Repressive Epigenetic Changes at the *mGlu2* Promoter in Frontal Cortex of 5-HT_{2A} Knockout Mice

Mitsumasa Kurita,¹ José L. Moreno, Terrell Holloway, Alexey Kozlenkov, Giuseppe Mocci, Aintzane García-Bea, James B. Hanks, Rachael Neve, Eric J. Nestler, Scott J. Russo, and Javier González-Maeso

Departments of Psychiatry (M.K., J.L.M., T.H., A.K., G.M., A.G.B., J.B.H., E.J.N., J.G.M.), Neurology (J.G.M.), Neuroscience (E.J.N., S.J.C.), Pharmacology and Systems Therapeutics (E.J.N.), and Friedman Brain Institute (E.J.N., S.J.R., J.G.M.), Icahn School of Medicine at Mount Sinai, New York, New York; and Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts (R.N.)

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ABSTRACT

Serotonin 5-HT_{2A} and metabotropic glutamate 2 (*mGlu2*) are G protein-coupled receptors suspected in the pathophysiology of psychiatric disorders, such as schizophrenia, depression, and suicide. Previous findings demonstrate that *mGlu2* mRNA expression is down-regulated in brain cortical regions of 5-HT_{2A} knockout (KO) mice. However, the molecular mechanism responsible for this alteration remains unknown. We show here repressive epigenetic changes at the promoter region of the *mGlu2* gene in frontal cortex of 5-HT_{2A}-KO mice. Disruption of 5-HT_{2A} receptor-dependent signaling in mice was associated with decreased acetylation of histone H3 (H3ac) and H4 (H4ac) and increased tri-methylation of histone H3 at lysine 27 (H3K27me3) at the *mGlu2* promoter,

epigenetic changes that correlate with transcriptional repression. Neither methylation of histone H3 at lysine 4 (H3K4me1/2/3) nor tri-methylation of histone H3 at lysine 9 (H3K9me3) was affected. We found that Egr1, a transcription factor in which promoter activity was positively regulated by the 5-HT_{2A} receptor agonist 4-bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide, binds less to the *mGlu2* promoter in frontal cortex of 5-HT_{2A}-KO, compared with wild-type mice. Furthermore, expression of *mGlu2* was increased by viral-mediated gene transfer of FLAG-tagged Egr1 in mouse frontal cortex. Together, these observations suggest that 5-HT_{2A} receptor-dependent signaling epigenetically affects *mGlu2* transcription in mouse frontal cortex.

Introduction

In eukaryotic cells, the DNA is packaged into chromatin. The basic repeating unit of chromatin is the nucleosome, which consists of 147 base pairs of DNA organized in approximately two superhelical turns of DNA wrapped around an octamer of core histone proteins (H2A, H2B, H3, and H4). The four core histones are predominantly globular except for their unstructured amino-terminal tails (Kouzarides, 2007; Borrelli et al., 2008; Dulac, 2010). The status of chromatin organization depends on epigenetic factors, such as DNA methylation (Suzuki and Bird, 2008) and histone modifications that primarily occur on their amino-terminal tails (Tsankova et al., 2007). Some of these events alter chromatin

structure and play an important role in regulating transcription. Thus, DNA cytosine methylation at CpG sites is often associated with transcriptional gene silencing, and there are various histone posttranslational modifications that correlate with open or closed states of chromatin. For example, acetylation of histone H3 (H3ac) and acetylation of histone H4 (H4ac) loosens DNA-histone interactions and allows the transcriptional machinery to bind and increase transcription. Histone methylation, on the contrary, can correlate with either transcriptional activation (methylation of lysine 4 on histone H3 [H3K4me] and methylation of lysine 36 on histone H3 [H3K36me]) or repression (methylation of lysine 9 on histone H3 [H3K9me] and methylation of lysine 27 on histone H3 [H3K27me]), depending on the histone and amino acid sequence being methylated. These epigenetic processes of DNA methylation and posttranslational histone modifications are fundamental for embryonic development and cellular differentiation (Ptak and Petronis, 2008; Orkin and Hochedlinger, 2011). Recent observations also suggest that environmental and pharmacological factors influence

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M.K. and J.L.M. contributed equally to this work.

¹Current affiliation: Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan.
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ABBREVIATIONS: ChIP, chromatin immunoprecipitation; GFP, green fluorescent protein; H3ac, acetylation of histone H3; H4ac, acetylation of histone H4; H3K4me1/2/3, mono/di/tri-methylation of lysine 4 on histone H3; H3K27me3, tri-methylation of lysine 27 on histone H3; H3K36me, methylation of lysine 36 on histone H3; H3K9me3, tri-methylation of lysine 9 on histone H3; KO, knockout; *mGlu2*, metabotropic glutamate 2 receptor; NG108-15 cells, mouse neuroblastoma × rat glioma cells; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; qPCR, quantitative real-time PCR; qRT-PCR, reverse-transcription quantitative real-time PCR; TCB-2, 4-bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide.

