# Title: Environment rapidly upregulates serotonin 2A receptor expression via immediate early gene *Egr3*

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**Abstract:**

Serotonin 2A receptors (5-$HT_{2A}$Rs) mediate the effects of hallucinogenic drugs and antipsychotic medications, and are reduced in schizophrenia patients' brains. However, how 5-$HT_{2A}$R expression is regulated remains poorly understood. We show that 5-$HT_{2A}$Rs are rapidly upregulated in the mouse frontal cortex (FC) in response to an environmental stimulus, sleep deprivation. This induction requires the immediate early gene transcription factor early growth response 3 (*Egr3*). Further, EGR3 binds to the *Htr2a* promoter in the FC *in vivo*, and drives reporter construct expression *in vitro* via two *Htr2a* promoter binding sites. These findings suggest that EGR3 directly regulates FC *Htr2a* expression in response to physiologic stimuli, providing a mechanism by which environment rapidly alters levels of a brain receptor that mediates symptoms, and treatment, of mental illness.

Serotonin 2A receptors (5-$HT_{2A}$Rs) have been implicated in the pathogenesis and treatment of psychiatric disorders ranging from depression to schizophrenia (1). The hallucinogenic properties of drugs such as lysergic acid (LSD), psilocybin, and mescaline, are mediated by the 5-$HT_{2A}$R (2). Binding to the 5-$HT_{2A}$R is also a critical characteristic that distinguishes the antipsychotic medication clozapine, from all prior treatments, and established the "second generation" antipsychotics (SGAs) that are now the standard of care for treating psychotic disorders (3). The affinity of these medications for the 5-$HT_{2A}$R is thought to contribute to both the improved efficacy of SGAs, and their absence of extra-pyramidal side effects, compared with prior medications (4).

Numerous post-mortem and *in vivo* studies have revealed that 5-$HT_{2A}$Rs are reduced in the brains of schizophrenia patients (5-15). Together, these findings underscore the critical role of the 5-$HT_{2A}$R in the symptoms, and treatment, of psychiatric illnesses, particularly those with psychotic features. Although it is well established that agonists and antagonist of the receptor modulate levels of 5-$HT_{2A}$Rs in the cell membrane (16, 17), relatively little is known about the molecular mechanisms that regulate the expression of this critical receptor (18, 19).

Our prior studies revealed that reduced 5-$HT_{2A}$R levels appears to underlie resistance to the sedating effects SGAs, a phenotype displayed by mice that lack function of the immediate early gene (IEG) *Egr3* (*Egr3*-/- mice) (20, 21). This phenotype parallels a characteristic of schizophrenia patients, who tolerate much higher doses of antipsychotic medications before developing side effects, including sedation, than do non-psychiatrically ill individuals (22). Indeed, 5-$HT_{2A}$R-/- mice display the same resistance to sedation by SGAs as do *Egr3*-/- mice and schizophrenia patients (23). Together these findings led us to hypothesize that EGR3, an activity-dependent transcription factor, may regulate expression of the 5-$HT_{2A}$R gene (*Htr2a*).

Here we report that levels of the 5-$HT_{2A}$R in the mouse frontal cortex (FC) can be significantly upregulated following a brief environmental stimulus of 6 to 8 hours (h) of sleep deprivation (SD). This is evident both at the level of mRNA expression as well membrane-bound receptor protein. This upregulation occurs in the FC, but not in more posterior regions of the cortex where 5-$HT_{2A}$R levels are normally low. We demonstrate that this environmental induction requires function of the activity-dependent IEG transcription factor *Egr3*. Furthermore, EGR3 protein binds to the promoter of the *Htr2a* gene in the mouse FC *in vivo*, and *in vitro* overexpression of EGR3 is able to drive expression of a reporter construct via binding to either *Htr2a* promoter sites, suggesting that EGR3 directly regulates 5-$HT_{2A}$R expression in the FC in response to environmental stimuli.

We hypothesized that, if EGR3 directly regulates *Htr2a*, then stimuli that upregulate EGR3 in the cortex should also increase expression of the 5-$HT_{2A}$R gene, *Htr2a*. To test this hypothesis we exposed mice to 6h of SD, a physiological stimulus that induces expression of *Egr3* (24). Our prior studies showed that 6h of SD significantly upregulated *Htr2a* mRNA isolated from whole mouse cortex, and that this induction required *Egr3* (25). However, since *Htr2a* is expressed in an anterior to posterior gradient in the mouse cortex (26, 27), it was not clear whether *Htr2a* was being upregulated in regions in which it is normally expressed, or if it is activated in regions where endogenous expression is low or absent. Moreover, it was unknown whether the increased expression resulted in increased receptor protein, or whether the EGR3 was directly regulating the *Htr2a* gene.

Published *in situ* hybridization images from the Allen Institute for Brain Science SD study suggest that SD upregulates *Egr3* expression throughout most of the cortex, but this does not appear to extend to most anterior regions of the frontal cortex (24). We therefore hypothesized that SD should similarly *not* increase *Htr2a* expression in the anterior frontal cortex (AFC), but should induce it in the region where Egr3 expression is increased, in the cortex at the coronal plane of the prefrontal cortex, an area we refer to as "posterior frontal cortex" (PFC) (see Fig 1A). Since published images show that *Htr2a* levels decrease in regions posterior to the PFC (26, 27), an area we refer to as mid-posterior cortex (MPC), we did not expect to see significant upregulation in this region.

