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ARTICLE

Chronic clozapine treatment restrains via HDAC2 the performance of mGlu2 receptor agonism in a rodent model of antipsychotic activity

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Preclinical findings in rodent models pointed toward activation of metabotropic glutamate 2/3 (mGlu2/3) receptors as a new pharmacological approach to treat psychosis. However, more recent studies failed to show clinical efficacy of mGlu2/3 receptor agonism in schizophrenia patients. We previously proposed that long-term antipsychotic medication restricted the therapeutic effects of these glutamatergic agents. However, little is known about the molecular mechanism underlying the potential repercussion of previous antipsychotic exposure on the therapeutic performance of mGlu2/3 receptor agonists. Here we show that this maladaptive effect of antipsychotic treatment is mediated mostly via histone deacetylase 2 (HDAC2). Chronic treatment with the antipsychotic clozapine led to a decrease in mouse frontal cortex *mGlu2* mRNA, an effect that required expression of both HDAC2 and the serotonin 5-HT_{2A} receptor. This transcriptional alteration occurred in association with HDAC2-dependent repressive histone modifications at the *mGlu2* promoter. We found that chronic clozapine treatment decreased via HDAC2 the capabilities of the mGlu2/3 receptor agonist LY379268 to activate G-proteins in the frontal cortex of mice. Chronic clozapine treatment blunted the antipsychotic-related behavioral effects of LY379268, an effect that was not observed in *HDAC2* knockout mice. More importantly, co-administration of the class I and II HDAC inhibitor SAHA (vorinostat) preserved the antipsychotic profile of LY379268 and frontal cortex mGlu2/3 receptor density in wild-type mice. These findings raise concerns on the design of previous clinical studies with mGlu2/3 agonists, providing the rationale for the development of HDAC2 inhibitors as a new epigenetic-based approach to improve the currently limited response to treatment with glutamatergic antipsychotics.

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INTRODUCTION

Schizophrenia affects up to 1% of the global population, and tends to manifest during adolescence and young adulthood [1, 2]. This early onset usually translates into life-long symptomatic management with antipsychotic medications. The symptomatology of schizophrenia is complex, but can be grouped into three major categories: psychotic or positive, negative, and cognitive symptoms. However, not all three domains of the disease respond equally to pharmacological intervention. In addition to impairments in cognitive domains associated with long-term use of antipsychotic medications [3–7], treatment discontinuation due to lack of therapeutic benefits and side effects is common [8]. These unmet clinical needs underline the necessity of novel and more efficient therapeutic targets for schizophrenia treatment.

All antipsychotic medications currently approved target principally dopamine D_2 and serotonin 5-HT_{2A} receptors [9, 10]. However, the abovementioned liabilities associated with these traditional antipsychotics targeting the monoaminergic system led to the development of novel compounds that regulate glutamatergic signaling as promising drug candidates for the treatment of schizophrenia and other psychiatric conditions [11, 12]. The metabotropic glutamate 2 and 3 (mGlu2 and mGlu3) receptor subtypes are phylogenetically and functionally related G protein-coupled receptors (GPCRs) classified within group II mGlu receptors. The similarity between the effect of *N*-methyl-D-aspartate (NMDA) non-competitive antagonists, such as ketamine, phencyclidine (PCP), and MK801, in healthy volunteers and some of the core symptoms of schizophrenia led to the development of animal models of the disease in which mGlu2/3 receptor agonists, such as LY354740, LY379268, and LY4040398, demonstrated antipsychotic-like properties, primarily via mGlu2 [13–18].

Despite the mounting preclinical evidence, and successful preliminary clinical studies on the mGlu2/3 receptor agonist prodrug LY2140023 (pomaglumetad) [19], follow-up studies failed to demonstrate the efficacy of LY2140023 in the treatment of

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positive and negative symptoms of schizophrenia [20–22]; thus, hindering the development of drugs targeting mGlu2/3 receptors for the treatment of schizophrenia.

The enzymatic action of histone deacetylases (HDACs) on lysine acetylation of histone H3 (H3ac) and H4 (H4ac) render a more packed chromatin architecture less accessible to genetic transcriptional modification [23–28]. In the central nervous system, HDAC inhibitors have shown promise as a treatment to combat cognitive deficits in rodent models of neuropsychiatric disorders [23, 24, 26]. Our work has focused on HDAC2, which we and others have shown to associate physically with the promoter region of genes implicated in synaptic plasticity and cognition, including the *mGlu2* [29–31].

We showed that chronic treatment with the serotonergic atypical antipsychotics clozapine or risperidone, but not with the primarily dopaminergic antipsychotic haloperidol, decreased both transcription and density of the mGlu2 receptor in the mouse frontal cortex [30, 32]. Our previous data also suggested that H3ac was strongly decreased at the *mGlu2* (*Grm2*) promoter in the frontal cortex of mice chronically treated with atypical, but not with typical, antipsychotics [30]. We proposed that this occurred via a signaling mechanism that required a 5-HT_{2A} receptor-dependent upregulation and increased binding of HDAC2 to the promoter region of the *mGlu2* gene. Based on these findings, we developed the hypothesis that repressive histone modifications induced at the *mGlu2* promoter by chronic atypical, but not typical, antipsychotic treatment would consequently restrict the mGlu2 receptor-dependent antipsychotic effects of mGlu2/3 receptor agonists [17, 27, 33]. Remarkably, this hypothesis raised by our previous preclinical and postmortem human brain studies was further supported by a recent post hoc analysis suggesting that the antipsychotic effects of LY2140023 were comparable to risperidone in schizophrenia patients previously treated with typical antipsychotics (haloperidol), but absent in patients with previous exposure to atypical antipsychotics (e.g., clozapine and olanzapine) [34].

Our previous findings demonstrated that downregulation of transcription of *mGlu2* by chronic atypical antipsychotic treatment was associated with increased binding of HDAC2, and decreased H3ac at the *mGlu2* promoter region in the mouse frontal cortex [29, 30]. Considering that class I HDACs exert their role as transcriptional regulators through interactions with multi-protein complexes [25], whether HDAC2 is necessary for the repressive histone modifications induced at the *mGlu2* promoter by chronic atypical antipsychotics remains unsolved. To tackle this challenge, here we examined the role of HDAC2 in the effect of chronic clozapine treatment on *mGlu2* mRNA expression and mGlu2 receptor-dependent G protein coupling. We also tested whether HDAC2 is necessary for the repressive histone modifications induced at the promoter regions of *mGlu2* and other genes involved in synaptic homeostasis in the frontal cortex of mice chronically treated with clozapine. To further evaluate pharmacological interventions aimed to preserve the target of the novel generation of glutamatergic-based antipsychotics abovementioned, we assessed the extent to which adjunctive treatment with the class I and II HDAC inhibitor SAHA (vorinostat) prevents (or reverses) the effect of chronic clozapine treatment on frontal cortex mGlu2 receptor density.

MATERIALS AND METHODS

Drugs

Clozapine and (1*R*, 4*R*, 5*S*, 6*R*)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268) disodium salt were purchased from Tocris. (5*R*, 10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d] cyclohepten-5,10-imine hydrogen maleate (dizocilpine, (+)-MK801) was purchased from Sigma-Aldrich. SAHA (vorinostat, *N*-hydroxy-*N*′-phenyloctanediamide) was purchased from Cayman Chemical. The injected doses (i.p.) were 10 mg/kg for clozapine, 5 mg/kg for LY379268, 0.5 mg/kg for MK801 and 20 mg/kg for SAHA. Doses and route of administration were selected based on previous findings [29, 30]. Saline was used as vehicle except in experiments involving clozapine and SAHA where saline with 2% DMSO as cosolvent was used to match the drug's vehicle.

Animals

mRNA expression, chromatin immunoprecipitation (ChIP), and [^{3}H] ketanserin and [^{3}H]LY341495 binding experiments were performed on adult (10–20 weeks old) male mice. [^{35}S]GTPγS binding and behavior assays were performed on adult (10–20 weeks old) male and female mice. Animals were housed at 12 h light/dark cycle at 23 °C with food and water ad libitum, except during behavioral testing. Experiments were conducted in accordance with NIH guidelines, and were approved by the Virginia Commonwealth University Animal Care and Use Committee. All efforts were made to minimize animal suffering and the number of animals used. Behavioral testing took place between 9:00 a.m. and 6:00 p.m.

mGlu2 (*Grm2*) knockout (*mGlu2-KO*) and 5-HT_{2A} (*Htr2a*) knockout (*5-HT_{2A}-KO*) mice have been described previously [35–38]. For experiments involving *mGlu2-KO* and *5-HT_{2A}-KO* mice, wild-type littermates in the 129S6/Sv background were used as controls.