TABLE 1
PCR primer pairs for ChIP assay in mouse samples

Gene Name	GenBank	Primer Pairs		Location	Nucleotide Location from TSS
		Forward	Reverse		
<i>Htr2c</i>	NT_039718	GCCATGGATGACCTCAGTTT	TACACTGCTCAGGGCCTGTT	Promoter	−238 to −94
<i>Grm2</i>	NT_039477	ATCCTGCTGCTACACGTCT	TGGACACAGAACTGGATGC	−1.4 kb upstream promoter	−1419 to −1299
<i>Grm2</i>	NT_039477	GCCACTGTCTCATCTGTTCC	ATCCCGCTCTTGACAGGT	Promoter	−340 to −188
<i>Grm2</i>	NT_039477	ATTGTCTGCTGGGTTTGGAG	TTAACC CGGTGAGACCTCTG	0.8 downstream promoter	+837 to +958
<i>Grm2</i>	NT_039477	TTAATGAGCACCGTGGCATA	CGTGTGTATCCTTGAGCAG	Exon 2	+1979 to +2113
<i>Grm2</i>	NT_039477	GGGAGGTAGCCTCAGCTCTT	GACCTCGTCGTCGGTATCTG	Exon 4	+7784 to +7918
<i>Grm3</i>	NT_039299	TTCACTCGCTCAGCTGCTC	AAGCTCTGCTAAGGCTCACG	Promoter	−694 to −555
<i>Actb</i>	NT_081055	GAGACATTGAATGGGGCAGT	ATGAAGAGTTTGGCGATGG	Promoter	−321 to −230

TSS, transcriptional start site.

processes of chromatin remodeling in adult human and mouse CNS (Bhaumik et al., 2007; Peter and Akbarian, 2011; Jakovcevski and Akbarian, 2012).

The serotonin 5-HT_{2A} receptor plays a primary role in behavioral functions related to cognition, perception, and sensory processing (Gonzalez-Maeso and Sealfon, 2009a,b). As an example, some of the cellular signaling and behavioral effects of hallucinogenic drugs, such as lysergic acid diethylamide (LSD), psilocybin, and mescaline, require expression of the 5-HT_{2A} receptor in cortical pyramidal neurons (Beique et al., 2007; Gonzalez-Maeso et al., 2007; Celada et al., 2008). Similarly, second generation, or atypical, antipsychotic drugs, such as clozapine, olanzapine, and risperidone, have in common a high affinity for the 5-HT_{2A} receptor and a lower affinity for the dopamine D2 receptor (Roth et al., 2004; Miyamoto et al., 2005; Lieberman et al., 2008). Radioligand binding assays in postmortem human brain samples and positron emission tomography (PET) studies suggest alterations in 5-HT_{2A} receptor binding and expression as potentially involved in neuropsychiatric disorders, such as schizophrenia (Gurevich and Joyce, 1997; Gonzalez-Maeso et al., 2008; Rasmussen et al., 2010; Muguruza et al., 2012), depression (Shelton et al., 2009), and suicidal behavior (Oquendo et al., 2006). The role of the 5-HT_{2A} receptor in these behavioral measures is further supported by previous observations showing that some of the effects of hallucinogenic and atypical antipsychotic drugs are absent in 5-HT_{2A} knockout (KO) mice (Gonzalez-Maeso et al., 2003, 2007; Fribourg et al., 2011).

Glutamate is the major excitatory neurotransmitter in the mammalian brain (Carlsson et al., 1999; Sodhi et al., 2008; Kantrowitz and Javitt, 2012). Previous findings convincingly demonstrate a functional interaction between 5-HT_{2A} and metabotropic glutamate 2 (*mGlu2*) receptors in vitro and in rodent models. Thus, drugs that activate the *mGlu2* modulate the cellular (Zhai et al., 2003; Benneyworth et al., 2007; Gonzalez-Maeso et al., 2008; Moreno et al., 2011a), electrophysiological (Marek et al., 2000; Fribourg et al., 2011; Kurita et al., 2012), and behavioral (Gewirtz and Marek, 2000; Benneyworth et al., 2007; Moreno et al., 2011a, 2012) responses that require expression of the 5-HT_{2A} receptor in cortical neurons. Of interest, we previously reported that 5-HT_{2A}-KO mice show reduced cortical expression of *mGlu2* mRNA (Gonzalez-Maeso et al., 2008), which further supports the cross-modulation of a diverse array of functions between 5-HT_{2A} and *mGlu2* receptors. However, the molecular mechanism responsible for this alteration in frontal cortex of 5-HT_{2A}-KO mice remains unknown. We investigated here the patterns of epigenetic modifications at the promoter region of

the *mGlu2* gene (also known as *Grm2*) in frontal cortex of wild-type and 5-HT_{2A}-KO littermates.

Materials and Methods

Chemicals. (4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide (TCB-2) was purchased from Tocris Bioscience (Minneapolis, MN). 3-[2-[4-(4-Fluorobenzoyl)-1-piperidinyl]ethyl]-2,4[1H,3H]-quinazolinedione tartrate (ketanserin tartrate) was obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were obtained from standard sources.

Transient Transfection of NG108-15 Cells. NG108-15 (mouse neuroblastoma × rat glioma) cells were obtained from the American Type Culture Collection (ATCC# HB-12317; Manassas, VA), and grown in Dulbecco's modified Eagle's medium supplemented with dialyzed 10% (v/v) fetal bovine serum, 0.4 μM aminopterin, 100 μM hypoxanthine, and 16 μM thymidine. Cells were maintained at 37°C in a 5% CO₂ humidified atmosphere. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY) according to the manufacturer's instructions.