**Figure 1.**

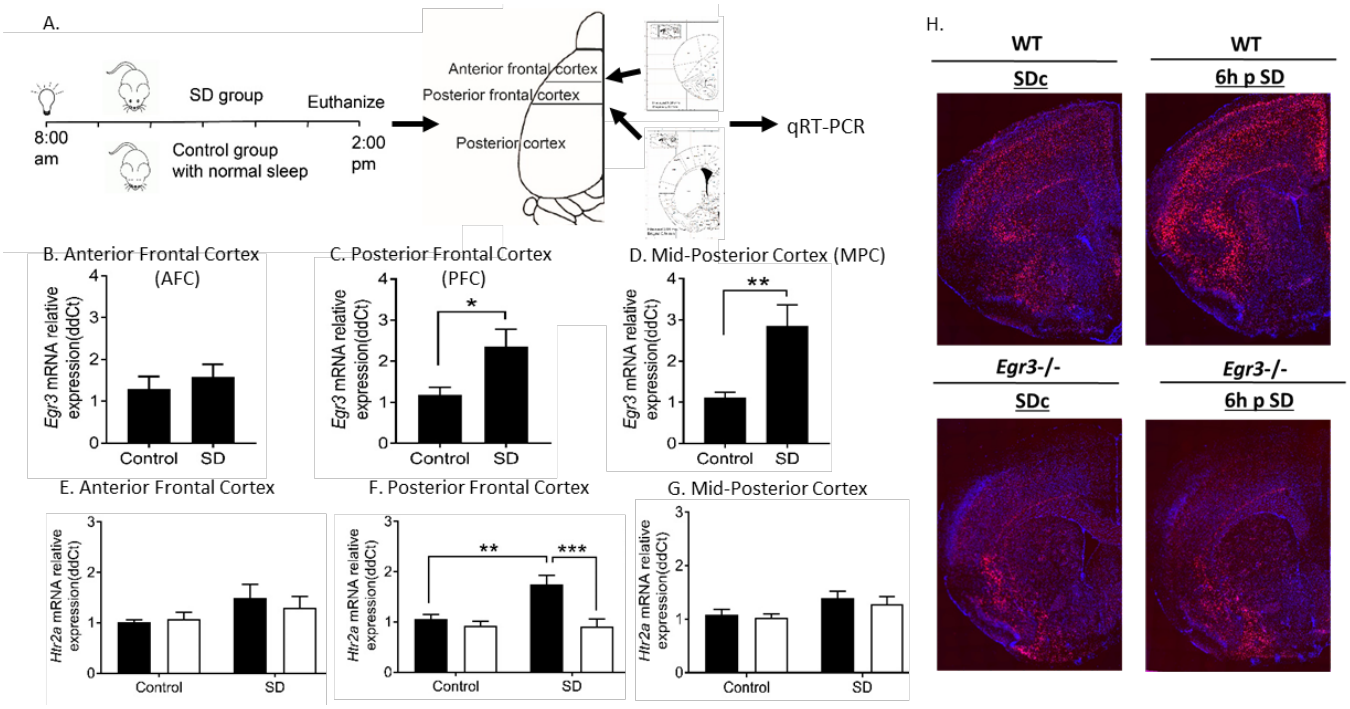


**Figure 1. Sleep deprivation upregulates *Htr2a* in an *Egr3*-dependent, and region-specific, manner.** (**A**) SD protocol and regional brain dissection. In WT mice quantitative RT-PCR shows that 6h of SD (**B**) does not increase *Egr3* expression in AFC regions ($t_{27} = 0.6679$; $p = 0.510$), but significantly upregulates *Egr3* mRNA in (**C**) PFC ($t_{28} = 2.615$, $p = 0.0142$) and (**D**) MPC ($t_{27} = 3.5$; $p = 0.0016$) regions, compared to non-SD controls. (**E** – **G**) 6h of SD in WT compared with *Egr3*-/- mice (**E**) does not increase expression in either genotype in AFC (ANOVA; no sig. main effect of SD ($F_{1, 52} = 3.488$, p = 0.0675) or genotype ($F_{1, 52} = 0.1129$, p = 0.7382)), but (**F**) significantly upregulates *Htr2a* expression in the PFC of WT, but not *Egr3*-/-, mice (ANOVA: sig. main effect of SD ($F_{1, 54} = 5.857$, $p = 0.0189$), and sig. main effect of genotype ($F_{1, 54} = 11.95$, $p = 0.0011$); post-hoc analyses showed a sig. increase of *Htr2a* mRNA after SD compared with SD controls in WT mice ($p < 0.01$), but not in the *Egr3* -/- mice ($p = 0.9998$)). (**G**) In the MPC, SD increased *Htr2a* overall when both genotypes were analyzed (ANOVA, sig. main effect of SD ($F_{1, 49} = 5.976$, $p = 0.0181$)) but *Htr2a* mRNA increases were not sig. when

comparing either genotype alone (post-hoc analyses showed no significant comparisons between genotypes or SD conditions). (**H**) RNAscope *in situ* hybridization demonstrating *Htr2a* expression in SDc and SD WT and *Egr3*-/- mice. Bonferroni-corrected comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 12\text{-}15$. Values represent means ± SEM. (AFC: anterior frontal cortex; h: hours; PFC: posterior frontal cortex; SD: sleep deprivation; sig: significant, MPC: mid to posterior cortex; WT: wildtype).

Figure 1A shows the SD protocol and coordinates for regional brain dissection. We first tested whether we could replicate the *in situ* hybridization results of Thompson and colleagues (24), showing that SD upregulates *Egr3*, using quantitative reverse transcription (qRT) PCR. Figures 1B – 1D show that, in WT mice, compared with non-SD control (SDc) mice, 6h of SD did not increase *Egr3* expression in the AFC. However, this stimulus significantly upregulated *Egr3* mRNA levels in cortex from the PFC, as well as in more posterior regions of cortex (an area we refer to as "mid-posterior cortex (MPC)).

We next examined whether 6h of SD can upregulate expression of *Htr2a* mRNA in the same cortical regions in WT and in *Egr3*-/- mice (Fig.s 1E – 1G). In the AFC, SD did not significantly alter expression of *Htr2a* in either WT or *Egr3*-/- mice compared with non-SD controls (Fig. 1E). However, in the PFC of WT mice, SD induced significantly upregulated *Htr2a* mRNA compared to non-SD controls, a result not seen in *Egr3*-/- mice (Fig. 1F). In the MPC, SD induced a small increase in *Htr2a* expression when both genotypes were analyzed together (ANOVA revealed a main effect of genotype), but the increase was not significant in either genotype alone (post-hoc tests were not significant) (Fig. 1G). Figure 1H shows the upregulation of cortical *Htr2a* mRNA in WT mice following 6h of SD (sections from coronal plane of the PFC). In *Egr3*-/- mice *Htr2a* expression is significantly lower than in WT mice at baseline (SDc), and is not increased following 6h SD.

To determine if the induction of *Htr2a* by SD is paralleled by an increase in 5-$HT_{2A}$R protein expression, we performed autoradiography of brain tissue sections labeled with $^3$H-M100907, a selective 5-$HT_{2A}$R antagonist. To allow for translation of mRNA, we continued the SD protocol for an additional 2 h, for a total of 8h (Fig. 2A). We then compared densitometry of 5-$HT_{2A}$R binding in WT and *Egr3*-/- mice at baseline versus 8h post SD. Figure 2B shows that, in the AFC, SD did not significantly increase 5-$HT_{2A}$R binding in either WT or *Egr3*-/- mice compared to SDc conditions. However, it augmented WT expression sufficiently to produce a significant difference in 5-$HT_{2A}$R levels between WT and *Egr3*-/- mice that is not present in SDc animals.

In contrast, in the PFC region, 8h of SD induced a significant increase in 5-$HT_{2A}$R levels in WT mice that was absent in *Egr3*-/- mice (Fig. 2C). In addition, 5-$HT_{2A}$R levels are significantly greater in WT than *Egr3*-/- mice in this region both at baseline (replicating our prior findings using radioligand binding assay (21)), and following SD.