HDAC2 conditional knockout (*HDAC2-cKO*) mice were used based on our previous findings showing the crucial role of pyramidal HDAC2 in synaptic plasticity and cognition [29]. Deletion of HDAC2 expression in forebrain pyramidal neurons was performed as previously described [29]. Briefly *HDAC2$^{loxP/loxP}$* mice (C57BL/6 background) were bred with *CaMKIIα-Cre* transgenic line which expresses Cre recombinase under the control of the *CaMKIIα* promoter. The genotype denoted as HDAC2 conditional knockout (*HDAC2-cKO*) corresponds to *HDAC2$^{loxP/loxP}$*:*CaMKIIα:Cre$^{+/-}$*. The selective deletion of *Hdac2* in CaMKIIα-expressing neurons was validated elsewhere [29]. For experiments involving *HDAC2-cKO* mice, control littermates in the C57BL/6 background were used. In experiments that involved the use of both sexes, the ratio of females relative to males was neither superior to 0.6 nor inferior to 0.4 in each group.

Mouse brain samples

The day of the experiment, mice were sacrificed by cervical dislocation, and bilateral frontal cortices (bregma 1.90–1.40 mm) were 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 C57BL/6 and 129S6/Sv mouse strains [39].

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) assays were carried out in quadruplicate as previously described [29], using a QuantStudio 6 Flex Real-Time PCR System (ThermoFisher Scientific). See Supplementary Table 1 for primer pair sequences.

Chromatin immunoprecipitation assay

ChIP experiments were performed using the EZ-Magna ChIP Kit (Millipore) as previously reported [29, 30]. The following primary antibodies were used: acetyl-histone H3 (Millipore 06-599, 1:200), HDAC2 (Abcam 12169, 1:200). See Supplementary Table 2 for primer pair sequences and location in the promoter relative to transcription start sites.

Locomotor and stereotypical behavior

Horizontal activity, stereotypy and average speed were assessed using a computerized three-dimensional activity monitoring system (Omnitech) in 27 × 27 × 21 cm test chambers. The habituation phase comprised the first 90 min in which the mice were allowed to explore the test chamber to exclude novelty of the environment as a confounding factor. After habituation, mice

were injected (i.p.) with the tested drug(s), or vehicle(s). A 5-min lag in the test chamber followed (not shown in the analysis), after which MK801 was administered. Mice were monitored for an additional 120 min during the test phase. Horizontal activity was measured as interruptions of infrared beams traversing the *x* and *y* planes regardless the beam interruptions are consequence of ambulation or activity without displacement (such as stereotypy). Stereotypy episode count was calculated as the number of times that stereotypic behavior was observed in the animal. A break in stereotypy of 1 s or more is required to separate one stereotypic episode from the next. If the animal breaks the same beam (or set of beams) repeatedly then the monitor considers that the animal was exhibiting stereotypy. This typically happened during grooming or head bobbing (data not shown). Average speed was determined as the mean of the average velocities for each ambulatory episode. The representative heat-maps show cumulative locomotion distributed over the bi-dimensional area of the test chamber. All experiments were conducted in dim light.

Radioligand binding

[^{3}H]Ketanserin, [^{3}H]LY341495 binding assays in mouse frontal cortex were performed as previously reported with minor modifications [30, 32]. [^{3}H]Ketanserin non-specific binding was determined in the presence of 10 µM methysergide (Tocris), whereas [^{3}H]LY341495 non-specific binding was determined in the presence of 10 mM glutamate (Tocris). Our previous data show that, under these experimental conditions, specific [^{3}H]ketanserin binding is absent in the cortex of *5-HT$_{2A}$-KO* mice [35].

[^{35}S]GTPγS binding studies were adapted from a previously described protocol [40]. Dissected brain tissue was frozen at −80 °C for a minimum of 24 h. After thawing on ice, tissue was sheared with a syringe (23G needle) in assay buffer (20 mM HEPES, 10 mM $MgCl_2$, 2 mM EGTA, 100 mM NaCl) and further homogenated in a 5 mL Teflon-glass grinder. The homogenize was centrifuged at (1000 × *g*, 10 min, 4 °C) and the supernatant centrifuged at (40,000 × *g*, 20 min, 4 °C). The resulting pellet was washed with fresh assay buffer (40,000 × *g*, 20 min, 4 °C, twice) and stored at −80 °C. [^{35}S]GTPγS binding reaction contained, in a total volume of 200 µL, 20 mM HEPES, 10 mM $MgCl_2$, 2 mM EGTA, 100 mM NaCl, 30 µM GDP, 0.3 nM [^{35}S]GTPγS (Perkin Elmer), the test concentration of LY379268 or cold GTPγS (final concentration 10 µM) for non-specific binding, and 10 µg of membrane preparation as determined by the Bradford method. The reaction incubation (45 min at room temperature) started with the mixing of the master mix containing all the reaction components listed above, including membrane preparations, and LY379268 (or cold GTPγS). The reaction was stopped by harvesting (FilterMate Haverster, Perkin Elmer) on a glass fiber filter (Printed Filtermat A, Perkin Elmer) and washed six times with ice-cold assay buffer. The glass filter was dried at 55 °C for 1 h, soaked in scintillation liquid (Betaplate Scint, Perkin Elmer), and the retained filter radioactivity measured on a Microbeta2 counter (Perkin Elmer).

Statistical analysis

Radioligand binding data were analyzed using a nonlinear curve fitting software (GraphPad Prism software version 6). 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_t method. Statistical significance of [^{35}S]GTPγS binding was assessed by normalizing the fold changes to [^{35}S]GTPγS binding in the absence of the mGlu2/3 receptor agonist. Pharmacological parameters EC_{50}, E_{max} and area under the curve (AUC) were extracted from nonlinear fitting. Statistical significance of qRT-PCR and ChIP was assessed by normalizing the fold changes of logarithmic (log2) values. Statistical significance of experiments involving three or more groups and two or more treatments was assessed by two-way ANOVA followed by Fisher's least significant different (LSD) post hoc test. Statistical significance of experiments involving three or more groups was assessed by one-way ANOVA followed by Bonferroni's post hoc test. Statistical significance of experiments involving two groups was assessed by Student's *t*-test. The level of significance was chosen at $P = 0.05$. All data are presented as mean ± standard error of the mean (SEM).

RESULTS

Effect of chronic clozapine on *mGlu2* mRNA transcription

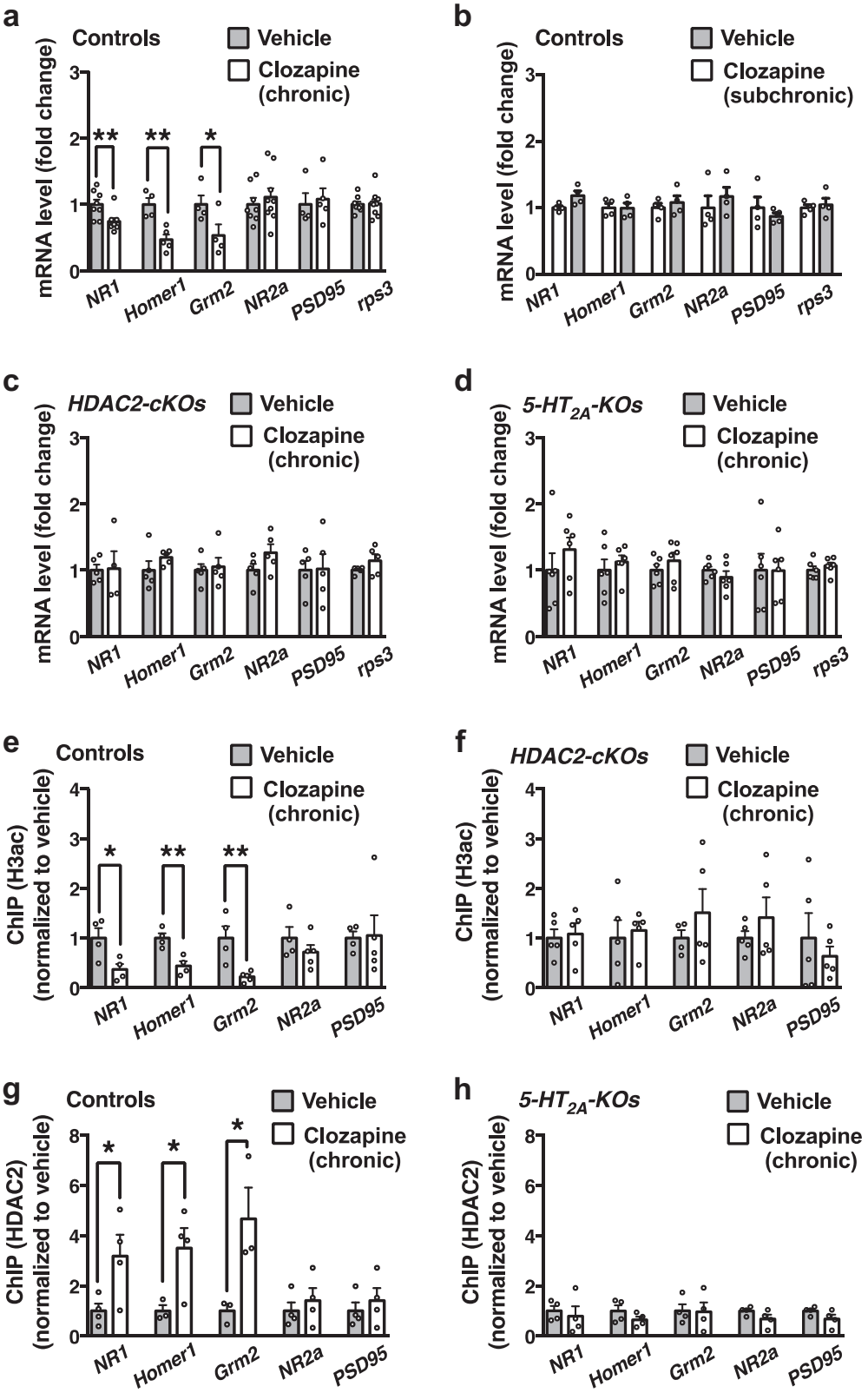
We first evaluated the effect of chronic (21 days) and sub-chronic (3 days) clozapine treatment (10 mg/kg) on expression of *mGlu2* mRNA, along with other markers of synaptic plasticity, in the mouse frontal cortex (Fig. 1a). Chronic atypical antipsychotic treatment led to a significant decrease in the expression of the *NR1*, subunit of the NMDA receptor (two-tailed Student's *t*-test, $t(15) = 2.964$, $P = 0.0096$); *Homer1*, an adaptor protein of the post-synaptic density ($t(7) = 4.345$, $P = 0.003$); and *Grm2*, the gene coding for *mGlu2* (Fig. 1a) ($t(6) = 2.166$, $P = 0.036$). No significant differences were found in the expression of *NR2a*, subunit of the NMDA receptor; or *PSD95*, a multimeric scaffolding protein for receptor clustering (Fig. 1a). The sub-chronic administration of clozapine to control mice did not produce significant differences in the expression of any of the genes tested (Fig. 1b) ($P > 0.05$).

