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Acute sleep deprivation upregulates serotonin 2A receptors in the frontal cortex of mice via the immediate early gene *Egr3*

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Conflicts of Interest

The authors have no conflicts of interest to report.

Supplementary information is available at MP's website.

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Abstract

Serotonin 2A receptors (5-HT_{2A}Rs) mediate the hallucinogenic effects of psychedelic drugs and are a key target of the leading class of medications used to treat psychotic disorders. These findings suggest that dysfunction of 5-HT_{2A}Rs may contribute to the symptoms of schizophrenia, a mental illness characterized by perceptual and cognitive disturbances. Indeed, numerous studies have found that 5-HT_{2A}Rs are reduced in the brains of individuals with schizophrenia. However, the mechanisms that regulate 5-HT_{2A}R expression remain poorly understood. Here we show that a physiologic environmental stimulus, sleep deprivation, significantly upregulates 5-HT_{2A}Rs levels in the mouse frontal cortex in as little as 6–8 hours (for mRNA and protein, respectively). This induction requires the activity-dependent immediate early gene transcription factor early growth response 3 (*Egr3*) as it does not occur in *Egr3* deficient (−/−) mice. Using chromatin immunoprecipitation, we show that EGR3 protein binds to the promoter of *Htr2a*, the gene that encodes the 5-HT_{2A}Rs, in the frontal cortex *in vivo*, and drives expression of *in vitro* reporter constructs via two EGR3 binding sites in the *Htr2a* promoter. These results suggest that EGR3 directly regulates *Htr2a* expression, and 5-HT_{2A}Rs levels, in the frontal cortex in response to physiologic stimuli. Analysis of publicly available post-mortem gene expression data revealed that both *EGR3* and *HTR2A* mRNA are reduced in the prefrontal cortex of schizophrenia patients compared to controls. Together these findings suggest a mechanism by which environmental stimuli alter levels of a brain receptor that may mediate the symptoms, and treatment, of mental illness.

Introduction

The serotonin 2A receptor (5-HT_{2A}R) is extensively expressed in the cerebral cortex where it is believed to play a critical role in perception, cognition, and psychosis. Much of the evidence for this comes from studies on the effects of drugs that bind to the 5-HT_{2A}R [1–3]. This includes agonists, such as lysergic acid (LSD), psilocybin, and mescaline, which cause hallucinations [4–6], as well as antagonists and inverse agonists, including second-generation antipsychotics, which reverse the perceptual disturbances of psychiatric illnesses such as schizophrenia [6–8]. Additional findings supporting a role for the 5-HT_{2A}R in the symptoms of psychosis include a long history of studies showing abnormal levels of 5-HT_{2A}Rs in the brains of patients diagnosed with schizophrenia. While some studies did not detect

differences [9–11], and one group identified increased levels [12, 13], the vast majority of these studies, including a meta-analysis [14], have revealed reduced 5-HT_{2A}R levels in schizophrenia subjects. These include post-mortem studies measuring 5-HT_{2A}R ligand binding and mRNA expression, and as well as *in vivo* Positron Emission Tomography (PET) scan investigations [14–24]. Notably, studies in antipsychotic-naïve patients indicate that the reduction in 5-HT_{2A}R levels is not simply a consequence of medication treatment [16, 18]. Despite the longstanding recognition of the importance of 5-HT_{2A}Rs in the response to psychedelic drugs, and the symptoms and treatment of psychotic disorders, the processes that regulate expression of this critical receptor remain unknown.

Our prior work identified that mice lacking the immediate early gene (IEG) *Egr3* (*Egr3*−/− mice) have reduced levels of 5-HT_{2A}Rs in the frontal cortex [25]. This suggested that *Egr3* is required for expression of *Htr2a*, the gene that encodes the 5-HT_{2A}R. If so, we reasoned that stimuli that activate expression of *Egr3* may also induce *Htr2a* expression. Sleep deprivation (SD) is a physiological stimulus that upregulates *Egr3* in the mouse cerebral cortex [26]. Using this approach, we found that just six hours of SD increases *Htr2a* mRNA levels in the mouse cortex and, moreover, this required *Egr3* [27].

This discovery that *Htr2a* expression can be rapidly induced in the rodent brain in response to a physiologic stimulus is supported by a prior study in humans. In 2012 Elmenhorst and colleagues reported that preventing healthy subjects from sleeping for a period of 24 hours resulted in a significant increase in 5-HT_{2A}R binding in the neocortex, detected on PET scan [28].

These findings raised the question of whether EGR3, an activity dependent transcription factor, directly regulates the *Htr2a* gene, and thereby mediates the environmental induction of *Htr2a* mRNA in the cortex. In the current study we have addressed the questions of where in the cerebral cortex SD is inducing *Htr2a* expression, does this also result in increased 5-HT_{2A}R protein, and is EGR3 directly regulating the *Htr2a* gene to effect this environmentally-induced receptor expression. We show that six hours of SD significantly upregulates *Htr2a* in the prefrontal cortex and adjacent sensorimotor cortex, and that 8 hours of SD upregulates 5-HT_{2A}R levels in the same regions. In both cases this upregulation requires *Egr3*. Further, we show that the EGR3 protein binds to the *Htr2a* promoter *in vivo* and drives expression of an *in vitro* reporter construct, suggesting that the activity dependent IEG transcription factor EGR3 directly regulates expression of *Htr2a* in response to the acute environmental stimulus of SD.

Materials and Methods

See online supplementary methods for details and primer sequences.

Animals

Previously generated *Egr3*−/− mice [29], backcrossed to a C57BL/6 background for >30 generations, were housed on a 14/10 h light/dark schedule with ad libitum access to food and water. Matched pairs of littermate *Egr3*−/− and Wildtype (WT) mice were designated at weaning and randomly assigned to experimental groups. Male mice ages 3–5 months

were used for qRT-PCR studies. Male and female mice we used for *in situ* hybridization (4 months old) and autoradiography (7–9 weeks old) experiments. WT male C57BL/6NTac mice (2.5–4 months old) were obtained from Taconic Biosciences for Western blot and chromatin immunoprecipitation (ChIP) experiments. Mice were acclimated to the facility for two weeks before experiments. Animal studies were performed in accordance with the University of Arizona Institutional Animal Care and Use Committee (IACUC) guidelines under an approved IACUC protocol.

Sleep deprivation (SD)

SD was initiated at the onset of "lights on" (8:00 a.m.) and conducted for 6h (for mRNA studies) or 8h (for protein), using the "Gentle handling" method as previously described [27]. Investigators were blinded to genotype during SD experiments. The number and type of stimulation required to keep each animal awake during the SD did not differ between WT and *Egr3*−/− mice (Supplementary Figure 1).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Tissue was dissected on ice, transferred to RNAlater (Thermo Fisher Scientific), frozen on dry ice and stored at −80°C. Tissue homogenization and RNA isolation was performed in TRI reagent (Life Technologies) using ceramic beads and a MagMAX express processor (Applied Biosystems). mRNA was reverse transcribed using M-MLV reverse transcriptase kit (Life Technologies) and qRT-PCR performed using FastStart SYBR green master mix (Roche). Each sample was amplified in triplicate for the gene of interest and the housekeeping gene phosphoglycerate kinase 1 (*Pgk1*). Fold changes in gene expression were calculated and data were plotted using the $2^{-\Delta\Delta}$Ct method [30]. Relative gene expression of all samples was first normalized to *Pgk1* (ΔCt). Then, average ΔCt data were normalized to WT SD controls for data points representing WT animals that underwent SD, or *Egr3*−/− SD controls for *Egr3*−/− animals that underwent SD.

In situ hybridization

In situ hybridization was performed on 12μm sections using the RNAscope Multiplex Fluorescent Reagent Kit V2 (Catalogue # 323100). The *Htr2a* probe was purchased from Advanced Cell Diagnostics, Inc (Probe-Mm-*Htr2a*-C3, 401291-C3) and developed with the fluorescent Opal dye 570 (Akoya Biosciences, Cat. # FP1488001KT) at a concentration of 1:750. Sections were counterstained with DAPI (Vector Laboratories). Qualitative analyses performed via thorough review of all tissue sections and in reference to anatomical regions described in The Allen Mouse Brain Atlas (Reference Atlas, Interactive P56, Coronal) [31, 32].