In the MPC, where *Htr2a* expression is lower than in more anterior cortical regions, SD did not increase 5-$HT_{2A}$R levels in WT or *Egr3*-/- mice (Fig. 2D). Levels of 5-$HT_{2A}$R were lower in *Egr3*-/- mice under both basal (SDc) and SD conditions in this region. Figures 2E – 2G show representative autoradiographic images from WT and *Egr3*-/- mice under SDc and SD conditions in coronal sections from AFC, PFC, and MPC regions.

In all regions, radioligand binding revealed significant differences in 5-HT$_{2A}$R levels between WT and *Egr3*-/- mice after SD, and in all but the most anterior region (AFC) in baseline (SDc) animals. In contrast, differences in *Htr2a* mRNA levels between WT and *Egr3*-/- mice were seen only in the PFC region following SD. This difference may be due to the longer perdurance of protein than mRNA.

These data show that 5-HT$_{2A}$Rs can be rapidly upregulated in the PFC in response to an environmental stimulus in an *Egr3*-dependent manner, and suggest that EGR3, an immediate early gene transcription factor, may directly regulate expression of *Htr2a* in response to environmental events.

**Figure 2.**

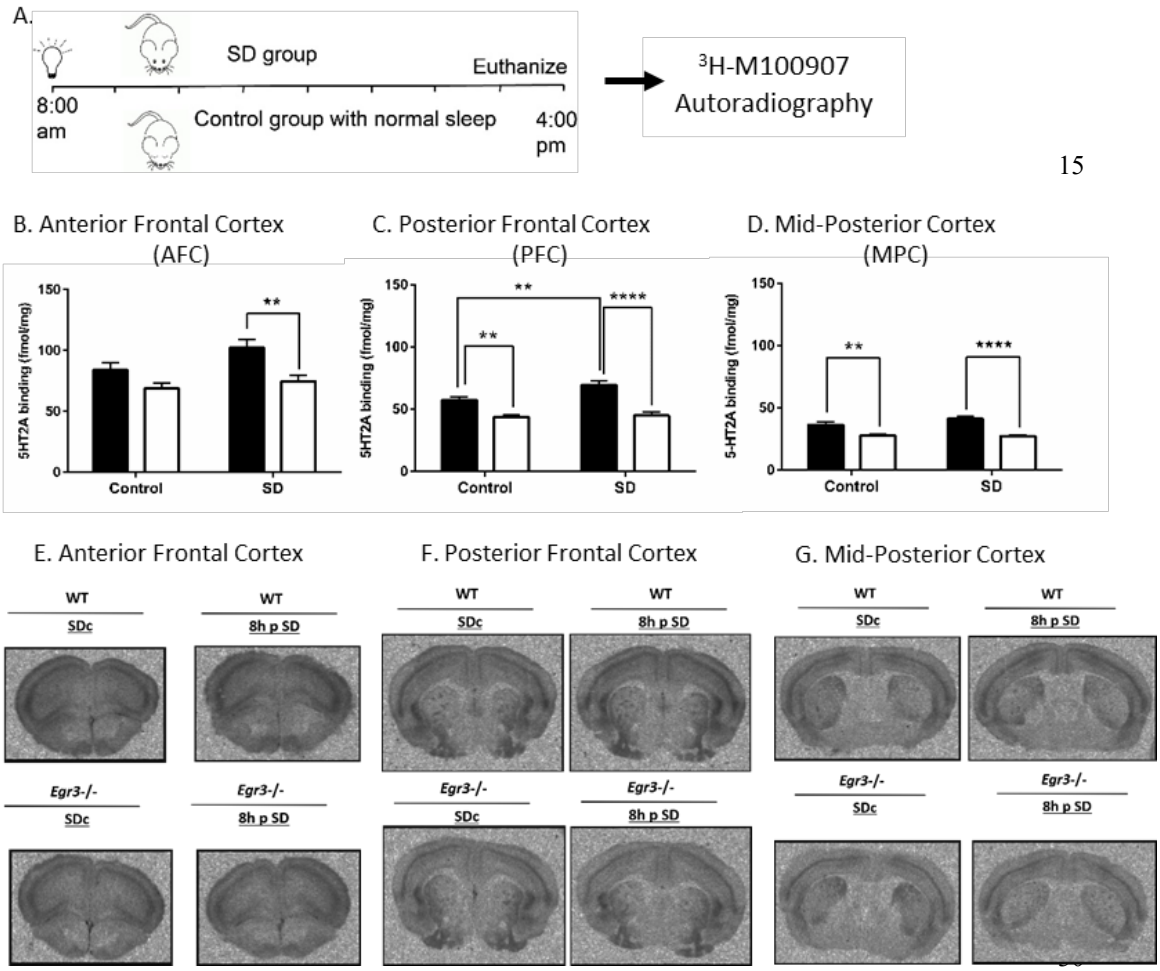


**Figure 2. SD increases 5-HT$_{2A}$R levels in the PFC of WT mice in an *Egr3*-dependent manner.** (**A**) 8h SD protocol. Quantification of $^3$H-M100907 binding autoradiography shows that SD, compared with non-SD control (SDc): (**B**) increased 5-HT$_{2A}$R levels in the WT AFC to cause significantly greater 5-HT$_{2A}$R levels in WT mice than *Egr3*-/- mice after SD (ANOVA: sig. main effects of SD ($F(1,62) = 4.61$, $p = 0.036$) and genotype ($F(1,62) = 14.78$, $p = 0.0003$);

(**C**) In the PFC SD significantly upregulates 5-HT$_{2A}$R levels in WT, but not *Egr3*-/-, mice (ANOVA: sig. interaction between SD and genotype ($F(1,62) = 4.18$, $p = 0.045$). (**D**) In the MPC, SD did not significantly increase 5-HT$_{2A}$R levels; notably, 5-HT$_{2A}$Rs were lower in *Egr3*-/- mice than WT under both basal (SDc) and SD conditions (ANOVA: no effect of SD ($F(1,62) = 1.371$, $p = 0.25$), but a sig. main effect of genotype ($F(1,62) = 38.79$, $p < 0.0001$). Representative $^3$H-M100907 autoradiography images of brain tissue sections from (**E**) AFC, (**F**) PFC, and (**G**) MPC. Bonferroni-corrected comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 16$-17. Values represent means ± SEM.