We next assessed the role of HDAC2 in the effect of chronic clozapine treatment on expression of these synaptic plasticity genes. Deletion of HDAC2 function in *HDAC2-cKO* mice prevented the effect of chronic clozapine treatment on transcriptional repression of *NR1*, *Homer1*, and *mGlu2* in the frontal cortex (Fig. 1c) ($P > 0.05$). Our previous findings showed that the effects of chronic atypical antipsychotic treatment on *mGlu2* expression are mediated via 5-HT$_{2A}$ receptor-dependent signaling [29, 30]. To further support this notion, here we found no significant changes in the expression of the studied genes in the frontal cortex of *5-HT$_{2A}$-KO* mice chronically treated with clozapine ($P > 0.05$), as compared to vehicle (Fig. 1d).

Chronic clozapine treatment leads to repressive histone modifications at the *mGlu2* promoter via HDAC2

We [30], and others [41], have shown a positive correlation between binding of H3ac to the *mGlu2* promoter and *mGlu2* mRNA expression, as well as a negative correlation between HDAC2 expression and *mGlu2* transcription [42]. Based on these findings, we tested the role of forebrain pyramidal HDAC2 in the effect of chronic clozapine treatment on H3ac binding to the promoter region of the *mGlu2* gene. Chronic clozapine treatment induced a significant decrease of H3ac binding to the *mGlu2* promoter in the mouse frontal cortex (Fig. 1e) (two-tailed Student's *t*-test, $t(7) = 3.623$, $P = 0.008$). A similar effect was observed at the promoter regions of *NR1* ($t(6) = 2.767$, $P = 0.032$) and *Homer1* ($t(6) = 4.202$, $P = 0.005$), whereas this epigenetic modification was not observed at the *NR2a* and *PSD95* gene promoters (Fig. 1e). Additionally, the effect of chronic clozapine treatment on binding of H3ac to the promoter regions of *mGlu2*, *NR1*, and *Homer1* was not observed in the frontal cortex of *HDAC2-cKO* mice (Fig. 1f) ($P > 0.05$).

We next tested whether 5-HT$_{2A}$ receptor-dependent signaling was involved in the repressive histone modifications at the promoter region of synaptic plasticity genes observed after chronic clozapine treatment in the mouse frontal cortex. Relative to vehicle-treated mice, animals chronically treated with clozapine showed a significant increase in the binding of HDAC2 to the promoter region of genes *NR1* (two-tailed Student's *t*-test,



$t(6) = 2.46$, $P = 0.049$), *Homer1* ($t(5) = 2.62$, $P = 0.047$) and *mGlu2* ($t(4) = 2.87$, $P = 0.045$) but not of *NR2a* or *PSD95* (Fig. 1g) ($P > 0.05$). This effect of chronic clozapine treatment on HDAC2 binding was not observed in the frontal cortex of *5-HT$_{2A}$-KO* mice (Fig. 1h) ($P > 0.05$).

Effect of chronic clozapine treatment on frontal cortex mGlu2/3 receptor-G protein coupling

To test the extent to which the effects of chronic (21 days) clozapine treatment (10 mg/kg) on *mGlu2* transcription are translated into functional outcomes, we examined the effect of

Fig. 1 HDAC2 and 5-HT_{2A} are responsible for the downregulation of *mGlu2 (Grm2)* and other genes involved in synaptic plasticity in the frontal cortex of mice chronically (21 days) treated (i.p.) with clozapine (10 mg/kg), as compared to vehicle (**a**–**d**). Shown are mRNA expression levels of test genes and housekeeping gene *rps3* relative to vehicle-treated group measured by qRT-PCR in chronically (**a**) sub-chronically-treated (3 days) (**b**) treated control mice, and *HDAC2-cKO* (**c**) and *5-HT_{2A}-KO* (**d**) mice chronically treated with clozapine. All mice were sacrificed 24 h after the last dose of clozapine, or vehicle ($n = 4$–9 mice per experimental condition). Chronic clozapine treatment decreases via HDAC2 the binding of H3ac at the promoter region of *mGlu2 (Grm2)* and other genes involved in synaptic plasticity in the frontal cortex (**e**, **f**). Fragmented chromatin was immunoprecipitated with antibody recognizing H3ac, and the level of association of the promoter region of the gene of interest was measured by qPCR. Control (**e**) and *HDAC2-cKO* (**f**) mice chronically (21 days) treated (i.p.) with clozapine (10 mg/kg), or vehicle, were sacrificed 24 h after the last injection ($n = 4$–5 mice per experimental condition). Chronic clozapine treatment decreases via 5-HT_{2A} the binding of HDAC2 at the promoter region of *mGlu2 (Grm2)* and other genes involved in synaptic plasticity in the frontal cortex (**g**, **h**). Fragmented chromatin was immunoprecipitated with antibody recognizing HDAC2, and the level of association of the promoter region of the gene of interest was measured by qPCR. Control (g) and *5-HT_{2A}-KO* (h) mice chronically (21 days) treated (i.p.) with clozapine (10 mg/kg), or vehicle, were sacrificed 24 h after the last injection ($n = 3$–4 mice per experimental condition). Two-tailed Student's *t*-test (*$P < 0.05$). Two-tailed Student's *t*-test (*$P < 0.05$, **$P < 0.01$)

the mGlu2/3 receptor agonist LY379268 on G protein coupling in frontal cortex plasma membrane preparations. As expected, LY379268 augmented the binding of the non-hydrolyzable GTP analog [^{35}S]GTPγS to plasma membrane preparations of the frontal cortex of control mice chronically treated with vehicle (Fig. 2). Control mice treated with chronic clozapine showed a distinct dose–response [^{35}S]GTPγS binding curve compared to that of vehicle-treated control mice (Fig. 2a) (least squares F-test, $F(3,154) = 18{,}63$, $P < 0.0001$), but not in *HDAC2-cKO* animals (Fig. 2b) ($F(3,162) = 1.591$, $P > 0.05$). Chronic clozapine treatment decreased both efficacy and potency of LY379268 in the frontal cortex of control mice (Fig. 2a and Table 1). This important effect of chronic clozapine treatment on LY379268-induced G-protein coupling was not observed in the frontal cortex of *HDAC2-cKO* mice (Fig. 2b and Table 1) (E_{max}, two-way ANOVA; genotype effect, $F(1,44) = 67.41$, $P < 0.0001$; treatment effect, $F(1,44) = 1.85$, $P > 0.05$; post hoc: controls (clozapine vs. vehicle) $P = 0.030$; *HDAC2-cKOs* (clozapine vs. vehicle) $P > 0.05$) (EC_{50}, two-way ANOVA; genotype effect, $F(1,44) = 3.415$, $P = 0.071$; treatment effect, $F(1,44) = 12.24$, $P = 0.001$; post hoc: controls (clozapine vs. vehicle) $P = 0.003$; *HDAC2-cKOs* (clozapine vs. vehicle) $P > 0.05$).