Radioligand Binding Analysis – Autoradiography

Six 12μm sections of anterior frontal cortex (Bregma 2.8 – Bregma 2.34) and posterior frontal cortex (Bregma 1.70 – Bregma 0.86), and two sections of mid-cortex (Bregma 0.02 – 0.82), and posterior cortex (Bregma −1.34 – 2.30) were thaw mounted on the same slide. A total of ten slides/brain were serially collected and stored at −80°C. Tissue was treated with selective $5HT_{2A}R$ antagonist 3-H-MDL100907 [R(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-

fluorphenyl)-ethyl]-4-piperidin-methanol] (specific activity; 59.22 Ci/mmol) and detected by autoradiography. Non-drug exposed control tissue was used to assess non-specific binding.

Western blot

Six hours after SD, the frontal cortex was dissected. Right and left hemispheres were used for Western blot and ChIP experiments respectively. Tissue was Dounce homogenized in RIPA buffer and centrifuged at 12,000 rpm, 4°C x 20 min. Supernatant protein was denatured at 95 °C x 5 min and 20ug of protein was loaded per lane on an Any kD TGX polyacrylamide gel (Bio-Rad Laboratories). Samples were transferred to nitrocellulose membrane and blocked overnight at 4°C in 5% non-fat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBS-T). Blocked membranes were incubated for 2h at room temperature (RT) with rabbit anti-EGR3 antibody (1:500, kindly donated by Dr. Mary Kay Lobo, University of Maryland School of Medicine, original source: Baraban Lab, Johns Hopkins University School of Medicine [33]) and mouse anti-β actin antibody (1:5000, Sigma-Aldrich, Cat. # A5441) diluted in 1% non-fat dry milk/TBS-T. Following washes, membranes were incubated with secondary antibodies IRDye800CW (1:10,000 for EGR3, Li-COR Biosciences, Cat. # 926–32211) and IRDye680RD (1:10,000 for β-actin, Li-COR Biosciences, Cat. # 926–68072) for 1h at RT. Blots were washed in TBS with 0.1% Tween 20, and imaged. Protein levels were determined as ratios of EGR3 to β-actin internal control and reported as percentage of the control group. For Western blot experiments, recombinant EGR3 protein was synthesized using the TnT Coupled Reticulocyte Lysate System (Promega, Cat. # L4611) using template pcDNA 3.1(−) mouse *Egr3*, a gift from Peter Johnson (Addgene plasmid # 107998; http://n2t.net/addgene:107998; RRID:Addgene_107998) [34].

Promoter Analysis

The genomic region, including 4 kb upstream of the *Htr2a* transcription start site (NM_172812, chr14:74636840–74640839), was obtained from the UCSC genome browser (https://genome.ucsc.edu/). Consensus binding sites for EGR3 were identified using the software ‘Find Individual Motif Occurrences’ (FIMO, http://meme-uite.org/tools/fimo) [35]. The motif occurrences with a p value less than 0.0001 were selected.

Chromatin immunoprecipitation

Following 6h of SD, frontal cortex was dissected, minced, incubated in 1% formaldehyde to crosslink DNA-protein, and quenched with 125 mM glycine. Right and left hemispheres were used for Western blot and ChIP experiments respectively. Samples were homogenized, and chromatin sheared to 200 bp-1000 bp at 4°C for 25 cycles. Fragment size was verified via agarose gel electrophoresis. Samples were incubated with 70μl of anti-EGR3 antibody [33] complexed to magnetic beads (Invitrogen) x 16h at 4°C. Control IgG IP was performed in parallel. Beads were sequentially washed with low salt, high salt, and LiCl wash buffers. Samples were reverse crosslinked at 65°C overnight and treated with 2μl RNase (Roche) at 37°C x 1h and 2μl proteinase K (Invitrogen) at 55°C x 2h. DNA was isolated via QIAGEN kit and qPCR was performed. Relative occupancy (aka. fold enrichment) of the EGR3 proteins at each *Htr2a* promoter site was estimated using the following equation 2^(ΔCt MOCK- ΔCt SPECIFIC), where ΔCT MOCK and ΔCT SPECIFIC are mean normalized

threshold cycles of PCR performed in triplicate on DNA samples from MOCK (anti-IgG antibody) and transcription factor EGR3 immunoprecipitations to the input IPs.

Promoter reporter vector design

The *Htr2a* promoter luciferase reporters were generated by Genecopeia Inc. using the dual-reporter system. The "*Htr2a* proximal promoter" reporter includes genomic DNA from 1061 bp upstream to 200 bp downstream of the *Htr2a* gene transcription start site (TSS) and contains the putative EGR3 binding sequence GCGCGGGGGAGGGG. The "*Htr2a* distal promoter" reporter includes genomic DNA from −2727 bp to −2841 bp upstream of the *Htr2a* TSS and contains the putative EGR3 binding site AGGAGGGGGAGTCT. The control *Arc* promoter luciferase reporter includes genomic DNA ranging from 1049 bp upstream to 200 bp downstream of the TSS of the *Arc* gene, which contains a previously validated EGR3 binding site [36]. The "non-promoter" luciferase reporter, containing non-promoter sequence (TGCAGATATCCTCGCCC), was used as a negative control.

Cell culture and transfection

Neuro2a cells (ATCC) were seeded into 6-well plates and grown to 70–90% confluence. 3h prior to transfection, culture medium was replaced with 3ml of fresh medium. For each promoter construct, 1.5μg of CMV-*Egr3* vector (or CMV vector control) was co-transfected with 1μg of promoter reporter, using 3.75μl of Lipofectamine 3000 reagent, 5μl of P3000 reagent and 250μl of Opti-MEM per well. Cells were incubated at 37°C in 5% CO_2: 95% air. Transfections were conducted in triplicate and replicated twice.

Luciferase signal measurement

Twenty-four hours after transfection, 0.2ml of medium from each cell culture was collected and luciferase activities were measured using the secrete-pair dual luminescence assay kit (Genecopoeia). Each sample was run in duplicate. For all measurements, the GLuc value was first normalized to the internal control SEAP luciferase value (GLuc/SEAP ratio) and then to the non-promoter luciferase reporter. Following collection of medium, cells were processed for protein isolation and Western blot analysis to determine EGR3 protein levels.

NCBI GEO Data Analysis

Published data were obtained from NCBI GEO, Gene Expression Omnibus [37] dataset GSE53987 [38]. The microarray platform file GPL570 was used to ascertain gene-specific probe IDs for *EGR3* and *HTR2A*, which were used to download gene expression data from prefrontal cortex (Brodmann Area 46).

Statistical Analyses

All statistical analyses were carried out using GraphPad prism with significance set at $p < 0.05$. For statistical analyses, unpaired, two-tailed, Student's t-tests, two-way analysis of variance (ANOVA) with Bonferroni *post-hoc* tests, or two-tailed Mann-Whitney U tests were performed to determine significance for conditions in which there were more than two groups or two factors. Outliers were identified and excluded using the ROUT method [39] in GraphPad Prism with a False Discovery Rate (FDR) of 1%. Data were examined

graphically within each group, and no strong deviation from normality was observed. Power analyses were performed based on previous data using G*Power (Heinrich-Heine Universität Düsseldorf). Data were plotted as means ± standard error of the mean (SEM).

Results

We had previously reported that SD rapidly upregulates *Htr2a* expression in the mouse cortex [27]. However, since this study was performed in cortical homogenates, it was unclear in what cortical regions this upregulation was taking place. This is important since levels of both *Htr2a* mRNA and 5-HT_{2A}R protein display a distinctive anterior-posterior gradient in the rodent cortex [40–42]. This raised the question of whether SD was increasing *Htr2a* expression in regions where the receptor is already expressed or inducing it *de novo* in regions where it is normally not expressed. We therefore examined the cortical region in which SD induces *Htr2a* expression.

We first tested whether we could replicate published *in situ* hybridization findings showing that SD upregulates *Egr3* [26] using quantitative reverse transcription (qRT) PCR. Figure 1A shows the SD protocol and coordinates for regional brain dissection. In WT mice, we found that 6h of SD did not increase *Egr3* expression in the most anterior region of frontal cortex (AFC) but did significantly upregulate *Egr3* mRNA in the posterior part of the frontal cortex (PFC), as well as in more posterior regions of cortex (labeled “mid-posterior cortex (MPC)) (Fig.s 1B – 1D).

