl staining of the coronal brain slice was taken from the mouse brain atlas with author's permission (Fig. 5a). All experiments were performed 3–4 after the viral infection, when transgene expression is maximal. Virally mediated HDAC1, HDAC2 and HDAC3 overexpression levels in frontal cortex were confirmed by western blotting and quantitative real-time PCR (Supplementary Fig. 5b and data not shown).

Immunohistochemistry. Immunohistochemistry experiments were performed as previously reported. GFP immunoreactivity was assayed by using a polyclonal antibody (Santa Cruz sc-8334; 1:100) and Alexa 488 dye-conjugated anti-rabbit antibody (1:500).

Electrophysiology. Whole-cell recordings were obtained from deep layer neurons of the frontal cortex in acute brain slices from 129Sv mice that had been stereotaxically injected into the frontal cortex with HSV-GFP or HSV-HDAC2 (see virally mediated gene transfer above). The preparation of acute brain slices of the frontal cortex and recordings from the frontal cortex were performed as described previously. Briefly, the artificial cerebrospinal fluid (aCSF) contained the following (in mM): NaCl, 128; KCl, 2; CaCl2, 2; MgSO4, 2; NaHCO3, 2; and O2, 95% and CO2, 5% (pH 7.35, 295–305 mOsm). Sucrose aCSF was defined by fully replacing NaCl with 254 mM sucrose in aCSF, and used during the slice cutting. Patch pipettes (3–5 MΩ) for whole-cell voltage clamp recordings were filled with an internal solution containing the following: 128 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 10 mM D-glucose, 24 mM NaHCO3, 2 mM CaCl2, and 2 mM MgSO4 oxygenated with 95% O2 and 5% CO2 (pH 7.35, 295–305 mOsm). The experiments were carried out at 30 °C. GFP-positive cells were visualized with an upright fluorescence microscope using infrared differential interference contrast illumination and identified by their pyramidal shape and size and prominent apical dendrite in the frontal cortex. The resting membrane potential and spontaneous EPSCs were recorded in voltage clamp mode, holding −70 mV using an amplifier Multiclamp 700B (Molecular Devices). Data acquisition was acquired with pClamp 10. Series resistance was monitored throughout experiments that were terminated whenever significant changes (>20%) in series resistance occurred. DOI and LY379268 were kept as concentrated stocks and diluted in aCSF immediately before experiments. After 2 min of stable baseline recording, DOI (5 μM) was applied for 2–4 min and subsequently DOI (5 μM) and LY379268 (1 μM) were applied for 2–4 min. These concentrations were chosen according to previous findings. The frequency and
amplitude of spontaneous EPSCs were measured for >2 min. All experiments were performed in the presence of picrotoxin (50 µM). Responses were obtained from 10 neurons from each animal, with means per animal then used to generate group means and perform statistical analyses (Origin 7.0). Data were analyzed by use of Clampfit 10.

Drug administration in mouse frontal cortex. Stereotaxic surgery was performed as described above. SAHA (10 µM), MS-275 (10 µM), or vehicle was delivered bilaterally with a Hamilton syringe at a rate of 0.1 µl/min for a total volume of 0.5 µl on each side. ChIP and quantitative real-time PCR experiments were performed 24 h after local administration of the tested drug in frontal cortex.

Head-twitch behavioral response. Head-twitch behavioral response (rapid lateral movements of the head similar to the pinna reflex) is reliably elicited by a variety of hallucinogenic drugs, such as DOI, DOM, DOB, mescaline, LSD and psilocin, and is absent in 5-HT2A knockout mice. 53, 54. Dissociative drugs, such as ketamine, PCP and MK801, induce head-weeping behavioral response (slow, side to side lateral head movement) in rodents. 55. Repetitive shaking of the body (wet-dog shake behavior) is commonly observed in rodents during morphine withdrawal. 56. We investigated the head-twitch behavior induced by DOI as a mouse behavior model of hallucinogenic 5HT2A agonist action. Experiments were carried out as previously. 20. Videotapes were scored for head-witches by an experienced observer blind to drug treatment and/or virally mediated gene transfer.