To further study the differences between control and *HDAC2-cKO* mice treated with chronic clozapine we compared the area under the curve (AUC) for each condition as a single variable analysis (Fig. 2c) (two-way ANOVA, treatment effect, $F(1,44) = 7.886$, $P = 0.0074$). In agreement with the previous analysis (Table 1), a notable decrease in AUC was observed in control mice treated with chronic clozapine relative to vehicle treated controls (post hoc: controls (clozapine vs. vehicle), $P < 0.0001$). This treatment effect was not observed in *HDAC2-cKOs* (post hoc: *HDAC2-cKOs* (clozapine vs. vehicle), $P > 0.05$). No differences in AUC were observed between control and *HDAC2-cKO* mice treated with vehicle (post hoc: controls vs. *HDAC2-cKOs,* $P > 0.05$).

Role of mGlu2 in the antipsychotic-like effects of LY379268

Pharmacological prevention of the hyperlocomotor activity induced by dissociative non-competitive NMDA receptor antagonists, such as PCP and MK801, are widely used as a preclinical model of antipsychotic drug action [43]. As discussed above, previous findings, including ours, suggest that the effects of mGlu2/3 receptor agonists, such as LY379268 and LY404039, on prevention of hyperlocomotor activity induced by PCP or MK801 are significantly decreased in *mGlu2-KO*, but not in *mGlu3-KO* mice [13, 16]. Our data here show that the increased horizontal activity displayed by wild-type mice treated with MK801 alone was associated with cumulative movement throughout the entire arena's bi-dimensional grid (Supplementary Fig 1a and 1b). Co-administration of LY379269 along with MK801 limited locomotor activity to the periphery of the corners of the test chamber (Supplementary Fig 1c). Nevertheless, considering that dissociative drugs also affect the level of stereotyped and repetitive behaviors, such as repetitive licking, chewing and grooming, in rodents [44, 45], these findings raise the question of whether mGlu2/3 receptor agonists affect the MK801-induced hyperlocomotor activity component alone, or together with stereotyped behavior.

To test this, we broke down the components of horizontal activity into two different measures: episodes of activity without ambulation (hence indicative of stereotyped behavior) and speed during ambulatory episodes (hence indicative of locomotive function). The individual traces of each genotype group showed a marked time-effect of MK801 on horizontal activity (beam interruptions/5 min), (Supplementary Fig 1d and 1e); (two-way ANOVA (MK801 with vs. w/o LY379268); Fig. 5d, Time, $F(23,239) = 3.963$, $P < 0.0001$; Fig. 5e, Time, $F(23,216) = 10.04$, $P < 0.0001$). This effect on horizontal activity was counteracted by LY379268 throughout the time course in wild-type mice (Supplementary Fig 1d), but not in *mGlu2-KO* mice (Supplementary Fig 1e); (two-way ANOVA (MK801 with vs. w/o LY379268); Supplementary Fig 1d, Treatment, $F(1,239) = 130.5$, $P < 0.0001$; Supplementary Fig 1e, Treatment, $F(1,216) = 2.954$, $P > 0.05$).

The summation of horizontal activity events during the test phase between $t = 15$ to $t = 120$ min revealed a very distinct effect of genotype on the ability of LY379268 to block the hyperlocomotive effect of MK801: wild-type mice showed a marked reduction in horizontal activity upon co-administration of MK801 and LY379268, whereas *mGlu2-KO* mice treated with MK801 alone showed an indistinguishable profile of horizontal activity as compared to *mGlu2-KO* mice co-administered with LY379268 and MK801 (Supplementary Fig 1f) (two-way ANOVA; Genotype: $F(2,33) = 17.14$, $P < 0.0001$, Treatment: $F(1,33) = 47.12$, $P < 0.0001$; post hoc: Controls (Veh. vs. MK801) $P < 0.0001$; Controls (Veh. vs. MK801 + LY379268) $P > 0.05$); Controls (MK801 with vs. w/o LY379268) $P < 0.0001$; *mGlu2-KO* (Veh. vs. MK801) $P < 0.0001$; *mGlu2-KO* (Veh. vs. MK801 + LY379268) $P < 0.0001$; *mGlu2-KO* (MK801 with vs. w/o LY379268) $P > 0.05$). Similarly, the antipsychotic-like effect of LY379268 on MK801-induced hyperlocomotion was also observed in control male (two-tailed Student's *t*-test MK801 with vs. w/o LY379268, $n = 3$ and 3, $t(4) = 5.278$, $P = 0.0062$) and female mice ($n = 3$ and 3, $t(4) = 4.844$, $P = 0.0084$), but not in *mGlu2-KO* male ($n = 2$ and 3, $t(3) = 0.005$, $P > 0.05$) or female ($n = 3$ and 3, $t(4) = 1.023$, $P > 0.05$). These findings validate that deletion of mGlu2 receptor-dependent function results in a nearly complete loss of the antipsychotic-like activity of the mGlu2/3 receptor agonist LY379268 in the MK801-induced hyperlocomotion paradigm.

Notably, and similarly to the general effect observed in global horizontal activity, LY379268 significantly reduced, in wild-type mice, both the number of MK801-induced counts of stereotypy-like episodes and the average speed during ambulatory episodes. However, the *mGlu2-KO* mouse group remained non-responsive to the effect of LY379268 on MK801-induced stereotypy (Supplementary Fig 2a) or speed (Supplementary Fig 2b) (Supplementary Fig 2a, two-way ANOVA; Genotype: $F(1,32) = 5.89$, $P = 0.021$; Treatment: $F(2,32) = 15.39$, $P < 0.0001$; post hoc: Controls (Veh. vs. MK801) $P = 0.0001$; Controls (Veh. vs. MK801 + LY379268)

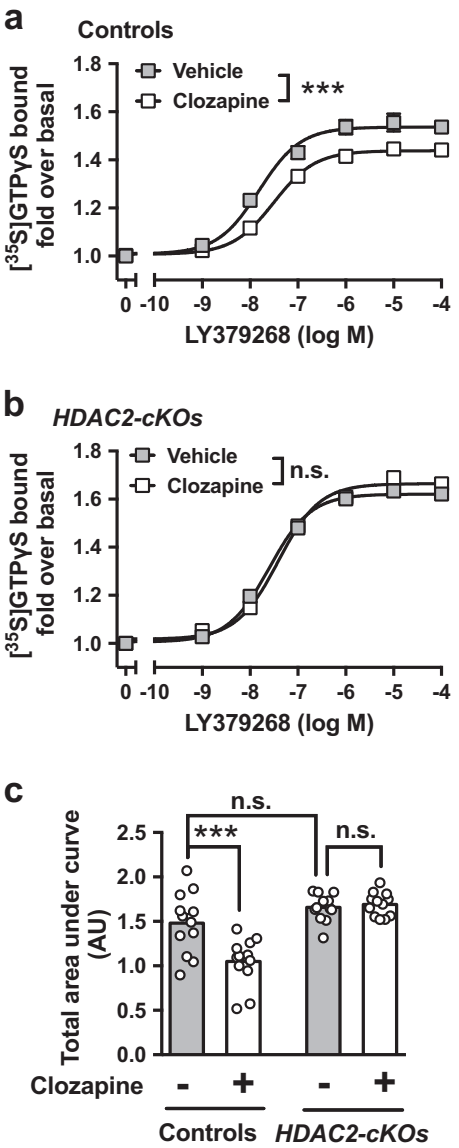


Fig. 2 Chronic clozapine decreases LY379268-stimulated [^{35}S]GTPγS binding in the frontal cortex of control mice, but not in *HDAC2-cKOs*. Concentration–response curve of LY379268 stimulating [^{35}S]GTPγS binding in frontal cortex membrane preparations of control mice (**a**) and *HDAC2-cKO* (**b**) mice chronically treated with clozapine, or vehicle. Control (**a**) and *HDAC2-cKO* (**b**) mice chronically (21 days) treated (i.p.) with clozapine (10 mg/kg), or vehicle, were sacrificed 24 h after the last injection ($n = 12$ mice per experimental condition). Area under the curve of bound [^{35}S]GTPγS in control and *HDAC2-cKO* mice chronically treated with vehicle or clozapine (**c**). Least squares F-test (**a**, **b**), see also Table 1. Two-way ANOVA with Bonferroni's post hoc test (**c**) (n.s., not significant; ***$P < 0.001$)

P > 0.05); Controls (MK801 with vs. w/o LY379268), $P = 0.0081$; *mGlu2-KO* (Veh. vs. MK801) $P = 0.0015$; *mGlu2-KO* (Veh. vs. MK801 + LY379268) $P = 0.062$); *mGlu2-KO* (MK801 with vs. w/o LY379268), $P > 0.05$) (Supplementary Fig 2b, two-way ANOVA; Genotype: $F(1,32) = 39.3$, $P < 0.0001$; Treatment: $F(2,32) = 8.953$, $P = 0.0008$; post hoc: Controls (Veh. vs. MK801) $P = 0.0495$; Controls (Veh. vs. MK801 + LY379268) $P > 0.05$); Controls (MK801 with vs. w/o LY379268), $P = 0.011$; *mGlu2-KO* (Veh. vs. MK801) $P = 0.0006$; *mGlu2-KO* (Veh. vs. MK801 + LY379268) $P = 0.002$); *mGlu2-KO* (MK801 with vs. w/o LY379268), $P > 0.05$). This highlights the required expression of mGlu2 receptor for LY379268 to elicit its suppressive effects not only on speed, as a surrogate of locomotor activity, but also on stereotyped behaviors in mice treated with MK801.