We next examined whether 6h of SD can upregulate *Htr2a* expression in the same cortical regions, and whether this requires *Egr3* (Fig.s 2A – 2C). In the AFC, SD increased *Htr2a* expression when both genotypes were analyzed together (two-way ANOVA), but not in either WT or *Egr3*−/− groups independently (post-hoc analyses were not significant) (Fig. 2A). However, in the PFC of WT mice, SD significantly increased *Htr2a* mRNA compared to SDc, a result that was absent in *Egr3*−/− mice (Fig. 2B). In the MPC, SD also increased *Htr2a* mRNA in WT mice, though the relative amount of the increase was less than in the PFC. Again, this effect did not occur in *Egr3*−/− mice (Fig. 2C).

To further characterize the regional distribution of *Htr2a* expression in response to SD, and in the absence of *Egr3*, we conducted RNAscope *in situ* hybridization. Figure 2D shows that 6h of SD increases *Htr2a* expression in WT mice in a region-specific manner. Compared to WT mice, *Egr3*−/− mice have lower levels of *Htr2a* mRNA throughout most of the cortex, sparing selected brain regions. SD may produce small increases in some regions of *Egr3*−/− mice, though this is less consistent than in WT mice.

In WT mice that did not undergo SD (SDc), RNAscope *in situ* hybridization showed a distribution pattern of *Htr2a* mRNA similar to prior studies in rats using radioactive *in situ* hybridization [40, 41]. This includes high levels of expression in the anterior frontal cortex, particularly in a thin layer (L) of cells at the L1/L2 border and in the deep L2/3 – L5 (L4 not present in anterior frontal cortex), with relatively lower signal in L6a. Also consistent with prior studies, *Htr2a* mRNA levels decrease with progression toward more posterior cortical regions, with notable reductions in L2/3, less drastic reductions in expression in L5, overall

minimal L6a expression, and strong expression in L6b bordering the corpus callosum. In the posterior frontal cortex (Bregma +2.1 – +0.14) expression of *Htr2a* is present in L2/3 in the somatomotor areas (MOs, MOp) and medial somatosensory cortex (SSp), but notably absent from the lateral SSp, and SSs, with posterior progression. This absence of *Htr2a* in L2/3 in the SSp/s is evident in prior studies in rat as well [40, 41].

In areas outside of the cortex, *Htr2a* expression in WT SDc animals is seen in the piriform cortex (PIR), olfactory tubercle (OT), taenia tecta dorsal and ventral (TTd/TTv), anterior olfactory nucleus (AON), and substantia innomata (SI) with strong expression in the claustrum (CL) and endopiriform cortex (dorsal) (EPd). Low level *Htr2a* expression is also seen in patches in the caudate/putamen (CP) as well as in the lateral and medial septum (LS, MS). Low level expression is also seen in the hypothalamus.

Following sleep deprivation in WT mice, the overall level of *Htr2a* in the anterior frontal cortex (anterior to Bregma +2.1) does not appear to be increased compared to SDc, however the laminar distribution that is apparent in SDc animals becomes less clearly delineated, suggesting a potential increase in expression in some regions and decrease in expression in others. In contrast to the cortex, in this anterior part of the brain SD produces a marked increase in expression in the olfactory regions, including the AON, OT, as well as the PIR (particularly the pyramidal layer, PIR2), EPd, and TTd & TTv, compared to WT SDc mice.

In the PFC (Bregma +2.1 – +0.14) SD increases overall *Htr2a* expression in WT mice, while also resulting in a more diffuse pattern, with loss of clear laminar signal, compared to SDc. Cortical areas in which SD produces the greatest increases in *Htr2a* levels include the anterior cingulate (ACAv, ACAd), & somatomotor areas (MOs, MOp), as well as more lateral regions, including the agranular insular area (AI). Notably, *Htr2a* expression in L6b is unchanged or only slightly increased by SD in WT mice. SD induces diffuse *Htr2a* expression in the CP, and potentially small increases in the MS and hypothalamus (this was not consistent across different animals in the same group).

Compared with WT mice, in *Egr3*−/− mice that did not undergo SD (SDc) *Htr2a* expression is reduced throughout the cortex, an effect that becomes more pronounced with progression in the posterior direction. The exceptions to this *Egr3*-depencence of *Htr2a* expression are the cells in L6b, as well as in the CL and EP and PIR, in which expression is only mildly reduced in *Egr3*−/− mice. Despite the reduced expression, a clear laminar distribution remains in the SDc mice, with most of the residual cortical expression in L5 & L4, and almost no expression evident in L2/3.

Despite the reduced expression of *Htr2a* in *Egr3*−/− mice, SD does appear to increase *Htr2a* mRNA in some brain regions of these animals. This induction is not consistently seen in the anterior frontal cortex but is clear in more posterior cortical regions (most evident in the ACA and MO, the same regions where SD increases expression in WT mice). L6b expression is only mildly increased, if at all, by SD in *Egr3*−/− mice. In regions outside of the cortex, SD increases *Htr2a* expression in the EPd, PIR, TT, AON, and OT, in *Egr3*−/− mice, though not to the levels seen in WT mice. Expression in the CL is not obviously affected by SD in *Egr3*−/− mice.

To determine if SD also increases 5-HT_{2A}R protein levels, we performed receptor autoradiography with the selective 5-HT_{2A}R antagonist ^{3}H-M100907 on brain sections from WT and *Egr3*−/− mice at baseline and following 8h SD (to allow time for translation of mRNA) (Fig. 3A). We found that, in the AFC, SD results in an overall increase in 5-HT_{2A}R binding that differs between genotypes, but this increase is not significant in either WT or *Egr3*−/− genotypes alone (Fig. 3B). However, SD increases 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 (Fig. 3B, comparison between WT and *Egr3*−/− in the SD groups). In the PFC, 8h of SD significantly increases 5-HT_{2A}R levels in WT mice but not in *Egr3*−/− mice (Fig. 3C). In addition, 5-HT_{2A}R levels are significantly greater in WT than *Egr3*−/− mice in this region both at baseline (replicating our prior radioligand binding assay findings [25]), and following SD. In the MPC, where endogenous *Htr2a* expression is lower than in more anterior cortical regions, SD does not increase 5-HT_{2A}R levels in WT or *Egr3*−/− mice (Fig. 3D), and levels of 5-HT_{2A}R are lower in *Egr3*−/− mice than WT mice under at baseline and following SD. Figures 3E–G show representative autoradiographic images from WT and *Egr3*−/− mice under SDc and SD conditions.

These data reveal the novel finding that 5-HT_{2A}Rs can be upregulated in the PFC in a matter of hours in response to an environmental stimulus, and that this requires *Egr3*. The results suggest that EGR3, an activity dependent IEG transcription factor, may directly regulate expression of *Htr2a* in response to environmental events. For this to be happening, EGR3 would have to be expressed in the same cells as *Htr2a* and EGR3 consensus binding sequences would have to be present in the *Htr2a* promoter. Our prior study demonstrated that the former is true [27]. To determine the other requirement, we used the FIMO program [35] which revealed two high probability EGR3 binding sites in the *Htr2a* promoter, a distal binding site at −2777 bp (site A, AGGAGGGGGAGTCT) and a proximal site at −61 bp (site B, GCGCGGGGGAGGGG) upstream of the start ATG (Fig. 4A).

To determine whether EGR3 protein binds to these sites in mouse cortex, we conducted chromatin immunoprecipitation (ChIP). As an IEG, *Egr3* is expressed in a stimulus dependent manner. Under basal, unstimulated conditions, levels of EGR3 protein in the brain are low, and little binding to the promoters of target genes would be expected. SD, which increases *Egr3* mRNA ([26, 27] and Fig. 1), should increase EGR3 protein levels and, we expected, binding to the *Htr2a* promoter. Figures 4B – 4C show that 6h of SD significantly increases EGR3 protein levels in the frontal cortex of WT mice, measured by Western blot. Next, we conducted ChIP on samples of chromatin from WT mice that underwent 6h of SD compared to SDc mice. We used the promoter region of activity-regulated cytoskeleton associated protein (*Arc*) that includes a validated EGR3 binding domain as a positive control [36]. Figure 4D shows that SD significantly increased EGR3 binding to the *Arc* promoter region, as well as to the distal *Htr2a* promoter, compared to SDc conditions. EGR3 binding to the proximal promoter was not significantly changed following SD.