Plasmid Construction. Polymerase chain reaction (PCR) was performed using Pfu Ultra Hotstart DNA polymerase (Agilent Technologies, Santa Clara, CA) in a Mastercycler Ep Gradient Auto thermal cycler (Eppendorf, Hauppauge, NY). For the *Egr1* promoter construct, mouse *Egr1* promoter (−410 to +10 bp) was PCR amplified from mouse genomic DNA (Clontech, Mountain View, CA) with use of the following primers: 5'-ACGCCATATAAGGAGCAGGA-3' and 5'-CGAATCGGCCTCTATTTC-3'. The amplicon was inserted into PCR-blunt vector (Invitrogen) and then sequenced. The product was reamplified using the primers 5'-GATctcgagACGCCATATAAGGAGCAGGA-3' and 5'-CTTaaagcttCGAATCGGCCTCTATTTC-3' and then digested with XhoI and HindIII and subcloned into the XhoI and HindIII sites of pGL4.11 [*luc2P*] plasmid (Promega, Madison, WI). For the pcDNA3.1-FLAG-*Egr1* plasmid, mouse *Egr1* cDNA was PCR amplified using the following primers: 5'-cttaagcttATGGCAGCGGCCAAGGCC-3' and 5'-ctttctagaTTAGCAAATTTCAATTGTCC-3'. The amplicon was inserted into PCR-blunt vector (Invitrogen) and then sequenced. The mouse *Egr1* was digested and subcloned into the HindIII and XbaI sites of pcDNA3.1 plasmid. The mouse *Egr1* fragment was digested with EcoRI and XhoI, the FLAG fragment was digested with BamHI and EcoRI from pcDNA3.1-FLAG-HDAC2 (Kurita et al., 2012), and these two fragments were simultaneously subcloned into the BamHI and XhoI sites of pcDNA3.1. For HSV-FLAG-*Egr1*, the FLAG-*Egr1* fragment was digested from pcDNA3.1-FLAG-*Egr1* (see cloning above) and subcloned into the BamHI and XhoI sites of the bicistronic p1005+ HSV plasmid expressing green fluorescent protein (GFP) under the control of the CMV promoter (Kurita et al., 2012). The pcDNA3.1-c-Myc-5HT_{2A} plasmid and the *mGlu2* promoter construct have been previously described (Kurita et al., 2012; Moreno et al., 2012). All the constructs were confirmed by DNA sequencing.

Luciferase Reporter Assay. Luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega) as previously reported with minor modifications (Kurita et al., 2012). In brief, NG108-15 cells were plated at a density of 1×10^5 in six-well dishes, cultured for 24 hours, and transfected with the corresponding plasmids. For the *Egr1* promoter assay, cells were transfected with the pGL4.11 [*luc2P*] plasmid (Promega) containing the mouse *Egr1* promoter (1.0 μ g), and the pcDNA3.1-c-Myc-5HT_{2A} plasmid (1.0 μ g). TCB-2 (0.01, 0.1, and 1.0 μ M), ketanserin (1.0 μ M), or vehicle was added to the medium without serum 5 hours after transfection. For the *mGlu2* promoter assay, cells were transfected with the pGL4.11 [*luc2P*] plasmid (Promega) containing the mouse *mGlu2* promoter (1.5 μ g) and the pcDNA3.1-FLAG-*Egr1* plasmid (1.0, 2.0, and 4.0 μ g). Transfected cells were incubated for 24 hours, and the luciferase activity was measured with a luminometer (TD-20/20; Turner Biosystems) with use of the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions. Transfection efficiency was normalized with coexpressed pGL4.75 [*hRluc/CMV*] (0.02 μ g; Promega).

Experimental Animals. Experiments in wild-type and 5-HT_{2A}-KO littermates were performed on adult (8–12 weeks old) male 129S6/SvEv mice. 5-HT_{2A}-KO (*Htr2a*^{-/-}) mice have been previously described (Gonzalez-Maeso et al., 2003). Wild-type and 5-HT_{2A}-KO were generated by interbreeding heterozygous mice. Animals were housed at 12 hour light/dark cycle (lights on, 8:00 to 20:00) at 23°C with food and water ad libitum. The day of the experiment, mice were sacrificed 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 biochemical or epigenetic assays. The coordinates were taken according to a published atlas of the mouse strain (Hof et al., 2000). The Institutional Animal Use and Care Committee at Mount Sinai School of Medicine approved all experimental procedures.

Quantitative Real-Time PCR. Quantitative real-time PCR (qPCR) and reverse-transcription quantitative real-time PCR (qRT-PCR) assays were performed in quadruplicate with use of SYBR green as previously described (Gonzalez-Maeso et al., 2007, 2008; Kurita et al., 2012). All reactions were confirmed to generate a single PCR

Fig. 1. Expression of *5HT2C*, *mGlu2*, *mGlu3*, and GAPDH mRNAs in the frontal cortex of wild-type and 5-HT_{2A}-KO mice assayed by qRT-PCR ($n = 6$ per group). *** $P < 0.001$; Student's t test. Error bars show S.E.M.

product by gel electrophoresis or melting curve analysis. See Kurita et al. (2012) for qRT-PCR primer pair sequences.

