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TITLE PAGE:

Repressive epigenetic changes at the *mGlu2* promoter in frontal cortex of 5-HT$_{2A}$ knockout mice

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Running title: Epigenetic changes at *mGlu2* in 5-HT_{2A}-KO mice

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Abbreviations: ChIP, chromatin immunoprecipitation; GFP, green fluorescent protein; H3ac, acetyl-histone H3; H4ac, acetyl-histone H4; H3K4me1/2/3, mono/di/tri-methyl-histone H3 (Lys4); H3K27me3, tri-methyl-histone H3 (Lys27); H3K9me3, tri-methyl-histone H3 (Lys9); KO, knockout; LSD, lysergic acid diethylamide; mGlu2 receptor, metabotropic glutamate 2 receptor; NG108-15, mouse neuroblastoma × rat glioma cell line; PBS, phosphate buffer saline; PET, positron emission tomography; qRT-PCR, quantitative real-time PCR; TCB-2, 4-bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide.

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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 whose promoter activity was positively regulated by the 5-HT_{2A} receptor agonist TCB-2, binds less to the *mGlu2* promoter in frontal cortex of 5-HT_{2A}-KO as compared to 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.

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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 post-translational 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 6 on histone H3 [H3K6me]) 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 post-translational 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 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; Gonzalez-Maeso

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and Sealfon, 2009b). 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, all have in common a high affinity for the 5-HT_{2A} receptor as well as 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; Gonzalez-Maeso et al., 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 also 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; Moreno et al., 2012) responses that require expression of the 5-HT_{2A} receptor in cortical neurons. Interestingly, 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-

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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. 3-[2-[4-(4-Fluorobenzoyl)-1-piperid-inyl]ethyl]-2,4[1H,3H]-quinazolinedione tartrate (ketanserin tartrate) was obtained from Sigma-Aldrich. 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), and grown in Dulbecco's modified Eagle's medium supplemented with dialyzed 10% (v/v) foetal bovine serum, 0.4 µM aminopterin, 100 µM hypoxanthine, and 16 µM thymidine. Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions.

Plasmid Construction. All PCR reactions were performed using Pfu Ultra Hotstart DNA polymerase (Stratagene) in a Mastercycler Ep Gradient Auto thermal cycler (Eppendorf). For the *Egr1* promoter construct, mouse *Egr1* promoter (-410 to +10 bp) was PCR amplified from mouse genomic DNA (Clontech) using the following primers: 5'-ACGCCATATAAGGAGCAGGA-3' and 5'-CGAATCGGCCTCTATTTCAA-3'. The amplicon was inserted into pCR-blunt vector (Invitrogen), and then sequenced. The product was re-amplified

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using the primers 5'-GATctcgagACGCCATATAAGGAGCAGGA-3' and 5'-CTTaagcttCGAATCGGCCTCTATTTCAA-3', and then digested with XhoI and HindIII and subcloned into the XhoI and HindIII sites of pGL4.11 [*luc2P*] plasmid (Promega). 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 (see [Kurita et al., 2012]), and these two fragments were simultaneously subcloned into the BamH1 and XhoI sites of pcDNA3.1. For HSV-Flag-Egr1, the Flag-Egr1 fragment was digested from pcDNA3.1-Flag-Egr1 (see above), and subcloned into the BamH1 and XhoI sites of the bicistronic p1005+ HSV plasmid expressing GFP under the control of the CMV promoter (see [Kurita et al., 2012]). The pcDNA3.1-c-Myc-5HT2A 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). Briefly, NG108-15 cells were plated at a density of 1×10^5 in six-well dishes, cultured for 24 h, 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 μg), and the pcDNA3.1-c-Myc-5HT2A plasmid (1.0 μg). TCB-2 (0.01, 0.1, and 1.0 μM), ketanserin (1.0 μM), or vehicle was added to the medium without serum 5 h after transfection. For the *mGlu2* promoter assay, cells were transfected with the pGL4.11 [*luc2P*] plasmid (Promega) containing the mouse *mGlu2* promoter (1.5 μg), and the pcDNA3.1-FLAG-Egr1

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plasmid (1.0, 2.0, and 4.0 µg). Transfected cells were incubated for 24 h, and the luciferase activity was measured with a luminometer (TD-20/20; Turner Biosystems) using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions. Transfection efficiency was normalized with coexpressed pGL4.75 [*hRluc*/CMV] (Promega) (0.02 µg).