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

[36], the distal *Htr2a* promoter, or the proximal *Htr2a* promoter, with either a CMV vector overexpressing EGR3, or a control empty CMV vector (Fig. 5A–C). Figure 5D shows Western blot results validating EGR3 overexpression in the cell culture assays. We found that both regions of the *Htr2a* promoter containing high-probability EGR3 binding sites (Fig. 4A) drive expression of luciferase in response to EGR3 expression. EGR3 expression induces a 4.9-fold increase in positive control *Arc* promoter-driven luciferase (Fig. 5A) and 3.9-fold increase in the *Htr2a* distal promoter-driven luciferase (Fig. 5B). In addition, although the proximal *Htr2a* promoter did not show a statistically significant increase in EGR3 binding in the *in vivo* ChIP assay (Fig. 4D), *in vitro* expression of EGR3 induced a significant 4.2-fold increase in *Htr2a* proximal promoter-driven luciferase signal, compared to CMV vector alone (Fig. 5C). These results suggest that the physiologic stimulus of SD upregulates EGR3, which directly binds to the *Htr2a* promoter and activates *Htr2a* expression, results in increased levels of cortical 5-HT_{2A}Rs in the mouse brain.

As discussed above, numerous studies have reported deficient levels of 5-HT_{2A}Rs in schizophrenia patients, as well as decreased levels of *HTR2A* in postmortem patient brain tissue. If our findings showing that EGR3 regulates *Htr2a* in mice are also true in humans, then the deficiency in *HTR2A* expression in the brains of schizophrenia patients could be a consequence of reduced expression of *EGR3*, a gene that has also been found to be reduced in schizophrenia patient brains. To further explore this possibility, we analyzed the results of a published gene expression dataset from postmortem prefrontal cortex tissue samples from schizophrenia patient and control brains in the NCBI GEO Database [38]. Figure 5E and 5F show that both *EGR3* and *HTR2A* expression are reduced in the schizophrenia subject samples compared to controls.

Discussion

The results we report here reveal several novel mechanisms of how 5-HT_{2A}R levels in the brain are regulated. Our findings demonstrate that a physiological stimulus, SD, rapidly upregulates expression of both *Htr2a* expression and levels of membrane bound 5-HT_{2A}R in the mouse brain in a matter of several hours. They also demonstrate that this environmentally induced upregulation requires the activity dependent IEG transcription factor EGR3, which binds to the *Htr2a* promoter in the frontal cortex *in vivo* and is able to activate expression of *Htr2a* promoter-driven reporter constructs *in vitro*. Thus, these results identify a direct transcriptional regulator of the 5-HT_{2A}R in the frontal cortex and reveal a previously unrecognized characteristic of 5-HT_{2A}R regulation; that it is environmental-stimulus responsive.

Our finding that 5-HT_{2A}Rs can be rapidly upregulated in the mouse frontal cortex in response to SD is supported by a study in humans that employed a similar stimulus. Elmenhorst and colleagues conducted a PET study using the 5-HT_{2A}R selective radioligand [^{18}F]altanserin to determine the effects on 24h of SD on 5-HT_{2A}R levels in the frontal cortex of healthy subjects. Comparing PET scans conducted following a night of normal sleep to scans conducted the following day, after 24h of total sleep deprivation, they found a 9.6% increase of [^{18}F]altanserin binding in neocortical regions, including the medial inferior frontal gyrus, insula, and anterior cingulate, parietal, sensomotoric, and

ventrolateral prefrontal cortices [28]. Their study was the first investigation of the effects of SD on cerebral cortex 5-HT_{2A}R levels in either humans or animals [28]. In discussing the possible mechanisms that may underlie their finding, the authors point to the fact that SD increases brain levels of serotonin (5-HT) in rodents [43]. However, the effects of serotonin, and other agonists, on 5-HT_{2A}R levels are complicated, with reports showing both upregulation and downregulation in different systems and in a manner that may be influenced by concentration or dosage [44]. Our findings suggest, instead, that this increase in 5-HT_{2A}R levels may result from direct upregulation of *Htr2a* by the IEG EGR3, which has been shown to be activated in the cortex of mice by 6h of SD by the Allen Institute for Brain Research group [26] and replicated by our laboratory here and in our prior study [27].

We chose to use SD as a stimulus in the current study because it is an environmental intervention that we and others have shown increases *Egr3* expression [26, 27] and thus allowed us to test our hypothesis that induction of EGR3 should upregulate expression of *Htr2a*. As a class, IEGs are activated in the brain in response to a wide range of stimuli, including many types of stress [45]. However, the expression of *Egr3* in response to specific stressors has been less well investigated than that of many other IEGs. So, regardless of whether SD causes mice to experience “stress”, it is an effective intervention that demonstrates how rapidly an environmental stimulus can upregulate 5-HT_{2A}R mRNA and protein levels in the brain; in just 6–8 hours.

Roles of 5-HT_{2A}Rs and Egr3 in stress response and memory

A significant body of research has suggested that stress influences 5-HT_{2A}R expression. Numerous types of stress, including physical stressors of immobilization, chronic forced swimming and toe pinch, social stress, including maternal separation, as well as *in utero* exposure to lipopolysaccharide (to mimic bacterial infection), have been found to elevate levels of 5-HT_{2A}R mRNA and protein, as well as to increase the frequency of 5-HT_{2A}R-mediated behaviors (head-twitch response to 5-HT_{2A}R agonists) (reviewed in Table 1 of [46]). Though not all types of stress produce this effect [46]. These findings suggest that increased expression of 5-HT_{2A}Rs may play a role in the brain’s response to environmental events, particularly stressful ones.

One of the critical responses of the brain to environmental events is the formation of memories. In fact, numerous studies suggest a role for 5-HT_{2A}Rs in memory formation. 5-HT_{2A}Rs facilitate associative learning (reviewed in Table 2 of [46]), play a role in the retrieval of recognition memory [47], and modulate reconsolidation of contextual recognition memory [48]. If EGR3 is regulating the increase in 5-HT_{2A}Rs expression in response to environmental stimuli, this suggests that the *Egr3* gene should also be required for memory formation. In fact, our prior studies showed that *Egr3* deficient (−/−) mice fail to habituate to a startling stimulus (demonstrating deficits in associative memory), display social interaction responses consistent with an inability to remember familiar mice (including persistent, non-habituating social investigation and increased aggressive behavior), and show deficits in Y-maze navigation indicative of defects in spatial memory formation [49].

We also found that *Egr3*−/− mice show a heightened response to stress [49]. While at first this may appear inconsistent with the literature indicating that 5-HT_{2A}Rs are upregulated in

response to stress, the findings are actually aligned. Without *Egr3*, mice fail to remember having experienced events, such as being handled by an investigator or having encountered mice they have lived with their whole lives, and they respond with the same high level of reactivity, and cortisol release, as mice being exposed for the first time [49]. Thus, the findings that increased 5-HT_{2A}R levels are important for associative and contextual learning, and the fact that *Egr3*−/− mice have deficient levels of 5-HT_{2A}R that fail to normally upregulate in response to environmental stimuli ([25, 27] and current results), provide a potential explanation for at least part of the heightened stress response of *Egr3*−/− mice [49]. More research will be necessary to determine if *Egr3* mediates the change in 5-HT_{2A}R levels in response to other types of stress.

Regulation of Htr2a expression

Studies examining the mechanisms by which 5-HT_{2A}R levels are regulated have largely focused on effects of agonist and antagonist actions. These studies demonstrate the complex nature of 5-HT_{2A}R regulation. The endogenous ligand 5-HT, as well as both agonists and antagonists, can trigger rapid internalization of 5-HT_{2A}Rs [44, 50, 51]. Though some studies have found that specific agonists can induce processes such as receptor desensitization without altering surface density [52], and other studies have shown a paradoxical increase in receptor density following antagonist exposure in specific cell types [53].

Yet, little is known about transcriptional regulation of *Htr2a*, or that environmental stimuli rapidly alter 5-HT_{2A}R levels. One study employed bioinformatic resources and structural equation modeling to analyze the putative effect of a polymorphism in the human *HTR2A* promoter on transcription factor binding in subjects with chronic fatigue syndrome [54]. Their findings suggested a diagnosis-specific interaction between methylation of an E47 transcription factor binding site and *HTR2A* expression levels in peripheral blood mononuclear cells in a manner influenced by cortisol level. However, the functionality of these transcription factor binding sites was not examined in that study [54]. Thus, our findings are novel in identifying direct regulation of *Htr2a* by an activity dependent IEG.