**Figure 3.**

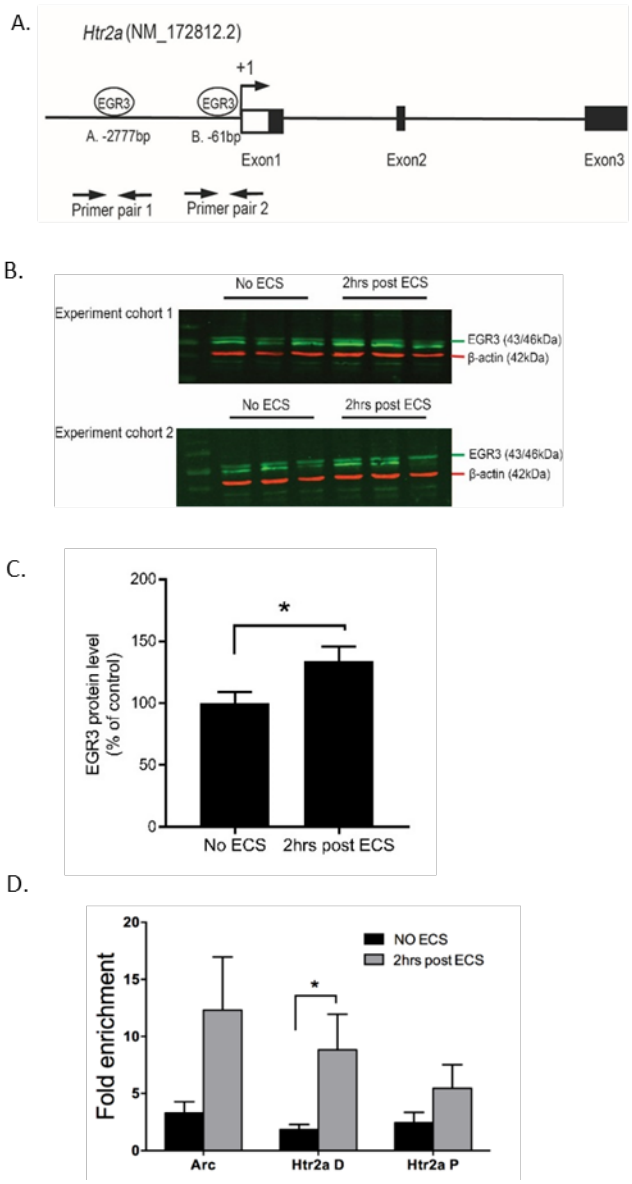


**Figure 3. EGR3 binds to the *Htr2a* promoter in frontal cortex.** (**A**) Schematic showing high probability EGR3 consensus binding sites in the *Htr2a* promoter. Western Blot **(B)** images and (**C**) average protein levels, show significant upregulation of activity dependent EGR3 protein 2h after electroconvulsive stimulation (ECS) in WT frontal cortex (n = 6 per group). **(D)** ChIP-qPCR shows ECS increases binding of EGR3 to *Htr2a* distal promoter in frontal cortex tissue (n = 11 per group). Unpaired student *t*-tests, * $p < 0.05$. Values represent means ± SEM.

We have previously shown that EGR3 is expressed in *Htr2a*-expressing pyramidal neurons in the mouse FC, an essential criterion for EGR3 to potentially directly regulate *Htr2a* expression. To test whether EGR3 binds to the *Htr2a* promoter in the FC *in vivo* we searched for EGR consensus binding sites in the 2 kb upstream DNA of the mouse *Htr2a* gene. Figure 3A shows two high probability EGR binding sites identified; a "distal" site located approximately 2800 bp upstream of the transcription start site, and a "proximal" site, approximately 70 bp upstream of the *Htr2a* transcriptional start site.

To determine whether EGR3 protein binds to these binding sites in mouse cortex we conducted chromatin immunoprecipitation (ChIP). As an activity dependent immediate early gene, *Egr3* is expressed in response to neuronal activity. We used electroconvulsive seizure (ECS) to induce neuronal activity and maximal *Egr3* expression in the brain. Figure 3B and 3C show that levels of EGR3 protein are significantly increased in the cortex from the PFC region of WT mice by 2 hrs following ECS. Compared to the positive control region, the promoter of activity-regulated cytoskeleton associated protein (*Arc*) (a validated EGR3 target gene (28)), binding of EGR3 to the distal *Htr2a* promoter was significantly increased following ECS, compared to non-stimulated controls (Fig. 3D).