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 h 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 –80ºC, 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 carried out in quadruplicate using SYBR green as previously described (Gonzalez-Maeso et al., 2007; Gonzalez-Maeso et al., 2008; Kurita et al., 2012). All reactions were confirmed to generate a single PCR 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

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(Millipore) as previously reported (Kurita et al., 2012). Briefly, mouse frontal cortex tissue samples were minced to less than 1 mm-sized pieces, and immediately cross-linked in 1% formaldehyde for 20 min 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), 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 by using 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 8.1) to a final volume of 1.0 ml. Primary antibodies were added to diluted lysates, and incubated at 4°C for 12 h with 20 µl of fully suspended protein A or G magnetic beads (Millipore). The following primary antibodies were used: acetyl-histone H3 (Millipore 06-599, 1:200), acetyl-histone H4 (Millipore 06-866, 1:200), mono/di/tri-methyl-histone H3 (Lys4) (Millipore 05-791,1:300), tri-methyl-histone H3 (Lys9) (Millipore 07-442, 1:250), tri-methyl-histone H3 (Lys27) (Millipore 07-449, 1:300), Egr1 (Santa Cruz sc-110, 1:200), and Egr2 (Santa Cruz, 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, and10 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 $NaHCO_3$) containing 0.1 µg/µl proteinase K for 2 h at 62°C. DNA was purified by using spin columns (Millipore). Input and immunoprecipitated DNAs were subjected to qPCR (see Table 1 for

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qPCR primer pair sequences). All assays included non-immune immunoglobulin (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 ng – 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) (Fig. 3B and Fig. 5A), as enrichment relative to that of the non-immune immunoglobulin (IgG) (Fig. 5B), or as fold enrichment relative to that of IgG for wild-type versus 5-HT_{2A}-KO mice (Fig. 2), depending upon 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 (Millipore) as previously reported (Kurita et al., 2012). Briefly, genomic DNA was isolated from mouse brain frontal cortex and liver tissue samples using the Non-Organic DNA Extraction Kit (Millipore) according to the manufacturer's instructions, and subjected to bisulfite modification to convert all non-methylated cytosines into thymidines. For sodium bisulfite treatment, the CpGenomie Fast DNA Modification Kit (Millipore) was used according to the manufacturer's instructions. Modified DNA was then amplified by PCR using bisulfite-treated DNA specific primer sets 5'- GGTATTAAGGGTTAATTTTATTTGG-3' and 5'-ACACTATAAACAACTCCAAACCCTAC-3'; and 5'-TGAGAGGTTGAGATAAAGATAGAGATATAG-3' and 5'- CCAAATAAAATTAACCCTTAATACC-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

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analyzed for possible methylated CpG sites using 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). Briefly, mice were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg) during the surgery. The virus was delivered bilaterally with a Hamilton syringe at a rate of 0.1 µl/min for a total volume of 0.5 µl on each side. The following coordinates were used: +1.6 mm rostral-caudal, -2.4 mm dorsal-ventral, +2.6 mm medial-lateral from bregma (relative to dura) with a 10° lateral angle. The coordinates were taken according to a published atlas of the 129/Sv mouse strain (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 over-expression levels in frontal cortex were confirmed by Western blotting and qRT-PCR (Fig. 7b and data not shown).

Immunohistochemistry. Experiments were performed as previously reported (Kurita et al., 2012). Briefly, 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 (PFA) in PBS at room temperature. Brains were removed and immersion-fixed in 4% PFA in 1 ml at 4ºC for 60 min and stored at 30% sucrose in PBS at 4ºC for an additional 12 h before to obtain the frontal cortex section. A series of 30 µm 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

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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 sc-8334; 1:100) were incubated overnight at 4ºC. After washing with PBS, the tissue sections were incubated with the secondary antibodies (Alexa 488 dye-conjugated anti-rabbit antibody; 1:500) for 60 min at room temperature. After washing (PBS, 6 $\times$ 2 ml), the tissue sections were mounted onto coverslips treated with antifade. Tissue samples were examined by 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; 1:1,000), mGlu2 (Abcam ab15672; 1:1,000), and α-tubulin (Cell Signaling 11H10; 1:1,000). Metabotropic glutamate receptors are G protein coupled receptors (GPCRs) 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; Gonzalez-Maeso et al., 2007; Gonzalez-Maeso et al., 2008). In order 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 following standard protocols at the Mount Sinai Mouse Genetics Shared Resource Facility. The embryos were then transferred to pathogen-free

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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.