5-HT_{2A}Rs in schizophrenia

5-HT_{2A}Rs are abundantly expressed in the neocortex and play important roles in cognition, mood and sleep, processes that are disrupted across numerous psychiatric disorders. Investigations of drugs that act as agonists at 5-HT_{2A}Rs have recently experienced a resurgence in the search for treatments for severe psychiatric symptoms ranging from depression [55] to anxiety disorders such as PTSD [56–58]. Yet decades of clinical practice and research have demonstrated a particular importance of 5-HT_{2A}Rs in schizophrenia.

Schizophrenia is characterized by abnormalities in perception, thinking and memory (exemplified by hallucinations, delusions and cognitive deficits). The fact that 5-HT_{2A}Rs mediate the hallucinogenic effects of numerous drugs including LSD, psilocybin, and mescaline [5, 6] suggest the possibility that this receptor may also influence the hallucinations and perceptual disturbances of schizophrenia. One of the most important discoveries that demonstrated a critical role for 5-HT_{2A}Rs in schizophrenia symptomatology was the discovery that clozapine, one of the most effective antipsychotic medications to date, binds

with high affinity to this receptor [59]. In fact, 5-HT_{2A}R binding is an essential feature of second-generation antipsychotics, which were modeled after clozapine and are the first-line of treatment for psychotic disorders [8, 59]. Furthermore, the 5-HT_{2A}R inverse agonist pimavanserin is effective for treatment of psychosis [7]. Finally, as far back as 1976 numerous post-mortem and *in vivo* studies have revealed that 5-HT_{2A}R (or 5-HT_2R) levels are reduced in schizophrenia patients' brains, and specifically in the prefrontal cortex [14, 15, 19, 22, 60, 61].

The prefrontal cortex has long been hypothesized to be a central brain region involved in schizophrenia pathogenesis [62, 63]. Activity in this region is essential for spatial working memory, dysfunction in which may contribute to the cognitive deficits that characterize schizophrenia (reviewed in [64]). Our results show that the environmental stimulus of SD increases levels of *Egr3* and 5-HT_{2A}R mRNA and protein in the antero-posterior domains of mouse cortex corresponding to the human prefrontal cortex, particularly the anterior cingulate cortex (ACAv, ACAd) [65]. These are also regions where we found that SD-induced 5-HT_{2A}R expression is *Egr3*-dependent.

The fact that this process is mediated by an activity-dependent IEG transcription factor suggests the intriguing possibility that the reduced 5-HT_{2A}R levels reported in schizophrenia patients may be a consequence of disrupted neural activity, resulting in insufficient activation of IEGs including *EGR3*. Such a hypothesis is intriguing in the context of recent findings that neural circuit connectivity, and the synchrony of neural oscillations, between the prefrontal cortex and other brain regions is disrupted in schizophrenia patients [66–68]. It is also supported by findings of abnormal IEG expression in patients' brains ([69] and Fig. 5E).

In further support of this hypothesis, numerous EGR family genes are associated with risk for schizophrenia [70, 71]. Although genome wide association studies (GWAS) have recently identified numerous loci believed to increase genetic risk for illnesses like schizophrenia, the mechanism by which environment may interact with these regions remains elusive. It is notable that, *EGR1, EGR4,* and *NAB2* (a transcriptional co-regulator that alters gene expression via binding to the EGRs) each map to one of the 145 GWAS loci for schizophrenia [71]. Although *EGR3* itself is not within a GWAS locus, it interacts in co-regulatory feedback loops with the EGRs and NAB2 [70, 72], and *EGR3* expression is reduced in the brains of schizophrenia patients [73, 74], including in our analyses of data from the NCBI GEO database, which shows reduced levels of both *EGR3* and *HTR2A* mRNA in the prefrontal cortex of schizophrenia patients compared with controls (Fig. 5E and 5F) [38].

Another feature of schizophrenia, although less commonly discussed, is sleep disruption, which includes fragmentation and overall decreased duration of sleep [75, 76]. Our finding that SD increases expression of *Egr3* and 5-HT_{2A}R mRNA and protein may initially appear inconsistent with the numerous reports that expression of these genes is reduced in schizophrenia patients, since sleep disruption and loss is a common characteristic of this mental illness [75, 76]. There could be several explanations for this. First, as an IEG, expression of *Egr3* is rapidly increased in response to a stimulus and returns to baseline within hours of the stimulus [77]. So, the timing of the study, or time of death (in the case of post-mortem studies), may influence whether the gene expression consequences of sleep disruption will still be detectable. However, an explanation we feel is more likely is that

the altered neural activity in the prefrontal cortex of schizophrenia patients, which may be related to documented deficiencies in synaptic density [78] and abnormal circuit function [64, 66–68], could result in deficient induction of *EGR3* in response to environmental stimuli, including SD. Our data suggest this would consequently result in deficient upregulation of 5-HT_{2A}Rs. Finally, genetic variations that affect expression of EGR family genes may also reduce the stimulus-dependent upregulation of 5-HT_{2A}Rs. The literature suggesting that this upregulation plays an important role in contextual and associative memory formation [46] suggests that such insufficient 5-HT_{2A}R expression might contribute to the cognitive deficits of schizophrenia.

Together, these findings suggest that dysfunction in activity dependent EGR family IEGs, which include, and result in, decreased activity of EGR3, may contribute to the reported deficits in 5-HT_{2A}R expression in schizophrenia patient brains. These findings thereby shed light on a potential mechanism whereby environment interacts with genetic variations to influence neurobiology that may contribute to the symptoms, and treatment, of neuropsychiatric illness.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Raote I, Bhattacharya A, Panicker MM. Serotonin 2A (5-HT2A) Receptor Function: Ligand-Dependent Mechanisms and Pathways. In: Chattopadhyay A (ed). Serotonin Receptors in Neurobiology: Boca Raton (FL), 2007.
2. Geyer MA, Vollenweider FX. Serotonin research: contributions to understanding psychoses. Trends Pharmacol Sci 2008; 29(9): 445–453. [PubMed: 19086254]
3. Lopez-Gimenez JF, Gonzalez-Maeso J. Hallucinogens and Serotonin 5-HT2A Receptor-Mediated Signaling Pathways. Curr Top Behav Neurosci 2018; 36: 45–73. [PubMed: 28677096]
4. Aghajanian GK, Marek GJ. Serotonin and hallucinogens. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 1999; 21(2 Suppl): 16S–23S. [PubMed: 10432484]
5. Nichols DE. Hallucinogens. Pharmacol Ther 2004; 101(2): 131–181. [PubMed: 14761703]
6. Halberstadt AL, Geyer MA. Multiple receptors contribute to the behavioral effects of indoleamine hallucinogens. Neuropharmacology 2011; 61(3): 364–381. [PubMed: 21256140]
7. Meltzer HY, Mills R, Revell S, Williams H, Johnson A, Bahr D et al. Pimavanserin, a serotonin(2A) receptor inverse agonist, for the treatment of parkinson's disease psychosis. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 2010; 35(4): 881–892. [PubMed: 19907417]
8. Meltzer HY, Huang M. In vivo actions of atypical antipsychotic drug on serotonergic and dopaminergic systems. Progress in brain research 2008; 172: 177–197. [PubMed: 18772033]