As expected based on previous findings [46], basal horizontal activity of *mGlu2-KO* mice was increased relative to the control littermates (two-tailed Student's *t*-test, $t(20) = 2.914$, $P = 0.009$).

Chronic clozapine treatment restricts mGlu2 receptor-dependent antipsychotic-like activity via HDAC2

Our data thus far suggest that chronic clozapine treatment significantly reduces the ability of LY379268 to activate frontal cortex G proteins in a HDAC2-dependent manner. Additionally, our current findings further demonstrate that the mGlu2 receptor plays a prominent role in the antipsychotic-like effects of the mGlu2/3 receptor agonist LY379268; using MK801-induced hyperlocomotor activity as a mouse model of psychosis. In light of these findings, we hypothesized that these effects of chronic clozapine treatment on *mGlu2* receptor expression and mGlu2 receptor functional state would then limit the antipsychotic potential of LY379268 in an HDAC2-dependent manner. Hence, we assayed the effect of LY379268 on MK801-induced hyperlocomotor activity in *HDAC2-cKO* mice and controls previously treated with chronic clozapine (21 days, 10 mg/kg), or vehicle. This effect of LY379268 on MK801-induced hyperlocomotor activity was tested 24 h after the last administration of clozapine, or vehicle (Fig. 3a).

As expected, after a 90-min habituation phase, administration of MK801 induced a time-dependent effect on horizontal activity in both *HDAC2-cKO* and control mice that was partially blocked by LY379268 (Fig. 3b, c) (two-way ANOVA; Fig. 3b, Time effect, $F(19,190) = 4.251$, $P < 0.0001$; Treatment effect, $F(1,10) = 20.64$; Fig. 3c, Time effect, $F(19,152) = 9.506$, $P < 0.0001$; Treatment effect, $F(1,8) = 15.37$, $P = 0.0044$). Remarkably, this antipsychotic-related effect of LY379268 on MK801-induced hyperlocomotor activity was reduced in control mice previously treated with clozapine (Fig. 3b); while the behavioral effect of LY379268 on MK801-induced hyperlocomotor activity was unaffected by previous chronic clozapine treatment in *HDAC2-cKO* mice (Fig. 3c) (two-way ANOVA; Fig. 3b, Treatment effect $F(1,11) = 1.706$, $P > 0.05$; Fig. 3c, Treatment effect $F(1,10) = 6.911$, $P = 0.0252$).

This is further supported by quantification of summation of horizontal activity events from $t = 15$ to $t = 120$ during the test phase (Fig. 3d) (two-way ANOVA; Treatment effect: $F(2,26) = 21.42$, $P < 0.0001$; Genotype effect: $F(1,26) = 2.488$, $P > 0.05$). As

Table 1. Pharmacological parameters relative to LY379268-induced [^{35}S]GTPγS binding in frontal cortex membrane preparations from control and *HDAC2-cKO* mice previously treated with chronic clozapine or vehicle

	Chronic vehicle			Chronic clozapine		
	n	E_{max} (fold-over basal ± SD)	EC_{50} (logM ± SD)	*n*	E_{max} (fold-over basal ± SD)	EC_{50} (logM ± SD)
Controls	12	1.557 ± 0.097	−7.733 ± 0.335	12	1.475 ± 0.077[a]	−7.357 ± 0.289[b]
HDAC2-cKOs	12	1.663 ± 0.047	−7.475 ± 0.135	12	1.691 ± 0.029	−7.353 ± 0.170

Two-way ANOVA with Bonferroni's post hoc test, [a]$P < 0.05$, [b]$P < 0.01$, compared to chronic vehicle-treated controls

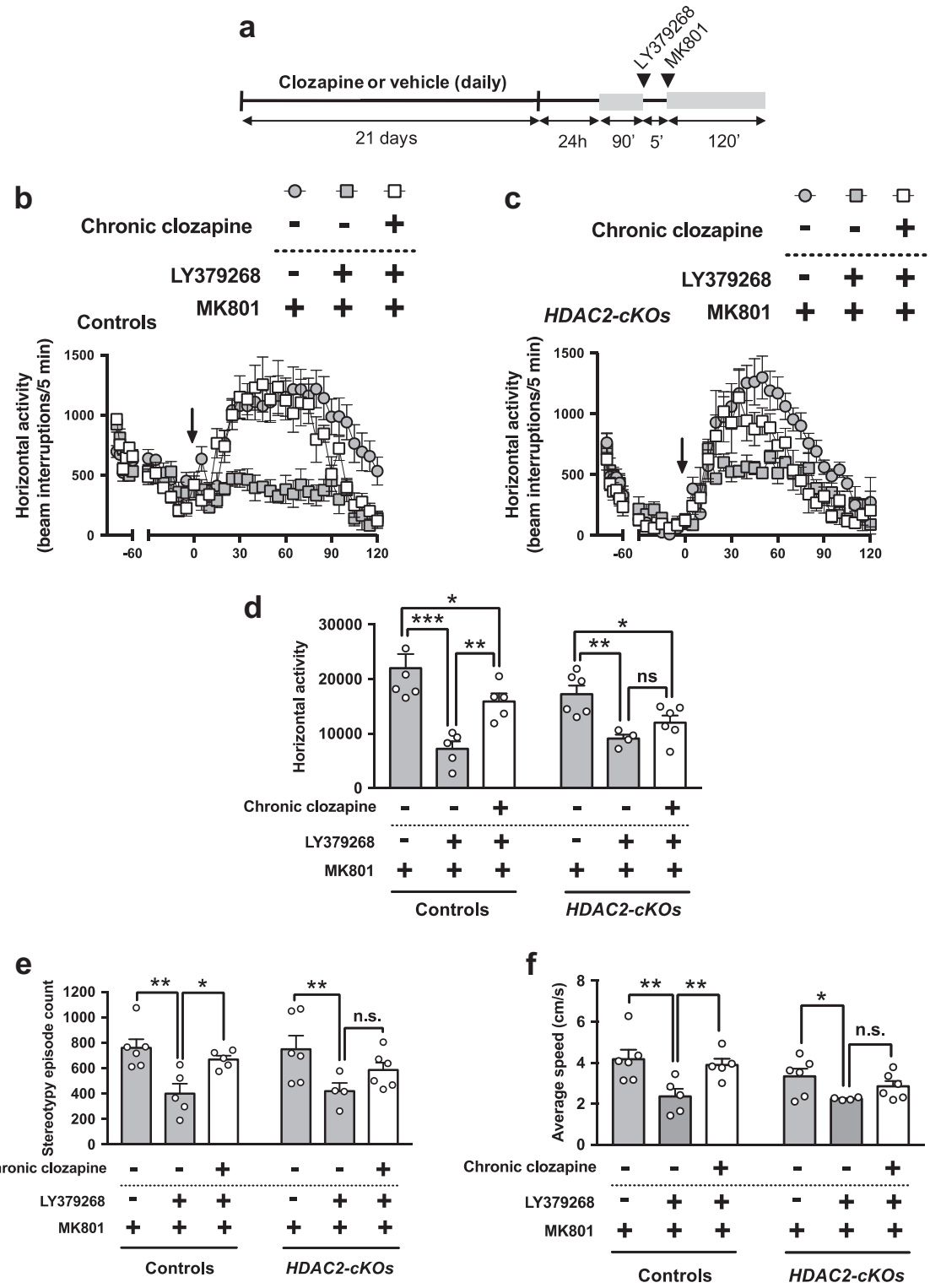


Fig. 3 Deletion of HDAC2 prevents the negative effect of chronic clozapine treatment on mGlu2 receptor-dependent antipsychotic-related behavior. Mice were chronically (21 days) treated (i.p.) with clozapine (10 mg/kg), or vehicle. Twenty-four hours after the last administration, mice were allowed to explore the locomotion chamber for 90 min (habituation phase) before pretreatment (i.p.) with LY379268 (5 mg/kg), or vehicle. After 5 min, MK801 (0.5 mg/kg) was administered (i.p.), and activity in the test chamber was recorded for additional 120 min (test phase). Experimental set up scheme showing the treatments and recorded phases (gray boxes) (**a**). Horizontal activity measured as beam breaks is shown in 5-min blocks for control (**b**) and *HDAC2-cKO* (**c**) mice over the whole experiment time course. Horizontal activity during the test phase was quantified from $t = 15$ and $t = 120$ for the different chronic treatments and pre-treatment for control and *HDAC2-cKO* mice (**d**). Cumulative stereotypy episodes (**e**) and average speed during ambulation episodes (**f**) from $t = 15$ to $t = 120$ after MK801 administration are shown for control and *HDAC2-cKO*. Time of injection indicated by arrow ($n = 4$–6 mice per experimental condition). Two-way ANOVA (**b**, **c**), two-way ANOVA with Fisher's LSD (**d**) post hoc test (n.s., not significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$)

anticipated based on the time course (Fig. 3b, c, above), a pronounced repressive effect of LY379268 on MK801-induced hyperlocomotor activity was observed in control and *HDAC2-cKO* mice chronically treated with vehicle (Fig. 3d, post hoc: Controls (MK801 with vs. w/o LY379268), $P < 0.0001$; *HDAC2-cKO* (MK801 with vs. w/o LY379268), $P = 0.0038$). Additionally, chronic clozapine treatment diminished the suppressive effect of LY379268 on MK801-induced hyperlocomotion in control mice, however, no significance differences were observed between the chronic clozapine vs. chronic vehicle in *HDAC2-cKO* groups co-administered LY379268 and MK801 (Fig. 3d, post hoc: Controls (chronic Veh. vs. Clo.) $P = 0.0019$; *HDAC2-cKO* (chronic Veh. vs. Clo.) $P > 0.05$). This shows that, in *HDAC2-cKO* mice chronically treated with clozapine, the mGlu2/3 receptor agonist LY379268 reproduced the antipsychotic-related effect observed in control mice chronically treated with vehicle.