Chromatin Immunoprecipitation Assay in Mouse Frontal Cortex. Chromatin immunoprecipitation (ChIP) experiments were performed using the EZ-Magna ChIP Kit (EMD Millipore, Billerica, MA) as previously reported (Kurita et al., 2012). In brief, mouse frontal cortex tissue samples were minced to less than 1-mm-sized pieces and immediately cross-linked in 1% formaldehyde for 20 minutes at room temperature. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M. The tissue was washed four times in cold phosphate-buffered saline (PBS) containing protease inhibitors (Complete; Roche, Branchburg, NJ) and homogenized in ice-cold cell lysis buffer (10 mM NaCl, 0.2% Nonidet P-40, and 10 mM Tris-HCl; pH 8.0) with protease inhibitor cocktail II. The nuclear fraction was lysed in ice-cold nuclear lysis buffer (10 mM EDTA, 1% SDS, and 50 mM Tris-HCl; pH 8.0) with protease inhibitor cocktail II and sonicated on ice with use of a Microson Ultrasonic Cell Disruptor. The lysate was centrifuged to remove insoluble material and then diluted 1:10 in ChIP dilution buffer (167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, and 16.7 mM Tris-HCl; pH

Fig. 2. Repressive histone modifications at the *mGlu2* promoter in frontal cortex of 5-HT_{2A}-KO mice. Fragmented chromatin was immunoprecipitated with antibody recognizing H3ac (A), H4ac (B), H3K4me1/2/3 (C), H3K27me3 (D), or H3K9me3 (E), and the level of association of the *5HT2C*, *mGlu2*, *mGlu3*, or β -actin promoters was measured by qPCR ($n = 8$ per group). * $P < 0.05$; *** $P < 0.001$; Student's t test. Error bars show S.E.M.

Fig. 3. Repressive histone modifications at the *mGlu2* promoter in frontal cortex of 5HT_{2A}-KO mice. (A) Map of the *mGlu2* (*Grm2*) gene showing position of exons (black rectangles) and primers used for PCR analysis. (B) ChIP analysis in mouse frontal cortex with antibody recognizing H3ac, H4ac, H3K4me1/2/3, H3K27me3, or H3K9me3. Results are shown as enrichment values (bound/input). Association of the *mGlu2* promoter was also measured ($n = 8$ per group). * $P < 0.05$; ** $P < 0.01$; Student's t test. Error bars show S.E.M.

8.1) to a final volume of 1.0 ml. Primary antibodies were added to diluted lysates and incubated at 4°C for 12 hours with 20 μ l of fully suspended protein A or G magnetic beads (EMD Millipore). The following primary antibodies were used: acetyl-histone H3 (EMD Millipore; 06-599, 1:200), acetyl-histone H4 (EMD Millipore; 06-866, 1:200), mono/di/tri-methyl-histone H3 (Lys4) (EMD Millipore; 05-791,1:300), tri-methyl-histone H3 (Lys9) (EMD Millipore; 07-442, 1:250), tri-methyl-histone H3 (Lys27) (EMD Millipore; 07-449, 1:300), Egr1 (Santa Cruz Biotechnology, Dallas, TX; sc-110, 1:200), and Egr2 (Santa Cruz Biotechnology; sc-20690, 1:200). The chromatin/immune complexes were washed with low salt immune complex wash buffer (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 20 mM Tris-HCl; pH 8.1), high-salt immune complex wash buffer (500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 20 mM Tris-HCl; pH 8.1), LiCl immune complex wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholic acid sodium salt, 1 mM EDTA, and 10 mM Tris-HCl; pH 8.1), and TE buffer (1 mM EDTA and 10 mM Tris-HCl; pH 8.0). The chromatin/immune complexes and input DNAs were reverse cross-linked by incubation with ChIP elution buffer (1% SDS and 0.1 M NaHCO₃) containing 0.1 μ g/ μ l proteinase K for 2 hours at 62°C. DNA was purified by using spin columns (EMD Millipore). Input and immunoprecipitated DNAs were subjected to qPCR (see Table 1 for qPCR primer pair sequences). All assays included nonimmune IgG to control for specificity of each antibody used. All reactions were

confirmed to generate a single PCR product by gel electrophoresis or melting curve analysis. Internal standard curves using known amounts of mouse genomic DNA (0.03–5 ng) were included to define the range in which DNA can be quantified. Data are shown as fold change of the DNA sequence enriched by immunoprecipitation with specific antibodies over the DNA sequence present in 2% of the supernatant after chromatin immunoprecipitation (defined as 2% input) (Figs. 3B and 5A), as enrichment relative to that of the nonimmune IgG (Fig. 5B), or as fold enrichment relative to that of IgG for wild-type versus 5-HT_{2A}-KO mice (Fig. 2), depending on the variable to be tested: genotype, different antibodies, and different regions across the gene of interest.