Importantly, we found that expression of *mGlu2* mRNA was also reduced in frontal cortex of 5-HT_{2A}-KO mice, as compared to wild-type littermates (Fig. 1). No differences were observed for expression of *5-HT_{2C}* or *mGlu3* mRNA (Fig. 1). Since 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 due 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 post-translational histone modifications at the *5HT2C*, *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 if histone modifications known to correlate with transcriptional repression are affected in 5-HT_{2A}-KO mice. A significant increase in histone H3 tri-methylation at lysine 27 (H3K27me3), a repressive histone modification marker, was found at the *mGlu2* promoter, with no apparent changes at the *5HT2C* and *mGlu3* promoters (Fig. 2D). Histone H3 tri-methylation

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at lysine 9 (H3K9me3), another histone modification that correlates with transcriptional repression, was not increased at any of these promoters in frontal cortex of 5-HT_{2A}-KO mice (Fig. 2E).

In order 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 as compared to 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 above, binding of H3K4me1/2/3 and H3K9me3 was not affected (Fig. 3B), and no significant changes were found at the promoter region of the *mGlu3* 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 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.

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; Moreno et al., 2013b) and frontal (Moreno et al., 2011a; Moreno et al., 2011b) cortex. These two genes encode

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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. Interestingly, 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 as compared to wild-type littermates (Fig. 5B). These differences were not observed upstream (-1 kb) or downstream (exon 5) 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 using 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, 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. Importantly, over-expression of Egr1 concentration-dependently increased *mGlu2* promoter function (Fig. 6B). Taken together with the findings in mouse frontal cortex (see Fig. 5 above), these data indicate that Egr1 is critical for regulating transcriptional activation of the *mGlu2* promoter.

Viral-mediated over-expression 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 intra-frontal cortical injections of HSV-2 viral

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particles expressing green fluorescent protein (GFP) and Flag-Egr1, or GFP alone (Fig. 7A). Over-expression of Flag-Egr1 in mouse frontal cortex was confirmed (Fig. 7B). Notably, such over-expression of Flag-Egr1 increases expression of mGlu2 in this brain region (Figs. 7B and 7C). 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 this study we demonstrate 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 as well as histone modifications at the *mGlu2* promoter that correlate with transcriptional repression. Our findings also demonstrate 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. Since 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 ultimately regulate distinct changes in gene transcription (Gonzalez-Maeso et al., 2003; Gonzalez-Maeso et al., 2007). Most of these genes, such as *Egr1*, *Egr2*, *c-Fos*, *Jun-B*, *N-10* and *I-κBα*, act as transcription factors or transcription regulators in numerous tissues including the CNS. While 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

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effects of other genes whose expression is modulated by 5-HT_{2A} receptor-dependent signaling pathways remain to be investigated. Similarly, our analysis as well as the data do not allow for deciding whether the repressive histone modification 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. Further 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 schizophrenia patients, 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 [^{3}H]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. Importantly, 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

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studies are needed to unravel the neuronal signaling pathways specifically activated by this serotonin receptor that could potentially be involved in affecting post-translational 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), to name a few.

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 post-translationally 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 demonstrate 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

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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, and González-Maeso.

Conducted experiments: Kurita, Moreno, Holloway, Kozlenkov, Mocci, García-Bea, and Hanks.

Contributed new reagents or analytical tools: Neve, Nestler, and Russo.

Performed data analysis: Kurita, Moreno, and González-Maeso.

Wrote or contributed to the writing of the manuscript: Kurita, Moreno, Nestler, Russo, and González-Maeso.

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Footnotes

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LEGENDS FOR FIGURES

Fig. 1. Expression of *5HT2C*, *mGlu2*, *mGlu3* and GAPDH mRNAs in 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 shown S.E.M.

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 shown 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 *mGlu3* promoter was also measured (n = 8 per group). **P* < 0.05, ***P* < 0.01; Student's *t*-test. Error bars shown S.E.M.

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, 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. Representative example is shown (B). Ten clones were sequenced for each mouse (n = 6 mice per group). Percentage of methylated clones is shown (C). N.S., not significant; Student's *t*-test. Error bars show S.E.M.