9. Laruelle M, Abi-Dargham A, Casanova MF, Toti R, Weinberger DR, Kleinman JE. Selective abnormalities of prefrontal serotonergic receptors in schizophrenia. A postmortem study. Archives of general psychiatry 1993; 50(10): 810–818. [PubMed: 8215804]
10. Reynolds GP, Rossor MN, Iversen LL. Preliminary studies of human cortical 5-HT2 receptors and their involvement in schizophrenia and neuroleptic drug action. J Neural Transm Suppl 1983; 18: 273–277. [PubMed: 6135746]
11. Eastwood SL, Burnet PW, Gittins R, Baker K, Harrison PJ. Expression of serotonin 5-HT(2A) receptors in the human cerebellum and alterations in schizophrenia. Synapse 2001; 42(2): 104–114. [PubMed: 11574947]
12. Gonzalez-Maeso J, Ang RL, Yuen T, Chan P, Weisstaub NV, Lopez-Gimenez JF et al. Identification of a serotonin/glutamate receptor complex implicated in psychosis. Nature 2008; 452(7183): 93–97. [PubMed: 18297054]
13. Muguruza C, Moreno JL, Umali A, Callado LF, Meana JJ, Gonzalez-Maeso J. Dysregulated 5-HT(2A) receptor binding in postmortem frontal cortex of schizophrenic subjects. Eur Neuropsychopharmacol 2013; 23(8): 852–864. [PubMed: 23176747]
14. Selvaraj S, Arnone D, Cappai A, Howes O. Alterations in the serotonin system in schizophrenia: a systematic review and meta-analysis of postmortem and molecular imaging studies. Neuroscience and biobehavioral reviews 2014; 45: 233–245. [PubMed: 24971825]
15. Rasmussen H, Frokjaer VG, Hilker RW, Madsen J, Anhoj S, Oranje B et al. Low frontal serotonin 2A receptor binding is a state marker for schizophrenia? Eur Neuropsychopharmacol 2016; 26(7): 1248–1250. [PubMed: 27179966]
16. Hurlemann R, Matusch A, Kuhn KU, Berning J, Elmenhorst D, Winz O et al. 5-HT2A receptor density is decreased in the at-risk mental state. Psychopharmacology (Berl) 2008; 195(4): 579–590. [PubMed: 17899021]
17. Dean B, Hayes W. Decreased frontal cortical serotonin2A receptors in schizophrenia. Schizophrenia research 1996; 21(3): 133–139. [PubMed: 8885041]
18. Ngan ET, Yatham LN, Ruth TJ, Liddle PF. Decreased serotonin 2A receptor densities in neuroleptic-naive patients with schizophrenia: A PET study using [(18)F]setoperone. The American journal of psychiatry 2000; 157(6): 1016–1018. [PubMed: 10831488]
19. Lopez-Figueroa AL, Norton CS, Lopez-Figueroa MO, Armellini-Dodel D, Burke S, Akil H et al. Serotonin 5-HT1A, 5-HT1B, and 5-HT2A receptor mRNA expression in subjects with major depression, bipolar disorder, and schizophrenia. Biological psychiatry 2004; 55(3): 225–233. [PubMed: 14744462]
20. Matsumoto I, Inoue Y, Iwazaki T, Pavey G, Dean B. 5-HT2A and muscarinic receptors in schizophrenia: a postmortem study. Neurosci Lett 2005; 379(3): 164–168. [PubMed: 15843056]
21. Serretti A, Drago A, De Ronchi D. HTR2A gene variants and psychiatric disorders: a review of current literature and selection of SNPs for future studies. Curr Med Chem 2007; 14(19): 2053–2069. [PubMed: 17691947]
22. Garbett K, Gal-Chis R, Gaszner G, Lewis DA, Mirnics K. Transcriptome alterations in the prefrontal cortex of subjects with schizophrenia who committed suicide. Neuropsychopharmacol Hung 2008; 10(1): 9–14. [PubMed: 18771015]
23. Kang K, Huang XF, Wang Q, Deng C. Decreased density of serotonin 2A receptors in the superior temporal gyrus in schizophrenia--a postmortem study. Progress in neuro-psychopharmacology & biological psychiatry 2009; 33(5): 867–871. [PubMed: 19389456]
24. Burnet PW, Eastwood SL, Harrison PJ. 5-HT1A and 5-HT2A receptor mRNAs and binding site densities are differentially altered in schizophrenia. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 1996; 15(5): 442–455. [PubMed: 8914117]
25. Williams AA, Ingram WM, Levine S, Resnik J, Kamel CM, Lish JR et al. Reduced levels of serotonin 2A receptors underlie resistance of Egr3-deficient mice to locomotor suppression by clozapine. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 2012; 37(10): 2285–2298. [PubMed: 22692564]

26. Thompson CL, Wisor JP, Lee CK, Pathak SD, Gerashchenko D, Smith KA et al. Molecular and anatomical signatures of sleep deprivation in the mouse brain. Front Neurosci 2010; 4: 165. [PubMed: 21088695]
27. Maple AM, Zhao X, Elizalde DI, McBride AK, Gallitano AL. Htr2a Expression Responds Rapidly to Environmental Stimuli in an Egr3-Dependent Manner. ACS Chem Neurosci 2015; 6(7): 1137–1142. [PubMed: 25857407]
28. Elmenhorst D, Kroll T, Matusch A, Bauer A. Sleep deprivation increases cerebral serotonin 2A receptor binding in humans. Sleep 2012; 35(12): 1615–1623. [PubMed: 23204604]
29. Tourtellotte WG, Milbrandt J. Sensory ataxia and muscle spindle agenesis in mice lacking the transcription factor Egr3. Nature genetics 1998; 20(1): 87–91. [PubMed: 9731539]
30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25(4): 402–408. [PubMed: 11846609]
31. Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A et al. Genome-wide atlas of gene expression in the adult mouse brain. Nature 2007; 445(7124): 168–176. [PubMed: 17151600]
32. Allen Mouse Brain Atlas. https://mouse.brain-map.org/static/atlas, 2004, Accessed Date Accessed 2004 Accessed.
33. O'Donovan KJ, Baraban JM. Major Egr3 isoforms are generated via alternate translation start sites and differ in their abilities to activate transcription. Molecular and cellular biology 1999; 19(7): 4711–4718. [PubMed: 10373520]
34. Salotti J, Sakchaisri K, Tourtellotte WG, Johnson PF. An Arf-Egr-C/EBPbeta pathway linked to ras-induced senescence and cancer. Molecular and cellular biology 2015; 35(5): 866–883. [PubMed: 25535333]
35. Grant CE, Bailey TL, Noble WS. FIMO: scanning for occurrences of a given motif. Bioinformatics 2011; 27(7): 1017–1018. [PubMed: 21330290]
36. Li L, Carter J, Gao X, Whitehead J, Tourtellotte WG. The neuroplasticity-associated arc gene is a direct transcriptional target of early growth response (Egr) transcription factors. Molecular and cellular biology 2005; 25(23): 10286–10300. [PubMed: 16287845]
37. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 2002; 30(1): 207–210. [PubMed: 11752295]
38. Lanz TA, Joshi JJ, Reinhart V, Johnson K, Grantham LE 2nd, Volfson D. STEP levels are unchanged in pre-frontal cortex and associative striatum in post-mortem human brain samples from subjects with schizophrenia, bipolar disorder and major depressive disorder. PloS one 2015; 10(3): e0121744. [PubMed: 25786133]
39. Motulsky HJ, Brown RE. Detecting outliers when fitting data with nonlinear regression - a new method based on robust nonlinear regression and the false discovery rate. BMC Bioinformatics 2006; 7: 123. [PubMed: 16526949]
40. Mengod G, Pompeiano M, Martinez-Mir MI, Palacios JM. Localization of the mRNA for the 5-HT2 receptor by in situ hybridization histochemistry. Correlation with the distribution of receptor sites. Brain research 1990; 524(1): 139–143. [PubMed: 2400925]
41. Pompeiano M, Palacios JM, Mengod G. Distribution of the serotonin 5-HT2 receptor family mRNAs: comparison between 5-HT2A and 5-HT2C receptors. Brain research Molecular brain research 1994; 23(1–2): 163–178. [PubMed: 8028479]
42. Weber ET, Andrade R. Htr2a Gene and 5-HT(2A) Receptor Expression in the Cerebral Cortex Studied Using Genetically Modified Mice. Front Neurosci 2010; 4.
43. Lopez-Rodriguez F, Wilson CL, Maidment NT, Poland RE, Engel J. Total sleep deprivation increases extracellular serotonin in the rat hippocampus. Neuroscience 2003; 121(2): 523–530. [PubMed: 14522011]
44. Van Oekelen D, Luyten WH, Leysen JE. 5-HT2A and 5-HT2C receptors and their atypical regulation properties. Life Sci 2003; 72(22): 2429–2449. [PubMed: 12650852]
45. Senba E, Ueyama T. Stress-induced expression of immediate early genes in the brain and peripheral organs of the rat. Neurosci Res 1997; 29(3): 183–207. [PubMed: 9436645]
46. Murnane KS. Serotonin 2A receptors are a stress response system: implications for post-traumatic stress disorder. Behav Pharmacol 2019; 30(2 and 3-Spec Issue): 151–162. [PubMed: 30632995]