DNA Methylation Assay. DNA methylation assay was performed using the Non-Organic DNA Extraction Kit (EMD Millipore) as previously reported (Kurita et al., 2012). In brief, genomic DNA was isolated from mouse brain frontal cortex and liver tissue samples with use of the Non-Organic DNA Extraction Kit (EMD Millipore) according to the manufacturer's instructions and subjected to bisulfite modification to convert all nonmethylated cytosines into thymidines. For sodium bisulfite treatment, the CpGenome Fast DNA Modification Kit (EMD Millipore) was used according to the manufacturer's instructions. Modified DNA was then amplified by PCR with use of bisulfite-treated DNA specific primer sets 5'-GGTATTAAGGGTT-AATTTTATTTTGG-3' and 5'-ACACTATAAACAACCTCAAACCTAC-3'

Fig. 4. Methylation status of the *mGlu2* (*Grm2*) gene CpG island is not affected in frontal cortex of 5-HT_{2A}-KO mice. (A) Schematic view of amplified region of the *mGlu2* gene. (B and C) Methylation status of 39 CpG sites close to the *mGlu2* promoter region obtained from bisulfite sequencing in mouse frontal cortex. Open and filled circles represent unmethylated and methylated cytosines, respectively. (B) Representative example. Ten clones were sequenced for each mouse ($n = 6$ mice per group). (C) Percentage of methylated clones. N.S., not significant; Student's *t* test. Error bars show S.E.M.

and 5'-TGAGAGGTTGAGATAAAGATAGAGATATAG-3' and 5'-CCA-AATAAATTAACCCCTTAATACC-3', which did not include any CpG sites where possible methylation could be present. The PCR products were cloned using TOPO-TA cloning kit (Invitrogen) and transformed into TOP10 competent cells; 9–12 different colonies from each DNA amplification reaction were then analyzed for possible methylated CpG sites with use of direct sequencing from the TOPO plasmids containing the insert.

Viral-Mediated Gene Transfer. FLAG-Egr1 was subcloned into a published bicistronic HSV-GFP virus vector (see above), and viral particles were then packaged as described before (Kurita et al., 2012). HSV-FLAG-Egr1, or control HSV-GFP, was injected into the frontal cortex by stereotaxic surgery according to standard methods (Kurita et al., 2012; Moreno et al., 2012). In brief, 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 μ l/min for a total volume of 0.5 μ l on each side. The following coordinates were used: +1.6 mm rostral-caudal, -2.4 mm dorsal-ventral, and +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 (Hof et al., 2000). All experiments were performed 3–4 days after the viral infection, when transgene expression is maximal (Kurita et al., 2012). Viral-mediated FLAG-Egr1 overexpression levels

in frontal cortex were confirmed by Western blotting and qRT-PCR (Fig. 7B and unpublished data).

Immunohistochemistry. Experiments were performed as previously reported (Kurita et al., 2012). In brief, mice were deeply anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Transcardiac perfusion was performed with 10 ml PBS, followed by 30 ml of freshly prepared 4% paraformaldehyde in PBS at room temperature. Brains were removed and immersion-fixed in 4% paraformaldehyde in 1 ml at 4°C for 60 minutes and stored at 30% sucrose in PBS at 4°C for an additional 12 hours before to obtain the frontal cortex section. A series of 30- μ m-thick coronal sections from frontal cortex were prepared on a sliding microtome (Leica, Bannockburn, IL) equipped with a freezing stage (Physitemp Instruments, Clifton, NJ). The free-floating sections were transferred to 24-well tissue culture plates containing PBS. The tissue sections were further washed with PBS and incubated in PBS containing 0.2% Triton X-100 and 5% bovine serum albumin to permeabilize the cells. Primary antibodies (anti-GFP; Santa Cruz Biotechnology; sc-8334; 1:100) were incubated overnight at 4°C. After washing with PBS, the tissue sections were incubated with the secondary antibodies (Alexa 488 dye-conjugated anti-rabbit antibody; 1:500) for 60 minutes at room temperature. After washing (PBS; 6 $\times$ 2 ml), the tissue sections were mounted onto coverslips treated with antifade. Tissue samples were examined using upright epifluorescence microscope (Zeiss Axioplan 2IE).

Immunoblot Assays. Western blot experiments in mouse brain samples were performed under reducing and denaturing conditions as previously reported (Fribourg et al., 2011). The following primary antibodies were used: FLAG (Sigma-Aldrich; F3165; 1:1000), *mGlu2* (Abcam, Cambridge, MA; ab15672; 1:1000), and α -tubulin (Cell Signaling, Danvers, MA; 11H10; 1:1000). Metabotropic glutamate receptors are G protein-coupled receptors that form covalently linked homodimers (Pin et al., 2003). We measured immunoreactivity of *mGlu2* as a monomer (~100 kDa).