**Figure 4.**

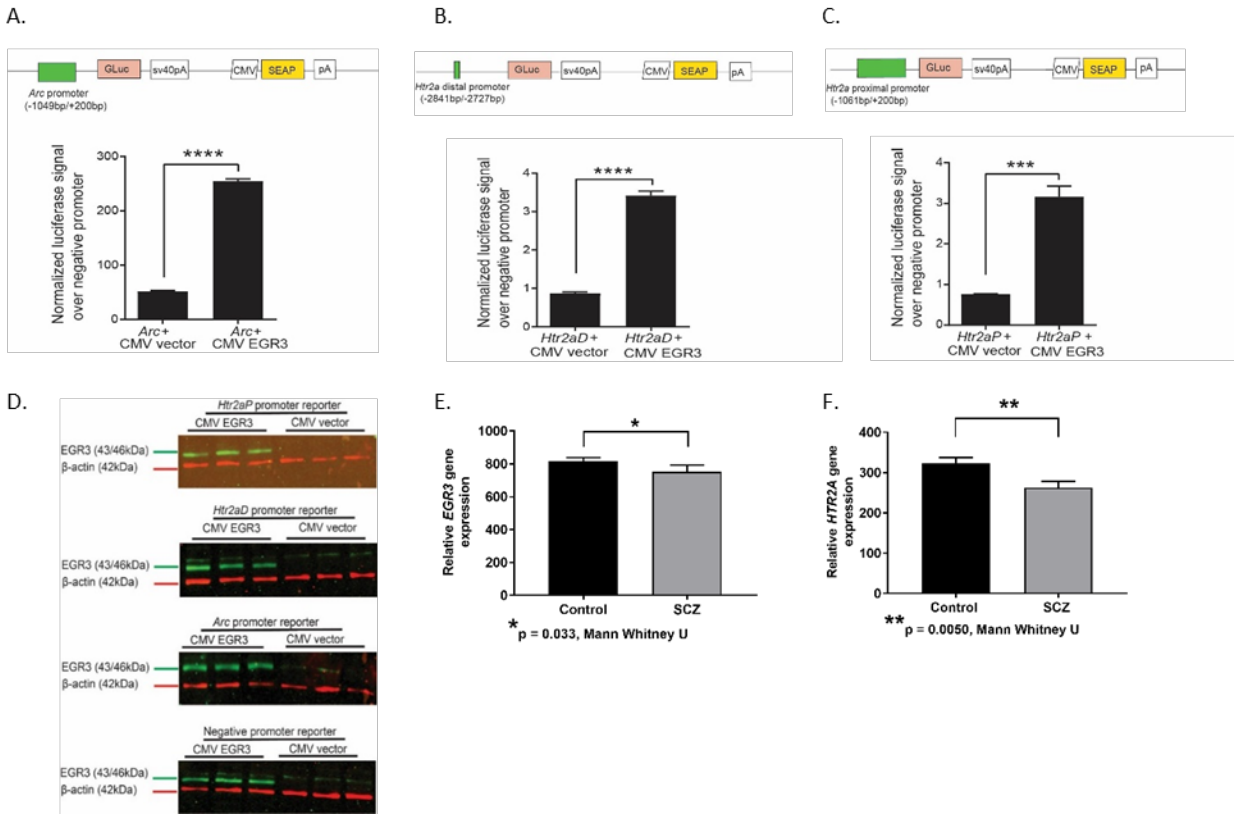


**Figure 4. EGR3 drives gene expression via binding sites in the *Htr2a* promoter.** (**A-C**) Schematic representations of dual luciferase/SEAP reporter constructs containing EGR consensus binding sites and results of *in vitro* assays in neuro2a cells. Compared to expression of CMV vector alone, CMV-driven EGR3 overexpression significantly upregulates expression of luciferase reporters driven by (**A**) *Arc* promoter region (-1049bp to +200bp), (**B**) *Htr2aD* distal promoter region (-2727 bp to -2841bp) ($t4 = 21.17$, $p < 0.0001$), and (**C**) *Htr2aP* proximal promoter region (-1061bp to +200bp) ($t4 = 8.977$, $p < 0.001$). (**D**) Western blot validation of EGR3 protein expression following transfection with CMV-EGR3 versus CVM vector alone, from cultures expressing reporter constructs driven by promoters from ARC, *Htr2aD*, *Htr2aP*, or negative promoter control vector. Unpaired student *t*-tests, ***$p < 0.001$, ****$p < 0.0001$, $n = 3$.

(**E, F**) *EGR3* and *HTR2A* mRNA levels are significantly decreased in brain tissue samples from the prefrontal cortex of schizophrenia patients compared to controls. Microarray (Robust Multi-Array Average) gene expression data derived from NCBI Geo database GSE53987 showing significant decrease in (**E**) *EGR3* (**p* < 0.033) expression and (**F**) *HTR2A* (***p* = 0.005) expression, in control (N = 19) vs. schizophrenia (N = 15) patients (Mann Whitney U test). Values represent means ± SEM. (Abbreviations: CMV - cytomegalovirus, GLuc - *Gaussia* luciferase, SEAP - secreted alkaline phosphatase.)

To confirm that the binding of EGR3 to the *Htr2a* promoter results in a change in gene expression, we conducted *in vitro* luciferase-reporter assays. We co-transfected neuro2a cells with luciferase/SEAP constructs driven by either the positive control *Arc* promoter, the distal *Htr2a* promoter, or the proximal *Htr2a* promoter, with either a CMV vector overexpressing *Egr3*, or a control empty CMV vector (Fig. 4A-C). Figure 4A shows that overexpression of EGR3 induces an approximately 4.9 fold increase in expression of luciferase, driven by the *Arc* promoter region previously reported to bind EGR3 (28). Figures 4B-C demonstrate that both regions of the *Htr2a* promoter containing high-probability EGR3 binding sites (Fig. 3A) drive expression of luciferase in response to EGR3 expression. Figure 4B shows that EGR3 expression induces an approximately 3.9-fold increase in the *Htr2a* distal promoter-driven luciferase signal compared to expression of the CMV vector alone (Fig. 4B; $t4 = 21.17$, $p < 0.0001$). Although the results of the ChIP for the proximal *Htr2a* promoter did not show a statistically significant increase in EGR3 binding following neuronal activity, overexpression of EGR3 *in vitro* resulted in an approximately 4.2-fold increase in *Htr2a* proximal promoter-driven luciferase signal compared to CMV vector alone (Fig. 4C). These results suggest that EGR3 directly binds to the *Htr2a* promoter in the cortex in response to neuronal activity, and activates *Htr2a* expression, which results in increased levels of cortical 5-$HT_{2A}$Rs.

Together, these findings demonstrate that an acute, physiological environmental stimulus can upregulate expression of 5-$HT_{2A}$Rs the frontal cortex in a matter of several hours, and the mechanism by which this occurs is through the activity-dependent immediate early gene transcription factor *Egr3*.

5-$HT_{2A}$Rs are abundantly expressed in the frontal cortex and play important roles in cognition and mood . They also mediate the hallucinogenic effects of numerous drugs including LSD, psilocybin, and mescaline. Investigation of these drugs has recently undergone a resurgence in the search for treatments for severe psychiatric symptoms including depression and anxiety disorders (29). 5-$HT_{2A}$Rs are also a key target of SGAs, which treat the symptoms of psychosis (4).

In recent years a range of post-mortem, as well as *in vivo* imaging, studies have revealed that 5-$HT_{2A}$R levels are reduced in schizophrenia patients (5-15). Such findings may lead to the assumption that brain neurotransmitter receptor levels represent a relatively stable characteristic. It is well established that drugs can alter 5-$HT_{2A}$R levels; both agonists and antagonists trigger receptor internalization and recycling (16, 17). However, the concept of 5-HT2AR levels being rapidly upregulated, within a matter of hours, in response to environmental events or stimuli, is a relatively novel concept in the field of psychiatry. Notably, our findings are supported by a recent human study reporting that 24 h sleep deprivation causes a significant increase in brain 5-$HT_{2A}$R levels detectably by Positron Emission Tomography (PET) scan (30).

*Egr3* is one of four transcription factors (*Egr1-4*) in the Early Growth Response family of immediate early genes. The family also includes the immediate early gene *Nab2*, which encodes a co-regulator that binds to *Egr1*, *2*, and *3*, to co-regulate downstream genes, including the *Egrs* themselves, completing regulatory feedback interactions among the family members (31, 32).

Each of these genes has been implicated in schizophrenia. Expression of *EGR1*, *2*, and *3* is reduced in the post-mortem brains of schizophrenia patients (33-38). *EGR1* and *NAB2* each map to one of the 108 genome-wide associated loci for schizophrenia (39), and *EGR4* resides at a locus that reached genome-wide significance in the most recent Psychiatric Genomics Consortium analysis (40). Our analyses of data from the NCBI GEO database identified additional findings of reduced expression levels of *EGR3* and *HTR2A* in the prefrontal cortex of schizophrenia patients, compared with controls (Fig. 4E and 4F) (41). These findings suggest that dysfunction in activity dependent EGR family immediate early genes, which include, and results in, decreased activity of EGR3, may underlie the reported deficits in 5-$HT_{2A}$R expression in schizophrenia patient brains.

In summary, our findings suggest a mechanism through which environmental events can alter expression, and membrane bound protein levels, of 5-$HT_{2A}$Rs in the FC in a matter of hours. The activity dependent immediate early gene *Egr3* is required for the process, and directly regulates expression of *Htr2a*. These findings have implications for the neuropsychiatric illness schizophrenia; a disorder influenced by both genetic and environmental factors, and characterized by reduced 5-$HT_{2A}$R levels. These findings thereby shed light on a potential mechanism whereby environment may interact with genetic variations to influence neurobiology that may contribute to the symptoms, and treatment, of neuropsychiatric illness.