Locomotor activity. Motor function (locomotor activity) was assessed as previously reported. 29

Prepulse inhibition of startle. Mice were placed in acoustically isolated startle chambers (MED Associates). The test started with a 10 min acclimation followed by three sessions of trials. Background noise was 70 dB throughout the acclimation and trial periods. Sessions 1 and 3 included 10 trials of startle stimuli (120 dB; 40 ms). Session 2 consisted of 56 trials in which startle response magnitude, peak latency, and onset latency to each stimulus were obtained for trials in which the startle stimulus was presented alone or preceded by 100 ms with a 15-ms prepulse. The prepulse amplitude was 2, 6, or 8 dB above background. The startle response was defined as changes in force on the floor (i.e., “displacement”) between 30 and 70 ms after the onset of the startle stimulus. Animals that had a peak response in less than 20 ms or after 80 ms of the presentation of the stimuli were excluded. PPI was calculated as 100 × (PPR/SR), where PPR is the average latency of startle response across trials presenting prepulse, and SR is the average latency of startle response from trials in which the startle was presented alone. Mice were injected with vehicle or MK801 (0.1 mg/kg) 15 min after injection with LY379268 (5 mg/kg), and 15 min before the start of the PPI experiment.

Discrete-trials rewarded delayed spatial alternation. Working memory was tested using a T-maze alternation task. The experiments were performed in a T-maze constructed of wood and painted black. The walls were 15 cm high, and the alley was 10.5 cm wide. The length of the mail alley was 28 cm, and the length of the side alley was 22.5 cm. The side alleys were closed off from the main alley by movable doors. A week before habituation, all animals were partially food-deprived (each animal received 2 g of food per day) and remained that way throughout the remaining part of the experiment. This maintained each animal above 85% of its free-feeding body weight. A video camera was situated ~1 m above the T-maze to videotape the test session. The T-maze was cleaned between different animals but not between different trials. The food reward was a 14 mg salt pellet (Bio-Serv F05684). The full experiment consisted of three parts: habituation, training, and testing. During habituation, all animals were placed on the T-maze until they ate two pieces of food or 90 s had elapsed. This was repeated three times a day for 5 d. During training, all animals received six trials per day. Each trial consisted of two runs, a forced run and a free run. On the forced run, mice were forced to obtain a piece of food from one goal arm of the T-maze with the other goal arm blocked by its door. Animals were then placed back into the start arm for 10 s delay period. At the beginning of the free run, the mice were allowed to choose either goal arm. If the mice chose the arm opposite to the one they had been forced into during the forced run, they received the food reward. If the mice chose the same arm that they had been forced into, they received no food reward. The training period ended after control animals made >70% correct choices on two consecutive days. Animals took ~7–12 d to reach the criterion. Animals that did not reach the criterion by 14 d were rejected from the study. In this study, ~5% of mice belonged to this latter group. Mice were then tested for their performance at 10 or 40 s delay periods. Mice were given three 10-s-delay and three 40-s-delay trials during the day of testing. For HSV-mediated transgene expression in frontal cortex, drug testing was performed 3–4 d after the viral infection. The sequence of delays and forced run food locations (left or right) were randomized, with the stipulation that the same delay or the same forced arm location could not be used for three trials in a row. Goal entries were defined as placing four paws in the arm.

Statistical analysis. Radioligand binding data were analyzed using a nonlinear curve fitting software (GraphPad Prism). An extra-sum-of-squares (F-test) was used to determine statistical difference for simultaneous analysis of binding saturation curves. For all ChIP and mRNA data, fold changes relative to controls were determined using the corrected C method. 57. In immunoblot assays in post-mortem human brain, the theoretical amount of protein in each sample was obtained by interpolation of the integrated optical density in the standard curve and compared with the real amount of protein loaded into the gel well. 58. Statistical significance of head-twitch experiments was assessed by normalizing (square root) the number of events. 59. Statistical significance of qRT-PCR, ChIP, and western blot experiments was assessed by normalizing (log) the fold changes. 59. Statistical significance of experiments involving three or more groups and two or more experimental conditions was assessed by two-way ANOVA followed by Bonferroni’s post hoc test. Statistical significance of experiments involving three or more experimental conditions was assessed by one-way ANOVA followed by Bonferroni’s post hoc test. Statistical significance of experiments involving two experimental conditions was assessed by Student’s t-test. The effects on mRNA expression and histone modifications of multiple genes was assessed by correcting the α value for multiple independent null hypothesis. This was performed by using the Holm’s sequentially rejective Bonferroni method as follows: 46, 60.

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