Results

***mGlu2* mRNA in Frontal Cortex of 5-HT_{2A}-KO Mice.** Our previous findings suggest that 5-HT_{2A}-KO mice show reduced cortical expression of *mGlu2* mRNA (Gonzalez-Maeso et al., 2008). These experiments were performed in samples of mice developed in 129S6/SvEv strain and maintained at Columbia University Medical Center (Gonzalez-Maeso et al., 2003, 2007, 2008). To establish a new breeding colony at Mount Sinai School of Medicine, we used sperm of 5-HT_{2A}-KO male mice to perform in vitro fertilization of wild-type mouse oocytes (C57BL/6 strain). Mouse in vitro fertilization was performed in accordance with standard protocols at the Mount Sinai Mouse Genetics Shared Resource Facility. The embryos were then transferred to pathogen-free hosts (C57BL/6 strain), after which 5-HT_{2A}-KO mice were backcrossed for at least ten generations onto 129S6/SvEv background. This new colony was maintained by interbreeding of heterozygous mice.

Of importance, we found that expression of *mGlu2* mRNA was also reduced in frontal cortex of 5-HT_{2A}-KO mice, compared with wild-type littermates (Fig. 1). No differences were observed for expression of 5-HT_{2C} or *mGlu3* mRNA (Fig. 1). Because experiments in mice bred at Columbia University Medical Center (Gonzalez-Maeso et al., 2008) and Mount Sinai School of Medicine (Fig. 1) were performed more than 5 years apart, these findings suggest that reduced *mGlu2* mRNA expression in cortical regions of 5-HT_{2A}-KO mice is attributable to disrupted 5-HT_{2A} receptor signaling capacity and not to housing conditions.

Repressive Histone Modifications at the *mGlu2* Promoter in 5-HT_{2A}-KO Mice. The notable down-regulation of *mGlu2* mRNA expression in frontal cortex of 5-HT_{2A} mice led us to investigate the epigenetic status of the *mGlu2* promoter. We first assayed the level of several posttranslational histone modifications at the 5HT_{2C}, *mGlu2*, and *mGlu3* promoter regions in mouse frontal cortex, including the β -actin (*Actb*) promoter as internal control. Histone H3 acetylation (H3ac), which correlates with transcriptional activation, was strongly decreased at the *mGlu2* promoter in 5-HT_{2A}-KO mice (Fig. 2A). Similar results were obtained with histone H4 acetylation (H4ac) (Fig. 2B). Histone H3 methylation at lysine 4 (H3K4me1/2/3), another marker of gene activation, was not affected in frontal cortex of 5-HT_{2A}-KO mice (Fig. 2C).

We next examined whether histone modifications known to correlate with transcriptional repression are affected in 5-HT_{2A}-KO mice. A significant increase in histone H3 trimethylation at lysine 27 (H3K27me3), a repressive histone modification marker, was found at the *mGlu2* promoter, with no apparent changes at the 5HT_{2C} and *mGlu3* promoters (Fig. 2D). Histone H3 tri-methylation at lysine 9 (H3K9me3), another histone modification that correlates with transcriptional repression, was not increased at any

Fig. 5. Binding of Egr1 to the *mGlu2* promoter is decreased in frontal cortex of 5-HT_{2A}-KO mice. (A) In wild-type mice, fragmented chromatin was immunoprecipitated with antibody recognizing Egr1, Egr2, or control IgG, and the level of association of the *mGlu2* or *mGlu3* promoters was measured by qPCR ($n = 8$ mice per group). * $P < 0.05$; Bonferroni's post hoc test of one-way analysis of variance. (B) Fragmented chromatin was immunoprecipitated with antibody recognizing Egr1 or control IgG, and the level of association of the -1 kb region, promoter region, and exon 4 region of the *mGlu2* gene was measured by qPCR ($n = 8$ mice per group). * $P < 0.05$; *** $P < 0.001$; Bonferroni's post hoc test of two-way analysis of variance. Error bars show S.E.M.

of these promoters in frontal cortex of 5-HT_{2A}-KO mice (Fig. 2E).

To further validate the specificity of these findings, additional ChIP experiments were performed at different regions across the *mGlu2* gene (Fig. 3A). Binding of H3ac was decreased at the promoter region of the *mGlu2* gene in frontal cortex of 5-HT_{2A}-KO mice, compared with wild-type littermates (Fig. 3B). These differences were not observed either 1.5 kb upstream of the *mGlu2* promoter or at three different locations downstream of the start site. Similar findings were obtained with H4ac (Fig. 3B). Binding of H3K27me3 was increased at the *mGlu2* promoter, but not upstream or downstream of the promoter region, in 5-HT_{2A}-KO mice (Fig. 3B). As shown in Fig. 2, binding of H3K4me1/2/3 and H3K9me3 was not affected (Fig. 3B), and no significant changes were found at the promoter region of the *mGlu2* gene (Fig. 3B).

DNA Methylation at the *mGlu2* Promoter in 5-HT_{2A}-KO Mice. Recent studies suggest that histone modifications cooperate with DNA methylation to affect gene inactivation (Dong et al., 2007). We found that the methylation pattern of the tested CpG sites at the *mGlu2* promoter is not affected in the frontal cortex of 5-HT_{2A}-KO mice (Fig. 4). Therefore, DNA methylation does not seem to contribute to the repression seen at the promoter region of the *mGlu2* gene.