We next analyzed the stereotypy and ambulatory components integrated within the summation of horizontal activity events. In both control and *HDAC2-cKO* mice, LY379268 decreased the total number of stereotypy episodes in animals chronically treated with vehicle (Fig. 3e). This effect of LY379268 on MK801-induced stereotypy was blocked by chronic clozapine in control mice, but not in *HDAC2-cKO* mice which showed a stereotypy episode count comparable to vehicle-treated mice (Fig. 3e, two-way ANOVA; Treatment effect: $F(2,26) = 10.25$, $P = 0.0005$; Genotype effect: $F(1,26) = 0.1593$, $P > 0.05$; post hoc: Controls (MK801 with vs. w/o LY379268) $P = 0.0020$; *HDAC2-cKO* (MK801 with vs. w/o LY379268) $P = 0.0064$; Controls (chronic Veh. vs. Clo.) $P = 0.0210$; *HDAC2-cKO* (chronic Veh. vs. Clo.) $P > 0.05$).

The effect of MK801 on average speed was also decreased by LY379268 in control and *HDAC2-cKO* mice chronically with vehicle (Fig. 3f). Analogously to stereotypy episode count, chronic clozapine diminished the ability of LY379268 to counterbalance MK801-induced increase on average speed in control mice, but not in *HDAC2-cKO* (Fig. 3f, two-way ANOVA; Treatment effect: $F(2,26) = 8.356$, $P = 0.0016$; Genotype effect: $F(1,26) = 5.114$, $P = 0.0323$; post hoc: Controls (MK801 with vs. w/o LY379268) $P = 0.0011$; *HDAC2-cKO* (MK801 with vs. w/o LY379268) $P = 0.0487$; Controls (chronic Veh. vs. Clo.) $P = 0.0063$); *HDAC2-cKO* (chronic Veh. vs. Clo.) $P > 0.05$). Together, these results support the notion that, after chronic clozapine treatment, genetic deletion of *Hdac2* expression preserves responsiveness to the mGlu2-dependent antipsychotic-related properties of LY379268.

During the habituation phase 90 min prior to drug administration, we found absence of significant differences between mice treated chronically with clozapine or vehicle (one-way ANOVA; $F(2,16) = 0.3041$, $P > 0.05$, post hoc: Controls (chronic Veh. vs. Clo.) $P > 0.05$). Similar findings were observed in *HDAC2-cKO* mice (one-way ANOVA; $F(2,16) = 0.3041$, $P > 0.05$, post hoc: *HDAC2-cKO* (chronic Veh. vs. Clo.) $P > 0.05$).

Adjunctive treatment with the HDAC inhibitor SAHA preserves mGlu2 receptor-dependent antipsychotic-like activity and receptor density

In light of our previous findings showing that daily co-administration of the class I and II HDAC inhibitor SAHA preserved mGlu2/3 receptor density after chronic clozapine treatment [30], and our current results showing HDAC2-dependent effects of chronic clozapine treatment on mGlu2 receptor-dependent antipsychotic-related behaviors, we next aimed to block the blunting effect of chronic clozapine on the antipsychotic-like effect of LY379268 by daily co-treatment with SAHA.

Control mice were treated chronically with vehicle, clozapine (10 mg/kg), or clozapine and SAHA (10 and 20 mg/kg, respectively) for 21 days. Twenty-four hours after the last dose of chronic treatment, mice were habituated to the locomotion chamber for 90 min, then pre-treated with LY379268, and finally challenged with MK801, with 120 min additional recording or horizontal activity during the test phase (Fig. 4a). As a control, a group of drug naive mice was treated with either vehicle or MK801 in between the habituation and test phases (horizontal activity traces not shown). In these mice, as expected, MK801 induced a pronounced effect of horizontal activity along the whole test phase timecourse (two-way ANOVA, treatment effect, $F(1,12) = 65.1$, $P < 0.0001$). The horizontal activity recorded during both the habituation and the test phase are shown in 5-min blocks for the chronically treated animals acutely administered with LY379268 and MK801 in the test (Fig. 4b). The horizontal activity profile of chronic vehicle-treated mice coadministered with LY379268 and MK801 was significantly less pronounced compared to drug naive animals that only received MK801 prior to the test phase (two-way ANOVA, acute treatment effect, $F(1,11) = 29.3$, $P = 0.0002$) and undistinguisable from that of drug naive animals that received vehicle instead of MK801 (two-way ANOVA, acute treatment effect, $F(1,9) = 4.756$, $P > 0.05$). Pair-wise two-way ANOVA analysis of the horizontal activity traces after co-administration of LY379268 and MK801 revealed a differential effect of the pre-existing chronic treatment during the test. The horizontal activity timecourse of mice chronically treated with vehicle was significantly different to that of chronic clozapine treated mice (two-way ANOVA, chronic treatment effect, $F(1,9) = 5.62$, $P = 0.0418$) but undistinguisable when compared to mice chronically treated with SAHA in addition to clozapine (two-way ANOVA, chronic treatment effect, $F(1,9) = 0.5097$, $P > 0.05$).

To further analyze the effect of chronic treatment on horizontal activity, beam-break events were quantified between $t = 15$ to $t = 120$ min during the phase test for the two groups of drug naive control animals treated with MK801 abovementioned along with all three groups of chronically treated animals acutely administered with LY379268 and MK801, (Fig. 4c) (one-way ANOVA, $F(4,26) = 1.514$, $P < 0.001$). MK801 induced a profound hyperlocomotive effect in drug naive control mice (post hoc: Drug naive (Veh. vs. MK801) $P < 0.0001$) that was significantly reduced by LY379268 in mice chronically treated with vehicle (post hoc: Drug naive and chronic vehicle (MK801 vs. LY379268 + MK801) $P < 0.0001$). As shown above (Fig. 3b), and anticipated in the horizontal activity traces (Fig. 4b), mice that received LY379268 and MK801 previously treated with chronic clozapine exhibited greater total horizontal activity compared to chronic vehicle-treated mice (Fig. 4c) (post hoc: LY379268 + MK801 (chronic vehicle vs. clozapine) $P = 0.0372$). Importantly, mice that received simultaneous administration of clozapine and SAHA showed undistinguishable total horizontal activity count relative to chronic-vehicle treated animals (Fig. 4c) (post hoc: LY379268 + MK801 (chronic vehicle vs. clozapine + SAHA) $P > 0.05$). Overall, LY379268 administration resulted in a blocking effect on MK801-induced hyperlocomotion that was blunted by previous treatment with chronic clozapine. However, such chronic clozapine-mediated resistance to the blocking effect of LY379268 on MK801-induced hyperlocomotion was prevented with co-administration of daily doses of SAHA.

We further aimed to determine whether the co-administration of clozapine and SAHA is able to preserve the expression of mGlu2/3 receptors with a less frequent dose regimen of the latter. We hence designed a paradigm that involved the administration of daily clozapine, or vehicle, chronically for 21 days alone, or together with SAHA administered every 3 days throughout the length of the treatment (Fig. 5a). As expected based on previous findings [30, 32], chronic clozapine treatment down-regulated mGlu2/3 receptor density in the frontal cortex of wild-type mice, as compared to wild-type mice chronically treated with vehicle (Fig. 5b). However, addition of SAHA every 3 days to the daily treatment with clozapine preserved the density of mGlu2/3 receptors in the mouse frontal cortex (Fig. 5b) (one-way ANOVA; $F(2,15) = 12.45$, $P = 0.0007$; post hoc: Veh. vs.