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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 ANOVA. (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 5 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 ANOVA. Error bars shown S.E.M.

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 over-expression 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 ANOVA. Error bars shown S.E.M.

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 µm). (B, C) Viral-mediated over-expression of Egr1 increases mGlu2 expression (n = 4). **P* < 0.01; Student's *t*-test. Error bars shown S.E.M.

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TABLES

Table 1. PCR primer pairs for ChIP assay in mouse samples

Gene name	GenBank	Primer pairs		Location	Nucleotide location from TSS[1]
		Forward	Reverse		
Htr2c	**NT_039718**	GCCATGGATGACCTCAGTTT	TACACTGCTCAGGGCCTGTT	Promoter	-238 to -94
Grm2	**NT_039477**	ATCCTGCTGCTACCACGTCT	TGGACACAGAAACTGGATGC	-1.4 kb upstream promoter	-1419 to -1299
Grm2	**NT_039477**	GCCACTGTCTCATCTGTTCC	ATCCCGCTCTTGACAGGT	Promoter	-340 to -188
Grm2	**NT_039477**	ATTGTCTGCTGGGTTTGGAG	TTAACCCGGTGAGACCTCTG	0.8 downstream promoter	+837 to +958
Grm2	**NT_039477**	TTAATGAGCACCGTGGCATA	CGTGTGTATCCTTGGAGCAG	Exon 2	+1979 to +2113
Grm2	**NT_039477**	GGGAGGTAGCCTCAGCTCTT	GACCTCGTCGTCGGTATCTG	Exon 4	+7784 to +7918
Grm3	**NT_039299**	TTCACTCGCTCACACTGCTC	AAGCTCTGCTAAGGCTCACG	Promoter	-694 to -555
Actb	**NT_081055**	GAGACATTGAATGGGGCAGT	ATGAAGAGTTTTGGCGATGG	Promoter	-321 to -230

[1]TSS: transcriptional start site

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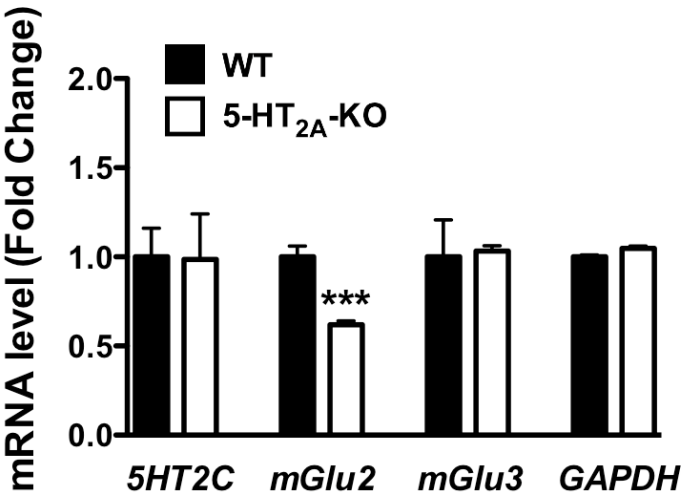