## Methods:

### Animals

*Egr3* -/- mice were generated by the deletion of sequences that encode the zinc fingers, the DNA-binding domain of the protein (Tourtellotte & Milbrandt, 1998). The mice were backcrossed to a C57BL/6 background for more than 30 generations. Animals were maintained as heterozygote × heterozygote mating pairs. *Egr3* -/- and their WT littermates were assigned as 'matched pairs' at the time of weaning. Male adult littermates were used and housed on a 14/10 h light/ dark schedule with *ad libitum* access to food and water.

### Sleep deprivation (SD)

Animals were single-housed for 5 days prior to the experimental procedure for habituation. For gene expression level study, 6hrs of SD was performed in two groups of animals (Fig.S1): 6 hrs SD group, time - matched controls allowed to sleep in their home cages (control group). For radioligand binding of protein level study, 8hrs of SD was performed in two groups of animals (Fig.S1): 8 hrs SD group, time - matched controls allowed to sleep in their home cages (control group).

SD started at the beginning of the light period (8:00 a.m. mountain standard time, MST). Mice were kept awake by "gentle handling" as previously described (Maple, Zhao, Elizalde, McBride, & Gallitano, 2015; Thompson et al., 2010). Briefly, animals were disturbed by a combination of cage tapping, introduction of novel objects (e.g., balled paper towels), cage rotation, and stroking of vibrissae and fur with an artist's paintbrush (Terao, Steininger, et al., 2003; Thompson et al.,

2010). The number and type of stimulation required to keep each animal awake during the SD procedure were recorded and compared between the WT and *Egr3* -/- mice.

**Quantitative reverse transcription polymerase chain reaction (qRT-PCR)**

Animals were sacrificed immediately after SD via isoflurane overdose. The brains were removed and the following regions were immediately dissected: the anterior frontal cortex, posterior frontal cortex, and the mid-posterior cortex. Brain regions were determined using the Coronal C57BL/6J Atlas from the Mouse Brain Atlas (Rosen et al, 2000). Collected cortical tissues were treated with RNA*later* solutions (ThermoFisher Scientific, Waltham, MA) for ribonucleic acid (RNA) stabilization and storage. For RNA isolation, the tissue was homogenized in 700µl of TRI reagent (Life Technologies, Carlsbad, CA) in the 2ml tubes (Bertin Corp, Rockville, MD) prefilled with ceramic beads (diameter 1.4 mm). Tissue was homogenized for 4 cycles at a speed of 6000 g for 30 s, in a Precellys 24 high-powered bead mill homogenizer (Bertin Corp, Rockville, MD). The samples were put on ice for 2 minutes between each cycle to prevent RNA degradation. Next, the homogenates were centrifuged at 12000g, 4°C for 5 min. The supernatant was saved and was separated into aqueous and organic phases by adding 70µl per sample bromochloropropane (BCP, Molecular Research Center, Inc. Cincinnati, OH). The RNA was precipitated in a MagMAX express magnetic particle processor (Applied Biosystems, Foster City, CA) with isopropanol and washed with ethanol. Next, RNA was dissolved in nuclease-free water and quantified with ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA) and further confirmed with Qubit 3.0 Fluorometer (Thermo Scientific, Waltham, MA). The mRNA was reverse transcribed into cDNA using M-MLV reverse transcriptase kit (Life Technologies, Carlsbad, CA). Quantitative RT-PCR was performed using FastStart SYBR green master mix (Roche Applied Science, Indianapolis, IN) with the reaction volume 20µl per well (10µl SYBR green master mix 2X, 8µl cDNA, 1µl of each forward and reverse primers of 10uM stock) in a 7500 Fast real-time PCR machine (Applied Biosystem, Foster City, CA). The thermal cycling parameters are 50 °C for 2 minutes, 95 °C for 10 minutes, 40 cycles of (95 °C for 15 seconds, 60 °C for 1 minutes). Quantitative RT-PCR primers were designed using primer3 (Koressaar & Remm, 2007) and blasted for targeting specific using the software primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences were ordered from Integrated DNA Technologies (IDT, Coralville, IA) with standard desalting purification. Primers used for the study are listed as below:

*Egr3* Forward primer: 5'-CTGGAGGCACTTTCTCCTTG-3'
*Egr3* Reverse primer: 5'-TGGATCAAGGCGATCCTAAC-3'
*Htr2a* Forward primer: 5'-TCACCATTGCGGGAAACAT-3'
*Htr2a* Reverse primer: 5'-ATCAGCTATGGCAAGTGACAT-3'
*Pgk1* Forward primer: 5'-TGTTAGCGCAAGATTCAGCTA-3'
*Pgk1* Reverse primer: 5'-CAGACAAATCCTGATGCAGTA-3'

**Tissue Collection/Sectioning for radioligand binding study**

Male WT and *Egr3* -/- mice (aged 55-62 days) were anesthetized with isoflurane and sacrificed. Tissue was flash frozen in -40°C methylbutane and stored at -80°C. Tissue was sectioned at a thickness of 12 microns on the cryostat (Leica). Six sections of anterior frontal cortex (Bregma 2.8 – Bregma 2.34), posterior frontal cortex (Bregma 1.70 – Bregma 0.86), two sections of mid-cortex (Bregma 0.02 - -0.82), and two sections of posterior cortex (Bregma -1.34 - -2.30) were

collected and freeze-thawed on the same slide. A total of ten slides/brain were serially collected and stored at -80°C.

**Radioligand Binding Analysis - Autoradiography**

Tissue was treated with 3-H-MDL100907 [R(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorphenyl)-ethyl]-4-piperidin-methanol] (specific activity; 59.22 Ci/mmol), a selective $5HT_{2A}$ antagonist, and was detected with autoradiography (Mikkelsen J.D., 2014). Control tissue lacking drug was exposed to assess non-specific binding.

**Autoradiography Analysis**

Standards of known activity were included in each cassette. Treated and non-treated tissue was exposed within the same cassette with the standards. Mean intensity values of the standards were determined by outlining the standard curve regions with the Quantity One, Bio-Rad program (Bio-Rad Laboratories, Hercules, CA). A derived equation of the standard curve was generated from standard mean intensity values. The mean intensity values of ligand-bound cortical and hippocampal regions of interest were generated by free hand contour tracing. Ligand-bound (fmol/mg) measurements were calculated from mean intensity values of the regions of interest extrapolated from the derived equation, divided by known activity of the drug. Mean intensity values for tissue with non-specific binding was determined and ligand bound values were extrapolated. Specific ligand bound activity was subtracted from non-specific activity to obtain corrected values.