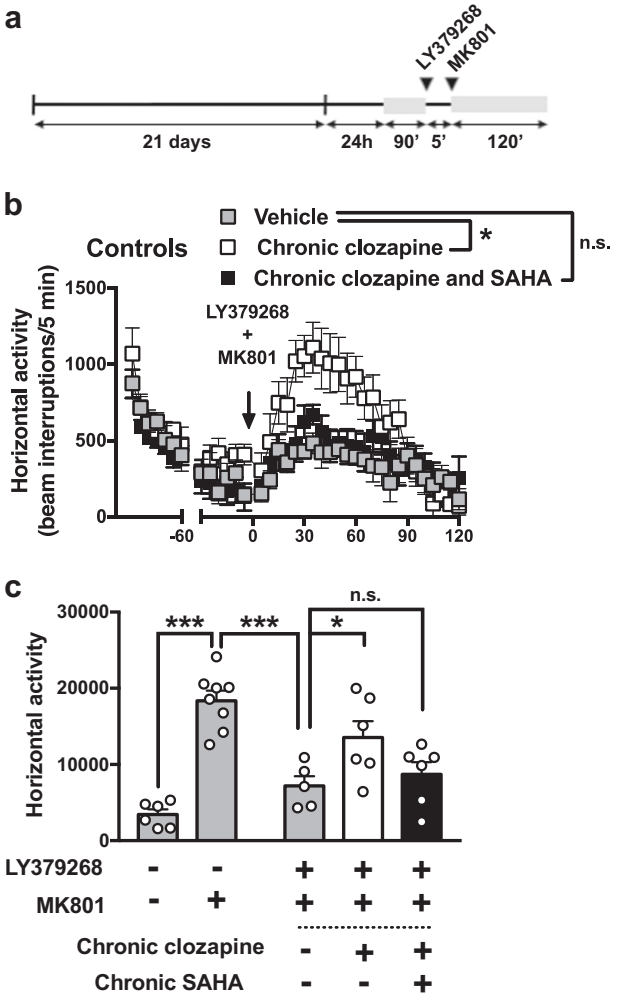


Fig. 4 Class I and II inhibitor SAHA co-treatment prevents the negative effect of chronic clozapine treatment on mGlu2 receptor-dependent antipsychotic-related behavior. Control mice were chronically (21 days) treated (i.p.) with vehicle ($n = 5$), clozapine (10 mg/kg, $n = 6$) or clozapine and SAHA (10 and 20 mg/kg, respectively, $n = 6$). Twenty-four hours after the last administration, mice were allowed to explore the locomotion chamber for 90 min (habituation phase) before pretreatment (i.p.) with LY379268 (5 mg/kg), or vehicle. After 5 min, MK801 (0.5 mg/kg), or vehicle was administered (i.p.), and activity in the test chamber was recorded for additional 120 min (test phase). Experimental set up scheme showing the treatments and recorded phases (gray boxes) (**a**). Horizontal activity measured as beam breaks is shown in 5-min blocks (**b**) over the whole experiment time course. Horizontal activity during the test phase was quantified from $t = 15$ and $t = 120$ for the different chronically treated animals and pre-treatment conditions (**c**); horizontal activity of drug naive animals treated with MK801 (0.5 mg/kg, $n = 8$) or vehicle ($n = 6$) included for magnitude of effect comparison purposes. Time of injection indicated by arrow. Two-way ANOVA (**b**), two-way ANOVA with Fisher's LSD (**c**) post hoc test (n.s., not significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$)

Clo. $P = 0.0006$; Veh. vs. Clo. + SAHA $P > 0.05$; Clo. vs. Clo. + SAHA $P = 0.012$).

To further test the therapeutic-related capabilities of adjunctive SAHA treatment, we assessed whether chronic SAHA administration could reverse the effect of chronic clozapine treatment on frontal cortex mGlu2/3 receptor density. To do so, mice were injected daily for 21 days with clozapine, or vehicle, after which mice received daily for another 21 days SAHA treatment along with clozapine, or vehicle (Fig. 5d). The density of mGlu2/3 receptors was decreased in the frontal cortex of mice treated solely with clozapine for 42 days, as compared to vehicle only (Fig. 5e). Importantly, addition of SAHA daily during the second half of the chronic clozapine treatment rescued the density of mGlu2/3 receptors in the frontal cortex beyond basal levels (Fig. 5e) (one-way ANOVA; $F(2,15) = 29.39$, $P < 0.0001$, post hoc: Veh. vs. Clo. $P = 0.0028$; Veh. vs. Clo. + SAHA $P = 0.0088$; Clo. vs. Clo. + SAHA $P < 0.0001$).

Chronic clozapine treatment reduced the binding of [^{3}H]-ketanserin in the frontal cortex of mice (Fig. 5c, f) (one-way ANOVA; Fig. 5c, $F(2,15) = 16.6$, $P = 0.0002$; post hoc: Veh. vs. Clo. $P = 0.0013$; Veh. vs. Clo. + SAHA $P = 0.0002$; Fig. 5f, $F(2,15) = 16.29$, $P = 0.0002$; post hoc: Veh. vs. Clo. $P = 0.0006$; Veh. vs. Clo. + SAHA $P = 0.0005$). Considering the greater cortical expression of 5-HT$_{2A}$ receptor, relative to 5-HT$_{2C}$ receptor, and overall concentration of 5-HT$_{2A}$ in the anterior cortical lobes, decreased [^{3}H]ketanserin binding after chronic clozapine is indicative of reduced 5-HT$_{2A}$ receptor density in the frontal cortex [47]. Such decrease in [^{3}H] ketanserin binding was not affected in any of the two paradigms of chronic clozapine and adjunctive SAHA administration (Fig. 5c, f). (Fig. 5c, post hoc: Clo. vs. Clo. + SAHA $P > 0.05$; Fig. 5f, post hoc: Clo. vs. Clo. + SAHA $P > 0.05$).

DISCUSSION

We recently proposed a mechanism by which chronic administration of atypical antipsychotics increase, via 5-HT$_{2A}$ receptor-dependent signaling, the expression and epigenetic function of HDAC2 in the frontal cortex, which ultimately restricts the therapeutic properties of chronic atypical antipsychotic medications [29, 30]. Here, we show an HDAC2-dependent association between the decrease in the fraction of H3ac bound to the promoter region of the *NR1*, *Homer1* and *mGlu2* (*Grm2*) genes and their transcriptional repression after chronic clozapine treatment. Additionally, this work shows how the epigenetic connection between HDAC2 and the repressive histone modifications induced at the *mGlu2* promoter upon chronic clozapine administration requires also 5-HT$_{2A}$ receptor-dependent signaling. The mechanism outlined above for the effect of chronic clozapine treatment on *mGlu2* mRNA transcription was also found to underlie the deleterious effect of this model of chronic antipsychotic action on several genes including *Homer1*, *mGlu2*, and different subunits of the NMDA receptor implicated in synaptic remodeling and cognitive processes [26]. Additionally, we demonstrate that the decrease in binding of H3ac is correlative to an increase of HDAC2 bound to the promoter of the genes *NR1*, *Homer1* and *mGlu2*. Our results are in agreement with a previous report showing that induction of HDAC2 expression following electroconvulsive seizures affected gene expression and promoter occupancy of genes involved in NMDA receptor expression and signaling [48]. Further studies will focus on how acetylation of specific lysine residues of H3 affects the expression of these genes and their implications in cognition. Similarly, further work will be necessary to assess whether covalent histone modifications, such as H4ac, H3K4me1/2/3, or H3K27me3 [30], are affected upon chronic antipsychotic treatment.

Effect of clozapine treatment on mRNA expression and H3ac or HDAC2 binding was analyzed as relative to the vehicle-treated group. Considering our previous findings showing that drug-naive *5-HT$_{2A}$-KO* mice exhibited repressive histone modifications at the *mGlu2* promoter in the frontal cortex [31], we do not exclude the possibility that expression of *NR1*, *Homer1*, or *mGlu2* is also affected in drug-naive *HDAC2-cKO* mice.

In addition to the marked effect of chronic clozapine treatment on *mGlu2* transcription in the mouse frontal cortex, our data showed that the repressive epigenetic state that emerges upon chronic clozapine treatment also translates into decreased mGlu2/3 receptor signaling via G proteins. Thus, we reported that