Figure 1

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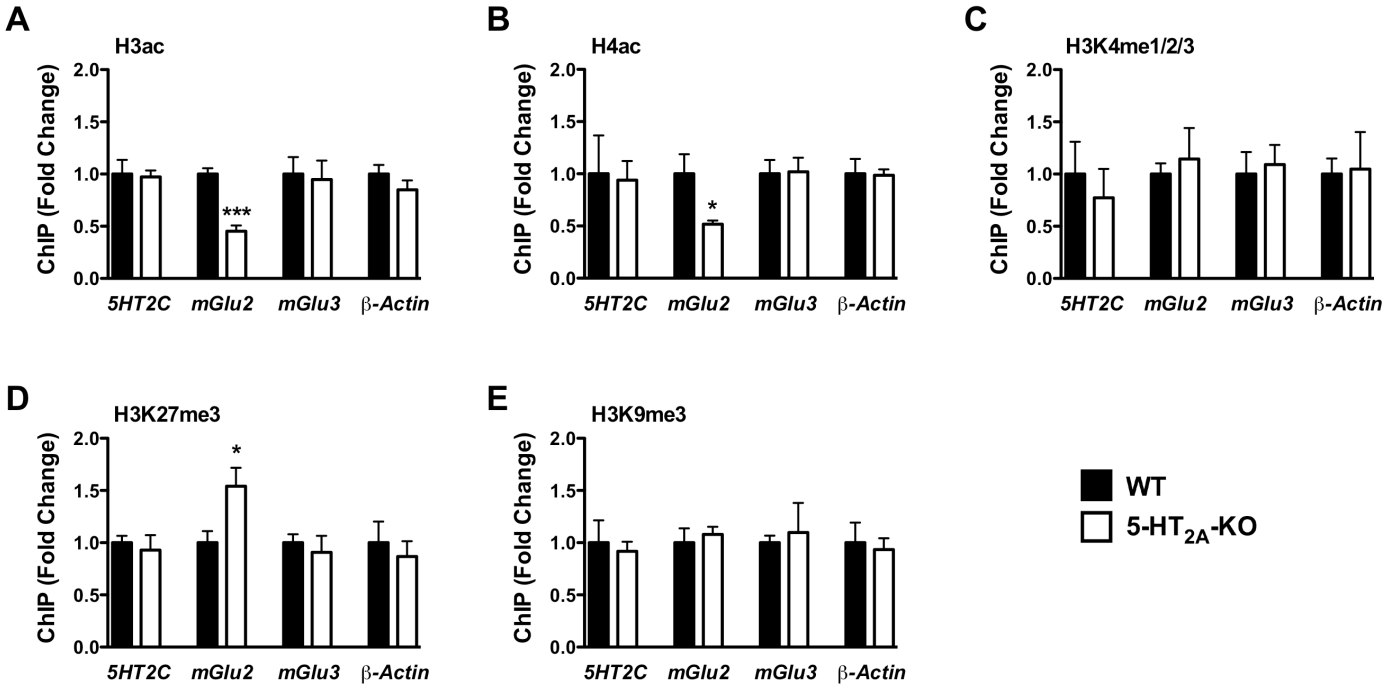

Figure 2

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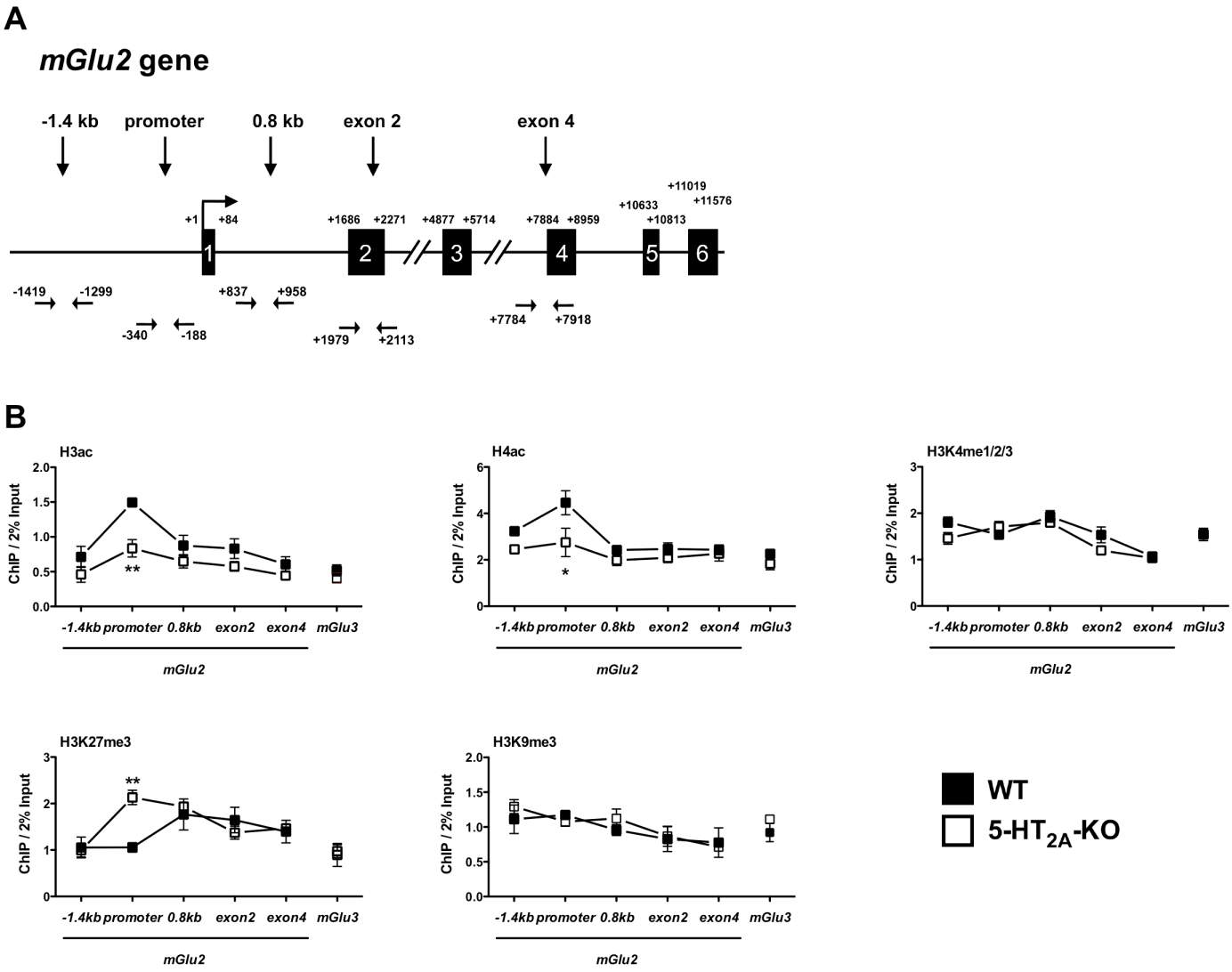