**Electroconvulsive seizure (ECS)**

Ten minutes after placing a drop of proparacaine hydrochloride ophthalmic solution USP, 0.5% (Akorn) onto each eye, the electroconvμlsive shock was delivered via silver trans - corneal electrodes previously damped with 0.9% sodium chloride (NaCl, Sigma-Aldrich). Animals were restrained by manual scruffing. The pulse generator (ECT Unit 57800 - 001; Ugo Basile, Comerio, Italy) delivered a stimulus of 220 - 250HZ (based on weight), 0.9 width square wave pulses for a duration of 0.2 seconds at a current of 20mAs (Ramanan, Shen et al. 2005, Ploski, Newton et al. 2006). Immediately following the shock, mice displayed tonic-clonic seizures with hind limb tonic extensions and were placed back in their home cages for recovery. Mice were sacrificed and their brains were dissected 2 hrs after ECS. Age-matched "no-ECS" control mice from the same litters were sacrificed at the same time of day as the mice experiencing seizures.

**Western blot to detect the expression pattern of EGR3 protein 2hrs after ECS**

2 hours after ECS, posterior frontal cortex was removed and homogenized in the 1% Nonidet P-40 lysis buffer containing 0.5% sodium deoxycholate with proteinase inhibitor (1:10,000; Sigma-Aldrich, St. Louis, MO) using the Wheaton Tenbroeck style tissue grinder (ThermoFisher Scientific, Waltham, MA). Cell debris was pelleted by centrifugation at 17,000 rpm for 5 min, 4 °C. The supernatant was saved and protein concentration was quantified using Pierce BCA protein assay kit (ThermoFisher Scientific, Waltham, MA). Protein samples were denatured for 5 min, 100 °C on block heater (VWR, Radnor, PA). 25ug of protein was loaded into homemade 4-8% gradient polyacrylamide gels. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) for 1hr using a semi-dry transfer apparatus (Bio-Rad Laboratories, Hercules, CA) which constantly delivers a voltage of 15 V. Then the membranes were blocked for 1hr with 3% (w/v) non-fat dry milk (LabScientific

Inc., Highlands, NJ). After washing with phosphate-buffered saline plus 0.1% of Tween-20 (PBST), the membranes were incubated overnight at 4°C with rabbit anti-EGR3 antibody (1:200, Santa Cruz Biotechnology, Dallas, TX) and mouse anti-beta actin antibody (1:5000, Sigma-Aldrich, St. Louis, MO). The next day, membranes were washed with PBST and incubated with IRDye800CW secondary antibodies (1:10,000 for EGR3, Li-COR Biosciences, Lincoln, NE) and the IRDye680 secondary antibodies (1:20,000 for beta actin, Li-COR Biosciences) dissolved in 3% non-fat dry milk for 1hr at room temperature with gentle shaking. After washing for 5 times, each time for 5 minutes, the membranes were visualized on an Odyssey instrument (Li-COR Biosciences). Protein expression levels were determined as the ratio of EGR3 to the internal control beta-actin and were reported as percentage of the control group.

**Bioinformatics analysis to identify putative EGR3 binding sites on the *Htr2a* gene promoter**

To identify potential EGR3 binding sites on the *Htr2a* gene promoter we downloaded the promoter sequences, including the region 4kb upstream of the *Htr2a* transcription start site (NM_172812, chr14:74636840-74640839) from genome browser of the University of California, Santa Cruz (UCSC genome browser, https://genome.ucsc.edu/). Then we scanned and identified matches of consensus binding sites for the EGR3 in the 4kb *Htr2a* promoter sequences using the software 'Find Individual Motif Occurrences' (FIMO, http://meme-uite.org/tools/fimo) (Grant, Bailey, & Noble, 2011). The motif occurrences with a $p$ -value less than 0.0001 were selected.

**Chromatin immunoprecipitation (ChIP)**

The ChIP protocol was modified from a previously reported study (H.-D. Kim et al., 2016). 2hrs after ECS, frontal cortex was removed and cut into 1mm pieces. The protein-DNA complexes of the frontal cortical tissue were crosslinked through incubation with 1% formaldehyde (Sigma-Aldrich, St. Louis, MO) on a rotator for 12 minutes. Formaldehyde was quenched with 125 mM glycine (Sigma-Aldrich, St. Louis, MO) for 5 minutes. Samples were homogenized using a Q125 sonicator (Qsonic, LLC, Newtown, CT) with a power of amplitude 40%, sonication duration 7, for 2 cycles. Samples were cooled on ice between each cycle. Chromatin was then sheared into 200bp-1000bp with a Bioruptor XL (Diagenode Inc., Denville, NJ) at 4°C at a sonication intensity for 30s on/30s off for 35 cycles. Fragment size was verified with an Agilent bioanalyzer (Agilent Technologies, Santa Clara, CA). 50µl of sheared chromatin were removed as input control with an input fraction of 5%. The magnetic beads-antibody complex was prepared by incubating 7.5ug of the anti-EGR3 antibody (Santa Cruz Biotechnology, Dallas, TX) with the magnetic sheep anti-rabbit beads (Invitrogen Corp., Carlsbad, CA) at 4°C overnight on a rotator. After washing the bead-antibody complex with 0.5% BSA blocking solution, 70µl of the beads-antibody complex was added into each ChIP sample, and incubated for 16hrs at 4°C on a rotator. To control for nonspecific binding, a normal IgG IP was performed in parallel. Beads were collected in sample tubes with a magnetic rack (ThermoFisher Scientific, Waltham, MA), and washed with the following buffer: low salt wash buffer (0.1%SDS, 1% TritonX100, 2mM EDTA, 150mM NaCl, 20mM Tris-HCl), high salt wash buffer (adjusted with 200 mM NaCl in place of the concentration of NaCl of the low salt wash buffer), LiCl wash buffer (150mM LiCl, 1%NP40, 1% NaDOC, 1mM EDTA, 10mM Tris-HCl). Cross-linked protein and DNA complex were reversed at 65°C overnight. RNA was stripped by 1hr incubation with 2µl of RNase A (Roche Applied Science, Indianapolis, IN) at 37°C. Proteins were digested with 2µl of proteinase K (20 mg/mL, Invitrogen Corp., Carlsbad, CA). DNA was purified with a DNA purification kit (QIAGEN Inc., Germantown, MD). qPCR was performed using FastStart SYBR green master

mix (Roche applied science) in a 7500 Fast real-time PCR machine (Applied Biosystem, Foster City, CA). The total reaction volume per well was 25µl reaction (2µl DNA, 9.5 µL nuclease-free water, 12.5 µL SYBR-Green Master Mix 2X, 0.5µl µL of each forward and reverse primers with 10uM stock). Thermal cycling parameters were designated as initial denaturation at 94°C for 10 minutes, 50 cycles of (denature at 94°C for 20 seconds, anneal and extension at 60°C for 1 minutes). The primers used are listed as follows:

*Arc* forward: 5'-TCGCTGCCCAGGACTAGGTA-3';
*Arc* reverse: 5'-TTCACAGCCCCGAGTGACTAA-3';
*Htr2a* proximal forward: 5'-CTTGGATAGAAGTGCTGGATGCT-3';
*Htr2a* proximal reverse: 5'-GGGTACATGGCAGTCATATTTTTAGG-3';
*Htr2a* distal forward: 5'-CTGGGCTCTAAAGGCAACTGA-3';
*Htr2a* distal reverse: 5'-TGCGCACGTGTATACAGAGTAGGT-3'

After performing ChIP- qPCR, the relative occupancy (aka. fold enrichment) of the EGR3 proteins at predicted binding loci of *Htr2a* putative regulation regions is estimated using the following equation 2^(ΔCt MOCK- ΔCt SPECIFIC), where ΔCT MOCK and ΔCT SPECIFIC are mean normalized threshold cycles of PCR done in triplicate on DNA samples from MOCK (anti-IgG antibody) and transcription factor EGR3 immunoprecipitations to the input IPs (Nelson, Denisenko, & Bomsztyk, 2006).