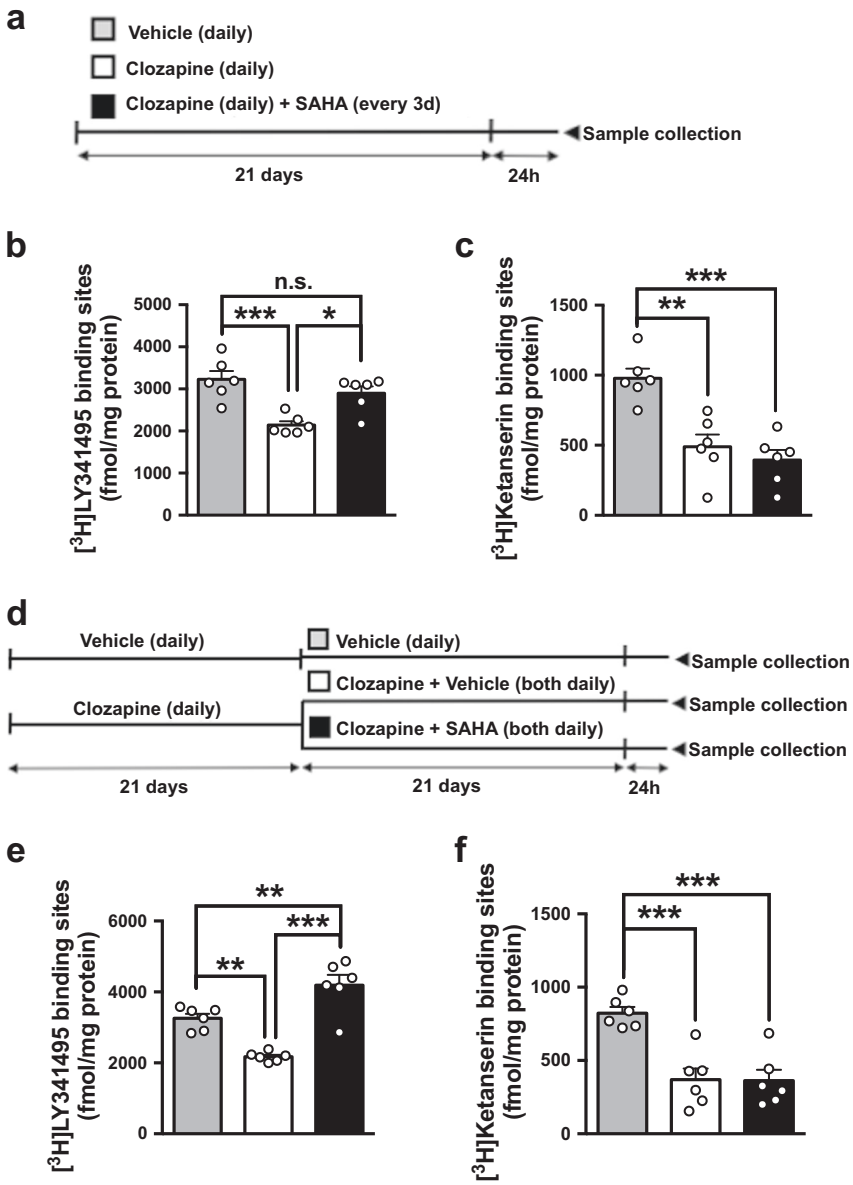


Fig. 5 Adjunctive treatment with the HDAC inhibitor SAHA prevents downregulation of frontal cortex mGlu2/3 receptor density upon chronic clozapine administration. Experimental set up of chronic treatment (i.p.) with clozapine (10 mg/kg) and SAHA (20 mg/kg), or vehicle (**a**, **d**). Maximum number of binding sites (B_{max}) of [^{3}H]LY341495 (**b**, **e**; mGlu2/3 receptor density) and [^{3}H]ketanserin (c,f; 5-HT$_{2A}$ receptor density) in frontal cortex membrane preparations ($n = 6$ mice per experimental condition). One-way ANOVA with Bonferroni's post hoc test (n.s., not significant; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

selective deletion of HDAC2 function in the forebrain preserved the effects of activation of mGlu2/3 receptors on frontal cortex G-protein coupling after chronic clozapine treatment. In addition to the phenotypes observed in *HDAC2-cKO* mice, we also demonstrate that pharmacological inhibition of HDACs is able maintain the expression of mGlu2/3 receptors in the frontal cortex of mice chronically treated with clozapine without affecting the chronic atypical antipsychotics signature of 5-HT$_{2A}$ receptor downregulation [29]. This further supports the notion that 5-HT$_{2A}$ is located up-stream in the signaling pathway whereby HDAC2 regulates mGlu2/3 receptor expression.

Available investigational orthosteric ligands targeting group II mGlu receptors with antipsychotic potential are mixed agonists for mGlu2 and mGlu3 [17]. Previous findings demonstrated that the effects of orthosteric mGlu2/3 receptor agonists on prevention of hyperlocomotor activity induced by dissociative drugs (PCP and MK801) or amphetamine are mGlu2, and not mGlu3, receptor dependent [13, 15, 18, 49]. In line with previous studies [13, 15, 16, 18], we showed that the mGlu2 receptor is necessary to prevent via administration of the orthosteric mGlu2/3 receptor agonist LY379268 the effects of MK801-induced locomotor activity. Stereotypy is characterized by repetitive behavior and has been noted in patients with psychosis and also in several pharmacological human and rodent models of psychosis [44, 45]. Our data further extend the involvement of mGlu2 on the antipscyhotic phenotype of mGlu2/3 agonists by showing that mGlu2 receptor is, at least in part, necessary for the effects of LY379268 on the stereotyped behavior induced by MK801. Overall, these findings add support to the hypothesis that mGlu2, and not mGlu3, mediates the effects of orthosteric mGlu2/3 receptor agonists preventing psychosis-like behaviors. Nevertheless, considering recent findings suggesting that activation of the mGlu3 receptor in the frontal cortex leads to cognitive improvement in rodent models [50, 51], further work will be needed to test whether

mGlu3 receptor is involved in the effects of LY379268 in rodent models of alterations in cognitive and sensorimotor gating processes induced by dissociative agents.

The serotonergic profile of atypical antipsychotics is suggested to contribute to their therapeutic advantage of atypical antipsychotics over typical dopaminergic antipsychotics [43, 52]. Thus, we and others have shown that chronic administration of atypical antipsychotic drugs, but not haloperidol, downregulates expression and density of 5-HT_{2A} receptor in mouse and human frontal cortex [43, 53]. Considering that hallucinogenic drugs, such as LSD and DOI, recruit specific cortical 5-HT_{2A} receptor-mediated signaling pathways to affect behavior in humans and rodents [35, 54], together, these findings suggest that downregulation and/or repression of 5-HT_{2A} receptor-dependent signaling may be one of the mechanisms underlying their therapeutic effects. On the other side, these effects of chronic atypical antipsychotic treatment on 5-HT_{2A} receptor downregulation and subsequent upregulation of HDAC2 have been proposed to be involved in the negative effects of chronic atypical antipsychotic treatment on synaptic plasticity and cognitive processes [29]. Recent observational studies have also reported an association between cumulative use of antipsychotics and deterioration of cognition, hence raising questions on the long-term efficacy of these medications, at least in terms of cognitive performance [3–7]. Here we have extended our previous findings showing the potential use of HDAC inhibitors as a pharmacological tool to induce antipsychotic-related phenotypes in mice and preserve mGlu2 expression [30]. Our current findings show that adjunctive administration of the HDAC inhibitor SAHA not only prevents glutamatergic antipsychotic drug resistance induced by chronic clozapine, but also preserves and restores mGlu2/3 receptor density in mouse frontal cortex. The persistent downregulation of 5-HT_{2A} receptor density after chronic co-treatment with SAHA and clozapine is also worth mentioning, as it suggests that other therapeutic properties of atypical antipsychotics related to their action on their primary target, the 5-HT_{2A} receptor, might not be interfered by the blockade of HDAC activity. Previous clinical studies with SAHA (vorinostat) in patients with cutaneous T-cell lymphoma showed severe adverse experiences that included, thrombocytopenia, pulmonary embolism, pyrexia, and sepsis, among many others [55]. Although these adverse effects may limit the clinical application of SAHA treatment in schizophrenia patients, our current findings showing that adjunctive administration of SAHA on intermittent regimen (every 3 days) throughout the length of the daily clozapine treatment prevents the effect of atypical antipsychotic treatment on mGlu2/3 receptor density point toward the translational potential of these preclinical findings into clinical protocols employing safer or more manageable HDAC inhibitors.

Targeting mGlu2/3 receptors has long been sought as new approach for the clinical treatment of schizophrenia and other psychiatric conditions. Indeed, recent intensive efforts by pharmaceutical firms led to the development of pomaglumetad—a well-tolerated mGlu2/3 receptor orthosteric agonist—and AZD8529—an mGlu2 receptor-selective positive allosteric modulator. However, their attrition due to lack of clinical efficacy draw a rather pessimistic scenario in the development of mGlu2/3 agonists for the treatment of schizophrenia. As discussed above, a recent meta-analysis of the phase II studies conducted with pomaglumetad identified two different schizophrenia patient groups based on their responsiveness to the tested treatment [34], suggesting that the over-represented population of recruited patients previously treated with atypical antipsychotics were refractory to the treatment, whereas a smaller population of untreated, or treated with dopaminergic typical antipsychotics prior to enrollment, were responsive to the antipsychotic action of pomaglumetad. Similar concerns on patient recruitment were raised in the case of AZD8529 [56]. Based on the available clinical evidence, our preclinical data identify prior treatment with clozapine or other atypical antipsychotic medications as a limitation in the clinical efficacy of mGlu2/3 receptor agonists as potential novel antipsychotics—a limitation mediated, for the most part, by the epigenetic effect via HDAC2 of chronic atypical antipsychotic drug administration on frontal cortex *mGlu2* transcription. If properly addressed, these findings may rescue the potential of mGlu2/3 receptor agonism as a novel treatment of schizophrenia and other psychiatric conditions.

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ADDITIONAL INFORMATION

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