Figure 3

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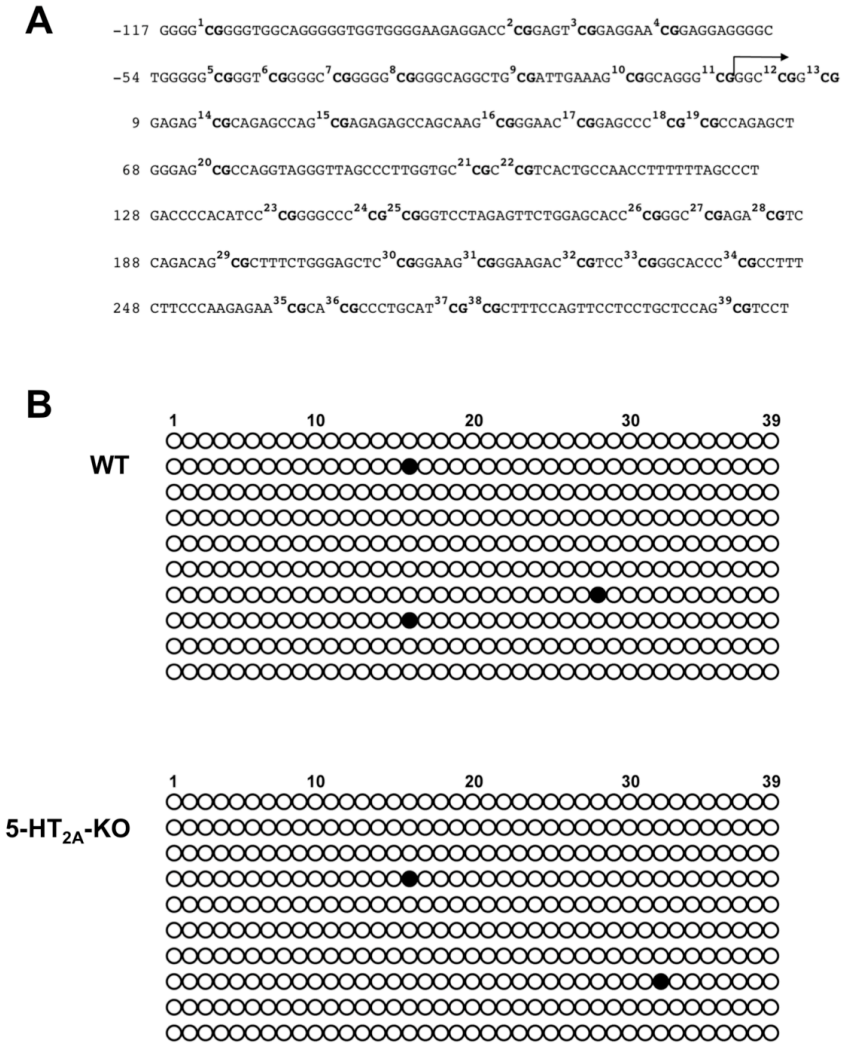