**Promoter reporter vector design**

The *Htr2a* proximal promoter luciferase reporter – the dual-reporter system contains a Gaussia luciferase gene (GLuc) which is driven by a *Htr2a* proximal promoter insert which corresponds to *Htr2a* promoter sequence located approximately 1061bp upstream and 200 bp downstream of the transcription start site (TSS) of the *Htr2a* gene. In the same vector, a secreted alkaline phosphatase (SEAP) is driven by a cytomegalovirus (CMV) promoter and serves as the internal control for signal normalization. This internal control SEAP exists in all the other promoter reporter clones in the present study. The *Htr2a* proximal promoter luciferase reporter contains the EGR3 putative binding site GCGCGGGGGAGGGG.

The *Htr2a* distal promoter luciferase reporter – the dual luciferase promoter reporter clone contains a GLuc gene and is driven by the insert, which is -2727 bp to -2841 bp upstream of the *Htr2a* TSS. This fragment contains the EGR3 putative binding site AGGAGGGGGAGTCT.

The *Arc* promoter luciferase reporter was used as a positive control for the illuminometer and the functionality of our CMV-EGR3 vector. We used the *Arc* promoter reporter clone containing an insert 1049bp upstream and 200bp downstream of the TSS of the *Arc* gene. This *Arc* promoter luciferase reporter contains the EGR3 binding site which was confirmed previously (L. Li et al., 2005a).

The non-promoter luciferase reporter was used as a negative control to detect the basic activity of the dual-reporter vector. This luciferase reporter contains an insert that is a non-promoter sequence (TGCAGATATCCTCGCCC).

All the promoter clones were generated by Genecopeia (Genecopeia Inc., Rockville, MD).

**Cell culture and lipofectamine transfection**

Neuro2a cells (mouse neuroblastoma cells; ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific) which contains 10% Fetal Bovine Serum (FBS, Gibco) and 1% Penicillin-Streptomycin (PS, Gibco), at 37°C, under a humidified atmosphere of 5% carbon dioxide ($CO_2$) : 95% air. Cells were seeded into a

6-well plate (Corning Inc., Corning, NY) with 2 ml of cell culture medium and were transfected at the point of 70-90% confluence. Cell culture medium was removed 3 hrs before transfection and 3ml of fresh medium were added to each well. 1.5 ug of CMV-Egr3 vector or CMV vector was co-transfected with 1 ug of each promoter reporter vector per well using Lipofectamine 3000 reagents (ThermoFisher Scientific). Transfection was performed with 3.75 µl of Lipofectamine 3000 reagent, 5 µl of P3000 reagent and 250 µl of Opti-MEM per well. Cells were incubated at 37°C with 5% $CO_2$ : 95% air following transfection until further processing.
Three separate transfections were performed. Transfections were conducted in triplicate.

**Luciferase signal measurement**

24 hrs after transfection, 0.2 ml of medium from each cell culture was collected and placed at room temperature. The duo luciferase activities were measured using the secrete-pair dual luminescence assay kit (Genecopoeia). Each sample was run in duplicate.
To detect the Gaussia luciferase (GLuc) signal, the 10X Gaussia luciferase stable buffer (GLuc-S) was diluted with distilled water to 1X GLuc-S (1:10). The GLuc assay working solution was made by diluting the Gaussia luciferase substrate with the 1X GLuc-S buffer (1:10) and was incubated in the dark at room temperature for 25 minutes. 100 µl of GLuc assay working solution was mixed with 10 µl of cell culture medium for each well. The mixture was incubated at room temperature for 1 minute in the dark. The signal was read with a Tecan Safire2 instrument (Tecan Group Ltd., Morrisville, NC).
50 µl of each culture medium was aliquoted and heated at 65°C for 15 min, and placed on ice. 1X SEAP buffer was prepared from the 10X SEAP buffer working stock The SEAP assay working solution was diluted with the SEAP substrate with 1X SEAP assay working solution (1:10) and incubated at room temperature for 10 minutes in the dark. 100 µl of SEAP assay working solution was mixed with 10 µl of each heated medium sample. The mixture was incubated at room temperature for 10 minutes in the dark, Secreted alkaline phosphatase levels were then read with the Tecan Safire2 instrument (Tecan Group Ltd.).
For all measurements, the GLuc value was first normalized to the intern control SEAP luciferase value (GLuc / SEAP ratio) and then to the non-promoter luciferase reporter.

**Western blot to measure the EGR3 protein level 24hrs after transfection**

After collecting the cell culture medium for luciferase measurement, the remaining medium in each well was discarded. Cells were washed 2 times with cold 1X PBS, 300 µl per well and were lysed with 300 µl per well of 1% Nonidet P-40 lysis buffer containing proteinase inhibitor (1:10,000; Sigma-Aldrich). Samples were further homogenized using a Q125 sonicator (Qsonica) with a power of amplitude 40%, 1 time for 5 seconds on ice. Protein concentrations were quantified with a Nanodrop 1000 spectrophotometer (Thermo Scientific). Protein samples were denatured and 25ug of proteins for each sample were loaded on gels. The western blot protocol follows that of our previously described method for ECS.

**NCBI GEO Data Analysis**

We used publicly available data on NCBI GEO, Gene Expression Omnibus, a public repository for gene expression studies mainly using RNA sequencing and microarray data (42). Data from dataset GSE53987 (41) were used in our study. We used the main microarray platform file GPL570 as a guide to ascertain gene -specific probe IDs for EGR3 and HTR2A. Subsequently, gene expression data for prefrontal cortex (Brodmann Area 46) EGR3 and HTR2A were

downloaded from individual sample files by using the probe IDs as a reference to get the corresponding gene expression values. We collated the gene expression data for different EGR3 and HTR2A probes per sample per diagnostic group and did pairwise comparisons for each probe comparing schizophrenia vs. control samples. Of these, data for probes 211616_s_at (HTR2A) and 206115_at (EGR3) showed significant differences and are shown in the manuscript.

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