47. Bekinschtein P, Renner MC, Gonzalez MC, Weisstaub N. Role of medial prefrontal cortex serotonin 2A receptors in the control of retrieval of recognition memory in rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 2013; 33(40): 15716–15725. [PubMed: 24089480]
48. Morici JF, Miranda M, Gallo FT, Zanoni B, Bekinschtein P, Weisstaub NV. 5-HT2a receptor in mPFC influences context-guided reconsolidation of object memory in perirhinal cortex. Elife 2018; 7.
49. Gallitano-Mendel A, Izumi Y, Tokuda K, Zorumski CF, Howell MP, Muglia LJ et al. The immediate early gene early growth response gene 3 mediates adaptation to stress and novelty. Neuroscience 2007; 148(3): 633–643. [PubMed: 17692471]
50. Bhattacharyya S, Puri S, Miledi R, Panicker MM. Internalization and recycling of 5-HT2A receptors activated by serotonin and protein kinase C-mediated mechanisms. Proceedings of the National Academy of Sciences of the United States of America 2002; 99(22): 14470–14475. [PubMed: 12388782]
51. Willins DL, Berry SA, Alsayegh L, Backstrom JR, Sanders-Bush E, Friedman L et al. Clozapine and other 5-hydroxytryptamine-2A receptor antagonists alter the subcellular distribution of 5-hydroxytryptamine-2A receptors in vitro and in vivo. Neuroscience 1999; 91(2): 599–606. [PubMed: 10366017]
52. Roth BL, Palvimaki EP, Berry S, Khan N, Sachs N, Uluer A et al. 5-Hydroxytryptamine2A (5-HT2A) receptor desensitization can occur without downregulation. The Journal of pharmacology and experimental therapeutics 1995; 275(3): 1638–1646. [PubMed: 8531139]
53. Akiyoshi J, Hough C, Chuang DM. Paradoxical increase of 5-hydroxytryptamine2 receptors and 5-hydroxytryptamine2 receptor mRNA in cerebellar granule cells after persistent 5-hydroxytryptamine2 receptor stimulation. Mol Pharmacol 1993; 43(3): 349–355. [PubMed: 8383800]
54. Falkenberg VR, Gurbaxani BM, Unger ER, Rajeevan MS. Functional genomics of serotonin receptor 2A (HTR2A): interaction of polymorphism, methylation, expression and disease association. Neuromolecular Med 2011; 13(1): 66–76. [PubMed: 20941551]
55. Carhart-Harris RL, Bolstridge M, Rucker J, Day CM, Erritzoe D, Kaelen M et al. Psilocybin with psychological support for treatment-resistant depression: an open-label feasibility study. Lancet Psychiatry 2016; 3(7): 619–627. [PubMed: 27210031]
56. Grob CS, Danforth AL, Chopra GS, Hagerty M, McKay CR, Halberstadt AL et al. Pilot study of psilocybin treatment for anxiety in patients with advanced-stage cancer. Archives of general psychiatry 2011; 68(1): 71–78. [PubMed: 20819978]
57. Griffiths RR, Johnson MW, Carducci MA, Umbricht A, Richards WA, Richards BD et al. Psilocybin produces substantial and sustained decreases in depression and anxiety in patients with life-threatening cancer: A randomized double-blind trial. Journal of psychopharmacology 2016; 30(12): 1181–1197. [PubMed: 27909165]
58. Nichols DE, Johnson MW, Nichols CD. Psychedelics as Medicines: An Emerging New Paradigm. Clin Pharmacol Ther 2017; 101(2): 209–219. [PubMed: 28019026]
59. Meltzer HY. The role of serotonin in antipsychotic drug action. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 1999; 21(2 Suppl): 106S–115S. [PubMed: 10432496]
60. Bennett JP Jr., Enna SJ, Bylund DB, Gillin JC, Wyatt RJ, Snyder SH. Neurotransmitter receptors in frontal cortex of schizophrenics. Archives of general psychiatry 1979; 36(9): 927–934. [PubMed: 37813]
61. Arora RC, Meltzer HY. Serotonin2 (5-HT2) receptor binding in the frontal cortex of schizophrenic patients. J Neural Transm Gen Sect 1991; 85(1): 19–29. [PubMed: 1678267]
62. Goldman-Rakic PS. Prefrontal cortical dysfunction in schizophrenia : the relevance of working memory. Psychopathology and the Brain 1991: 1–23.
63. Berman KF, Weinberger DR. The prefrontal cortex in schizophrenia and other neuropsychiatric diseases: in vivo physiological correlates of cognitive deficits. Progress in brain research 1990; 85: 521–536; discussion 536–527. [PubMed: 2094913]

64. Kupferschmidt DA, Gordon JA. The dynamics of disordered dialogue: Prefrontal, hippocampal and thalamic miscommunication underlying working memory deficits in schizophrenia. Brain Neurosci Adv 2018; 2.

65. Laubach M, Amarante LM, Swanson K, White SR. What, If Anything, Is Rodent Prefrontal Cortex? eNeuro 2018; 5(5).

66. Sheffield JM, Barch DM. Cognition and resting-state functional connectivity in schizophrenia. Neuroscience and biobehavioral reviews 2016; 61: 108–120. [PubMed: 26698018]

67. Bahner F, Meyer-Lindenberg A. Hippocampal-prefrontal connectivity as a translational phenotype for schizophrenia. Eur Neuropsychopharmacol 2017; 27(2): 93–106. [PubMed: 28089652]

68. Hu ML, Zong XF, Mann JJ, Zheng JJ, Liao YH, Li ZC et al. A Review of the Functional and Anatomical Default Mode Network in Schizophrenia. Neurosci Bull 2017; 33(1): 73–84. [PubMed: 27995564]

69. Gandal MJ, Zhang P, Hadjimichael E, Walker RL, Chen C, Liu S et al. Transcriptome-wide isoform-level dysregulation in ASD, schizophrenia, and bipolar disorder. Science 2018; 362(6420).

70. Marballi KK, Gallitano AL. Immediate Early Genes Anchor a Biological Pathway of Proteins Required for Memory Formation, Long-Term Depression and Risk for Schizophrenia. Frontiers in behavioral neuroscience 2018; 12: 23. [PubMed: 29520222]

71. Pardinas AF, Holmans P, Pocklington AJ, Escott-Price V, Ripke S, Carrera N et al. Common schizophrenia alleles are enriched in mutation-intolerant genes and in regions under strong background selection. Nature genetics 2018; 50(3): 381–389. [PubMed: 29483656]

72. Kumbrink J, Kirsch KH, Johnson JP. EGR1, EGR2, and EGR3 activate the expression of their coregulator NAB2 establishing a negative feedback loop in cells of neuroectodermal and epithelial origin. Journal of cellular biochemistry 2010; 111(1): 207–217. [PubMed: 20506119]

73. Yamada K, Gerber DJ, Iwayama Y, Ohnishi T, Ohba H, Toyota T et al. Genetic analysis of the calcineurin pathway identifies members of the EGR gene family, specifically EGR3, as potential susceptibility candidates in schizophrenia. Proceedings of the National Academy of Sciences of the United States of America 2007; 104(8): 2815–2820. [PubMed: 17360599]

74. Mexal S, Frank M, Berger R, Adams CE, Ross RG, Freedman R et al. Differential modulation of gene expression in the NMDA postsynaptic density of schizophrenic and control smokers. Brain research Molecular brain research 2005; 139(2): 317–332. [PubMed: 16122832]

75. Kaskie RE, Graziano B, Ferrarelli F. Schizophrenia and sleep disorders: links, risks, and management challenges. Nat Sci Sleep 2017; 9: 227–239. [PubMed: 29033618]

76. Ferrarelli F Sleep Abnormalities in Schizophrenia: State of the Art and Next Steps. The American journal of psychiatry 2021; 178(10): 903–913. [PubMed: 33726524]

77. Yamagata K, Kaufmann WE, Lanahan A, Papapavlou M, Barnes CA, Andreasson KI et al. Egr3/Pilot, a zinc finger transcription factor, is rapidly regulated by activity in brain neurons and colocalizes with Egr1/zif268. Learning & memory 1994; 1(2): 140–152. [PubMed: 10467592]

78. Glausier JR, Lewis DA. Dendritic spine pathology in schizophrenia. Neuroscience 2013; 251: 90–107. [PubMed: 22546337]