Figure 4

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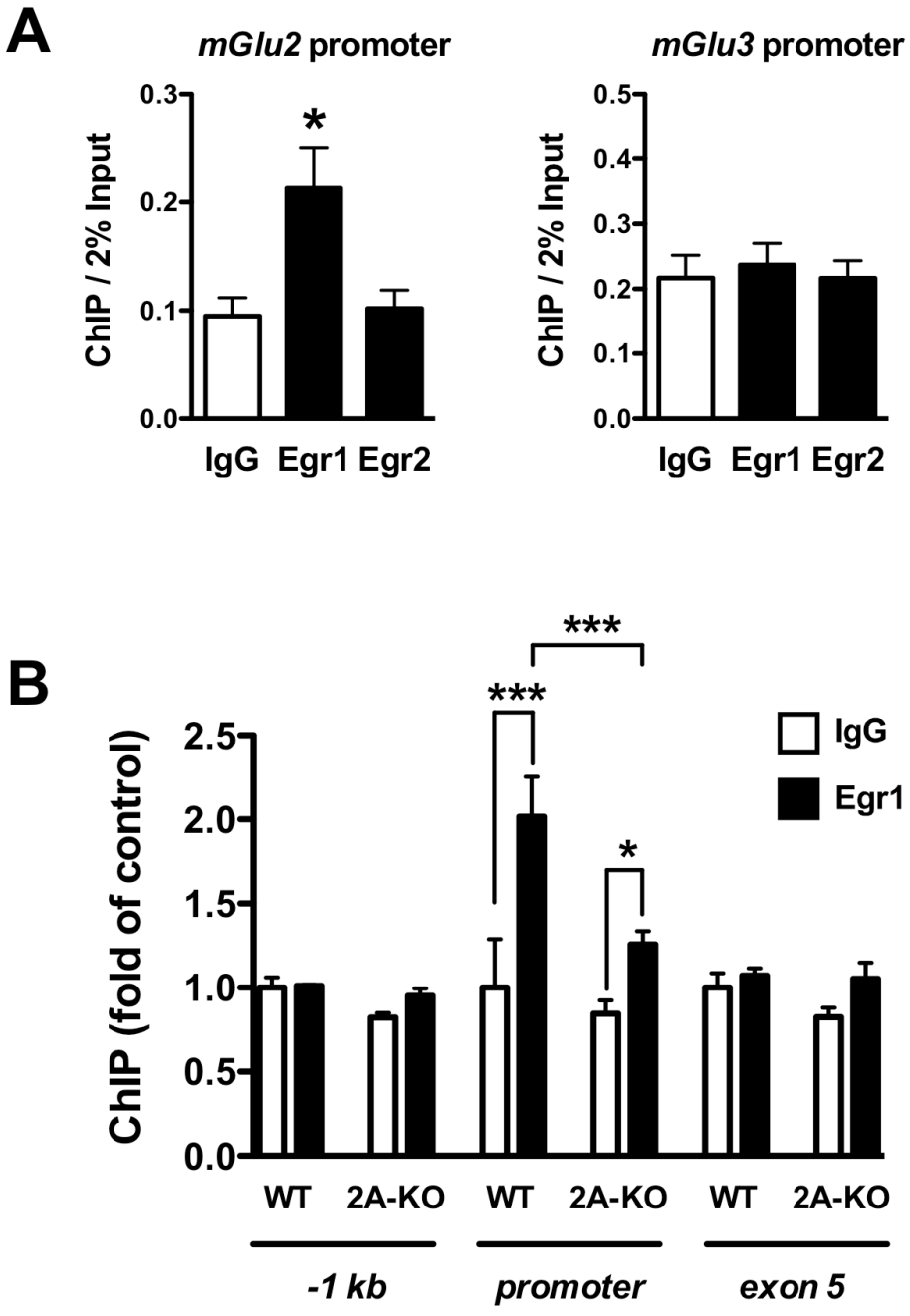