Fig. 6. (A) Activation of *Egr1* promoter activity by the 5-HT_{2A} agonist TCB-2. NG108-15 cells were transfected with an *Egr1* promoter-luciferase construct and/or with a plasmid encoding the 5-HT_{2A} receptor; treated with TCB-2, ketanserin, and/or vehicle; and then analyzed for luciferase activity ($n = 6$). (B) Activation of *mGlu2* promoter activity by overexpression of Egr1. NG108-15 cells were transfected with the *mGlu2* promoter-luciferase construct in combination with a plasmid encoding Egr1 and then analyzed for luciferase activity ($n = 6$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Bonferroni's post hoc test of one-way analysis of variance. Error bars show S.E.M.

Decreased Binding of Egr1 to the *mGlu2* Promoter in 5-HT_{2A}-KO Mice. Previous findings demonstrate that 5-HT_{2A} receptor agonists induce the expression of *Egr1* and *Egr2* in vitro in tissue culture (Gonzalez-Maeso et al., 2003), as well as in vivo in mouse somatosensory (Gonzalez-Maeso et al., 2007; Moreno et al., 2013a,b) and frontal (Moreno et al., 2011a,b) cortex. These two genes encode transcription regulatory factors (Egr1 and Egr2) that are implicated in gene expression and underlie mechanisms of neuronal plasticity and memory formation (Jones et al., 2001; Davis et al., 2003). We tested the association of Egr1 and Egr2 with the promoter regions of *mGlu2* and *mGlu3* genes in frontal cortex of wild-type mice. Of interest, Egr1 was more enriched than Egr2 at the *mGlu2* promoter (Fig. 5A), whereas no detectable binding was seen at the promoter region of the *mGlu3* gene (Fig. 5A). The binding of Egr1 to the *mGlu2* promoter was further investigated in wild-type and 5-HT_{2A}-KO mice. ChIP analysis revealed that, although Egr1 binds to the *mGlu2* promoter in frontal cortex of both wild-type and 5-HT_{2A}-KO mice, this binding was significantly reduced in 5-HT_{2A}-KO mice, compared with wild-type littermates (Fig. 5B). These differences were not observed upstream (−1 kb) or downstream (exon 4) of the promoter region of the *mGlu2* gene (Fig. 5B).

Egr1 Positively Regulates *mGlu2* Promoter Activity. The impact of Egr1 on the transcriptional activity of *mGlu2* was then examined in tissue culture with use of promoter-reporter gene constructs. As expected (see above), activation of the 5-HT_{2A} receptor by TCB-2 resulted in positive regulation of *Egr1* promoter activity, an effect that was reversed by the 5-HT_{2A} receptor antagonist ketanserin (Fig. 6A). Next, we tested the effect of Egr1 on *mGlu2* promoter activity. Of importance, overexpression of Egr1 concentration dependently increased *mGlu2* promoter function (Fig. 6B). Taken together with the findings in mouse frontal cortex (Fig. 5),

these data indicate that Egr1 is critical for regulating transcriptional activation of the *mGlu2* promoter.

Viral-Mediated Overexpression of Egr1 Increases *mGlu2* Expression in Mouse Frontal Cortex. We next determined whether selective alteration of Egr1 expression in frontal cortical neurons might regulate *mGlu2* expression. Mice received intrafrontal cortical injections of HSV-2 viral particles expressing GFP and FLAG-Egr1 or GFP alone (Fig. 7A). Over-expression of FLAG-Egr1 in mouse frontal cortex was confirmed (Fig. 7B). Of note, such over-expression of FLAG-Egr1 increases expression of *mGlu2* in this brain region (Fig. 7, B and C). The specificity of the antibody anti-*mGlu2* has previously been assessed in frontal cortex of *mGlu2*-KO mice (Fribourg et al., 2011; Moreno et al., 2012).

Discussion

In the present study, we revealed repressive epigenetic changes at the promoter region of the *mGlu2* gene in frontal cortex of 5-HT_{2A}-KO mice. Mice with global disruption of 5-HT_{2A} receptor signaling show reduced expression of *mGlu2* mRNA and histone modifications at the *mGlu2* promoter that correlate with transcriptional repression. Our findings also reveal positive regulation of the *Egr1* promoter activity by the 5-HT_{2A} receptor agonist TCB-2 in tissue culture and decreased binding of Egr1 to the *mGlu2* promoter in 5-HT_{2A}-KO mice. Because viral-mediated over-expression of Egr1 up-regulates cortical *mGlu2* expression, together, these findings suggest that 5-HT_{2A} receptor-dependent cellular signaling pathways modulate the chromatin state of the *mGlu2* promoter in mouse frontal cortex through mechanisms that involve patterns of repressive histone modifications and decreased binding of the transcription factor Egr1.