Figure 5

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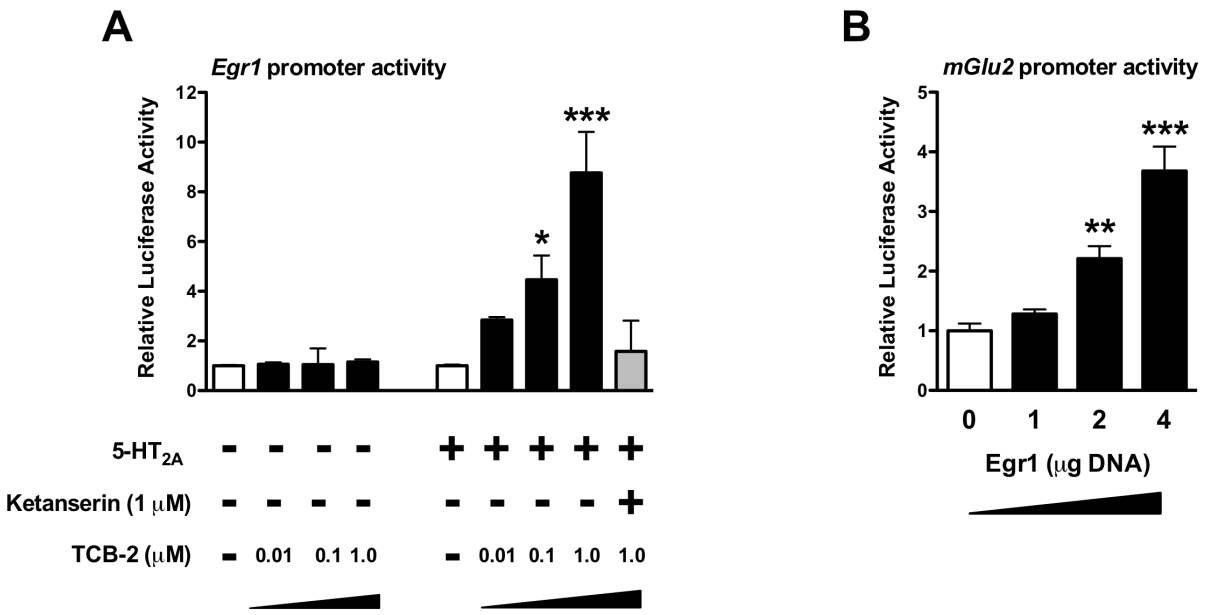

Figure 6

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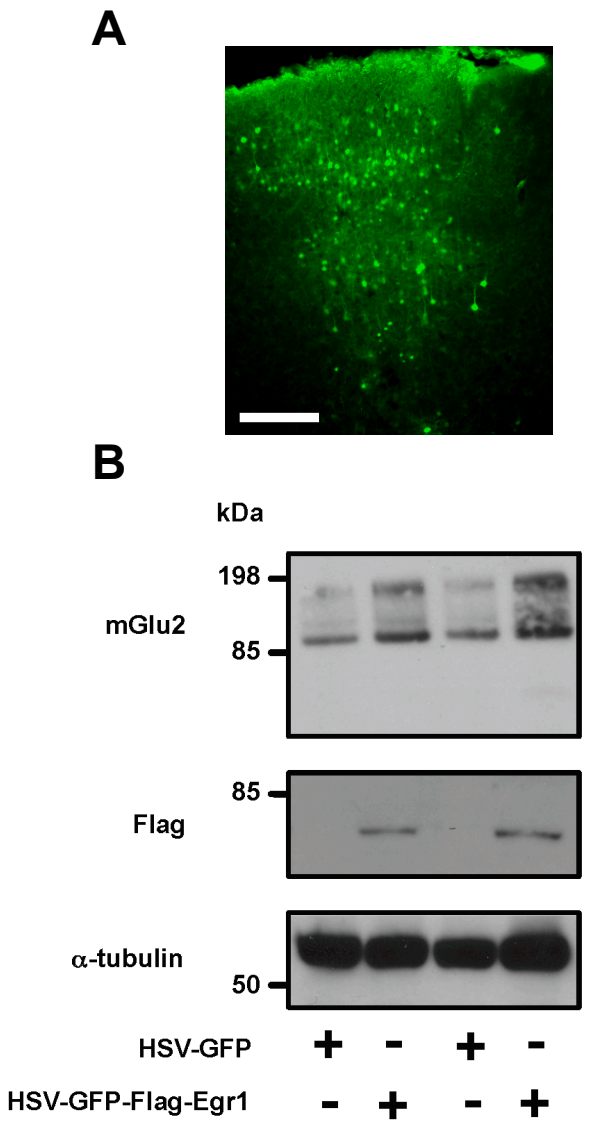

Figure 7