We have previously shown that different agonists acting at the 5-HT_{2A} receptor elicit patterns of cellular signaling that

Fig. 7. Viral over-expression of Egr1 increases expression of mGlu2 in mouse frontal cortex. (A) Representative image of HSV-mediated transgene expression in frontal cortex. HSV-FLAG-Egr1, which also expresses GFP, was injected into frontal cortex, and GFP expression was revealed by immunocytochemistry (scale bar, 200 μ m). (B and C) Viral-mediated over-expression of Egr1 increases mGlu2 expression ($n = 4$). * $P < 0.01$; Student's t test. Error bars show S.E.M.

ultimately regulate distinct changes in gene transcription (Gonzalez-Maeso et al., 2003, 2007). Most of these genes, such as *Egr1*, *Egr2*, *c-Fos*, *Jun-B*, *N-10*, and *I- κ B α* , act as transcription factors or transcription regulators in numerous tissues, including the CNS. Although our findings suggest a mechanism that requires 5-HT_{2A} receptor-dependent modulation of Egr1 binding to the *mGlu2* promoter as involved in the repressed transcriptional activity of the *mGlu2* gene in frontal cortex of 5-HT_{2A}-KO mice, the effects of other genes which expression is modulated by 5-HT_{2A} receptor-dependent signaling pathways remain to be investigated. Similarly, our analysis and the data do not allow for deciding whether the repressive histone modifications observed at the *mGlu2* promoter in frontal cortex of 5-HT_{2A}-KO mice are cause or consequence of the decreased binding of Egr1 to the promoter region of the *mGlu2* gene. Additional work is also needed to

determine the stages throughout embryonic and pubertal development at which absence of 5-HT_{2A} receptor-dependent signaling affects the epigenetic status of the *mGlu2* gene.

One of the virtually unique conditions of psychiatric medications is their requirement for chronic administration to achieve full clinical effects. In patients with schizophrenia, optimal treatment with antipsychotic drugs requires weeks to months of sustained drug therapy (Agid et al., 2003; Lieberman et al., 2008; Nestler and Hyman, 2010). It has been recently shown that chronic treatment with the atypical antipsychotics clozapine or risperidone, but not with the typical antipsychotic haloperidol, decreases the density of 5-HT_{2A} receptor in mouse frontal cortex (Gonzalez-Maeso et al., 2008; Kurita et al., 2012; Moreno et al., 2013b). Similar effects of chronic antipsychotic treatment have been reported in postmortem human brain of schizophrenic subjects. Thus, the number of [³H]ketanserin binding sites to the 5-HT_{2A} receptor was increased in antipsychotic-free schizophrenic subjects, but not in schizophrenic subjects treated with atypical antipsychotic drugs (Gonzalez-Maeso et al., 2008; Muguruza et al., 2012). These results suggest that down-regulation of 5-HT_{2A} receptor binding by chronic treatment with atypical antipsychotics may be one of the mechanisms underlying their therapeutic effects. Of importance, it has also been shown that chronic treatment with atypical antipsychotic drugs induces 5-HT_{2A} receptor-dependent repressive histone modifications at the *mGlu2* promoter in mouse and human frontal cortex (Kurita et al., 2012). These findings, together with the current results, indicate that signaling pathways downstream of the 5-HT_{2A} receptor modulate the function of epigenetic regulators that affect chromatin structure and *mGlu2* transcription. Further studies are needed to unravel the neuronal signaling pathways specifically activated by this serotonin receptor that could potentially be involved in affecting posttranslational histone modifications at the *mGlu2* promoter in mouse and human frontal cortex, including β -arrestin-2 (Schmid et al., 2008), PSD-95 (Abbas et al., 2009), HDAC2 (Kurita et al., 2012), and Src (Gonzalez-Maeso et al., 2007).

Some histone modifications are thought to influence nucleosome stability, but an exciting emerging theme is that histone modifications can influence one another such that one modification recruits or activates chromatin-modifying complexes to generate a different histone modification (Suganuma and Workman, 2008; Ruthenburg et al., 2011). Many of the enzymes that posttranslationally modify histones display a high degree of specificity not only toward a particular site but also toward the pre-existing modification state of their substrate. We showed that absence of 5-HT_{2A} receptor-dependent signaling results in a substantial decrease in H3ac and H4ac at the *mGlu2* promoter, which occurs in association with an enhancement of repressive H3K27me3 binding. These results support the notion of an apparent coupling between removal of permissive marks and repression of transcription to placement of H3K27me3, an interesting phenomenon that has been previously observed in other systems (Bernstein et al., 2005; Roh et al., 2006).

In conclusion, the results of the present study indicate that 5-HT_{2A} receptor-dependent signaling affects both the histone code at the promoter region of the *mGlu2* gene and binding of the transcription factor Egr1 to this promoter in mouse frontal cortex, further supporting the notion that serotonin

and glutamate systems are intimately interconnected. The repressive transcriptional regulatory mechanisms that we found at the *mGlu2* promoter in mouse frontal cortex suggest the existence of compensatory pathways driven by global disruption of 5-HT_{2A} receptor signaling. From a more general perspective, these findings also raise concerns about the possibility of mechanisms that conceal phenotypic variation in genetically engineered mouse models.

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Authorship Contributions

Participated in research design: Kurita, Moreno, González-Maeso.
Conducted experiments: Kurita, Moreno, Holloway, Kozlenkov, Mocci, García-Bea, Hanks.
Contributed new reagents or analytic tools: Neve, Nestler, Russo.
Performed data analysis: Kurita, Moreno, González-Maeso.
Wrote or contributed to the writing of the manuscript: Kurita, Moreno, Nestler, Russo, González-Maeso.

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Address correspondence to: Dr. Javier González-Maeso, Department of Psychiatry, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1229, New York, NY 10029. E-mail: Javier.Maeso@mssm.edu