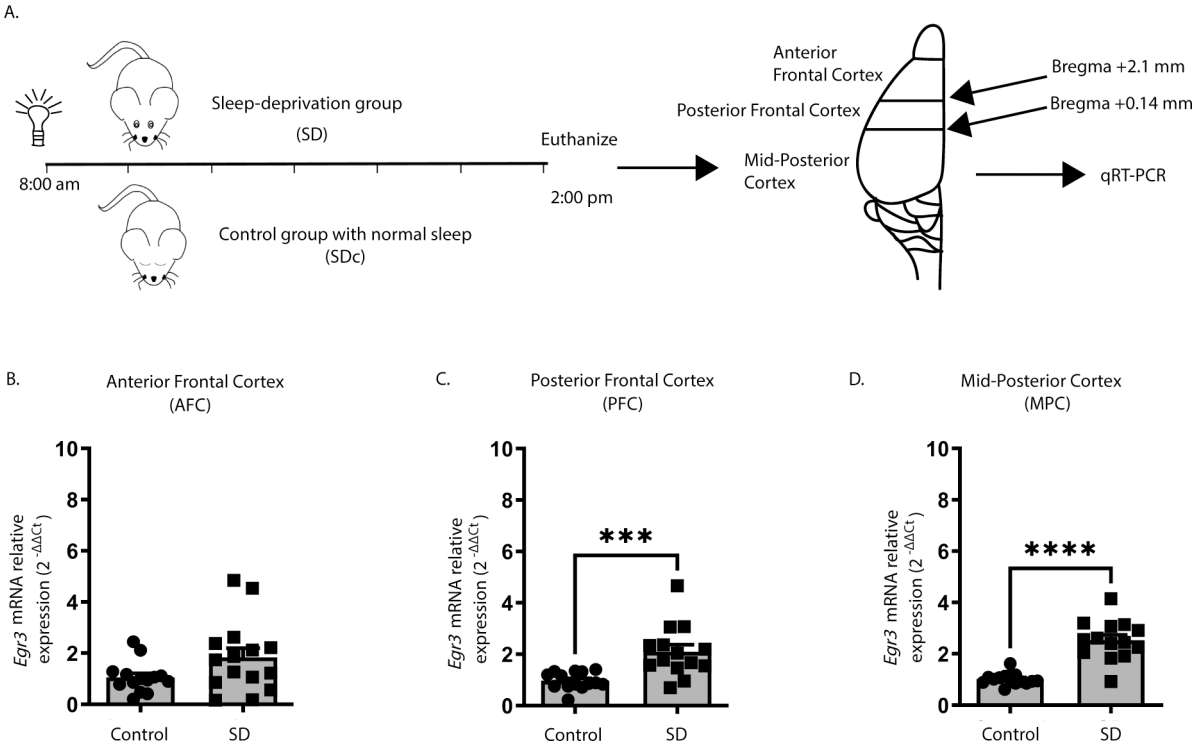

Figure 1. Sleep deprivation upregulates *Egr3* in a region-dependent manner in the frontal cortex.

(**A**) SD protocol. In WT mice quantitative RT-PCR shows that 6h of SD (**B**) does not increase *Egr3* expression in AFC regions ($t_{27} = 1.956$; $p = 0.0609$; SDc, $n = 14$; SD, $n = 15$), but significantly upregulates *Egr3* mRNA in (**C**) PFC ($t_{26} = 3.979$, $p = 0.0005$; SDc, $n = 14$; SD, $n = 14$) and (**D**) MPC ($t_{27} = 7.307$; $p < 0.0001$; SDc, $n = 15$; SD, $n = 14$) regions, compared to SDc. Unpaired student's *t*-test, *** $p < 0.001$, **** $p < 0.0001$. Values represent means ± SEM. (Abbreviations: AFC- anterior frontal cortex; PFC- posterior frontal cortex; MPC- mid to posterior cortex; SD- sleep deprivation; SDc- SD control; WT- wildtype; h: hours).

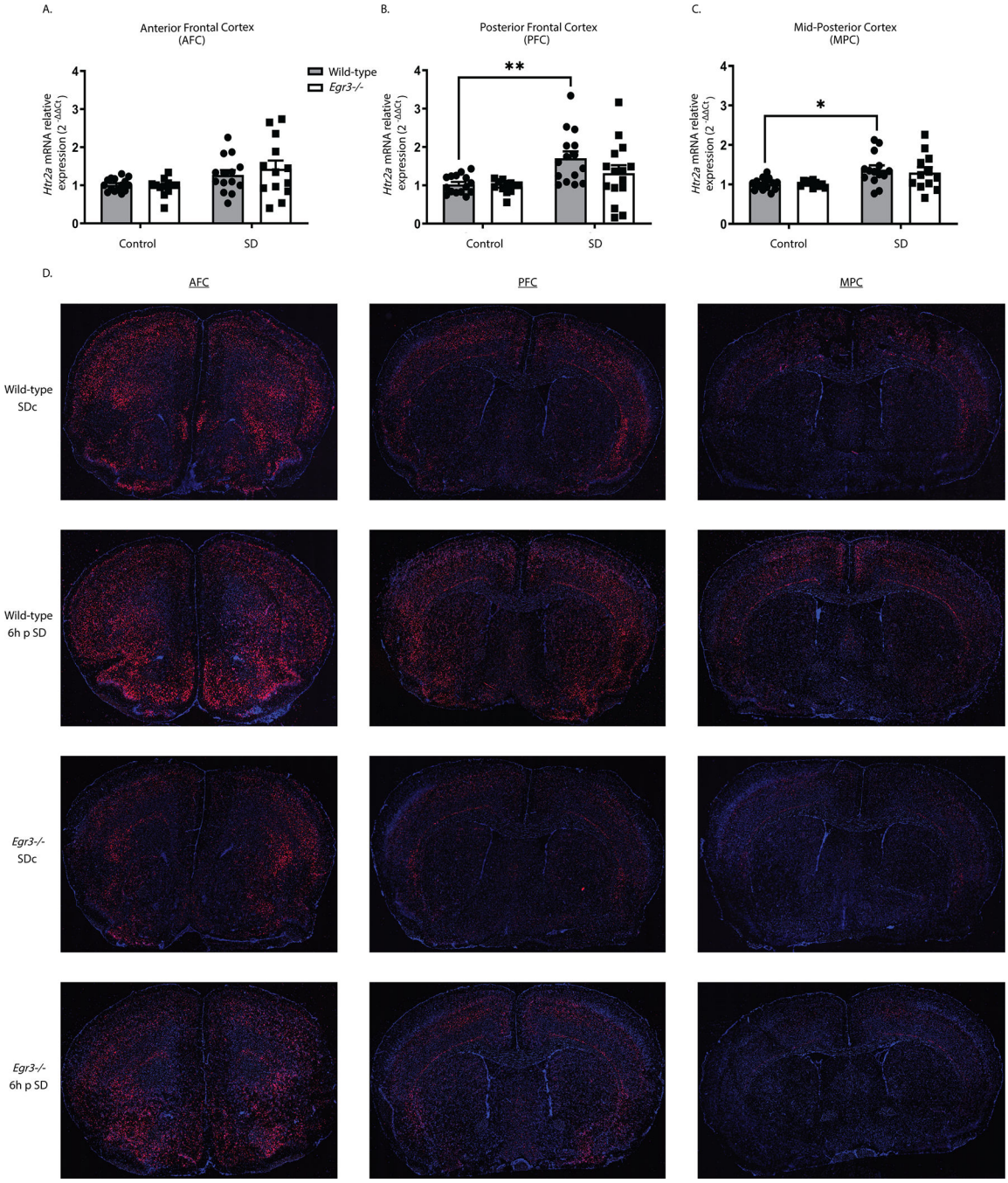

Figure 2. Sleep deprivation upregulates *Htr2a* in an *Egr3*-dependent, and region-specific, manner.

(**A** – **C**) In WT and *Egr3*−/− mice qRT-PCR shows that 6h of SD (**A**) increases *Htr2a* overall in the AFC when both genotypes were analyzed (two-way ANOVA, sig. main effect of SD ($F_{1, 50} = 8.279$, $p = 0.0059$; WT: SDc, $n = 15$; SD, $n = 14$; *Egr3*−/−: SDc, $n = 12$; SD, $n = 13$)) but not in either genotype alone (post-hoc analyses showed no sig. differences between genotypes or SD conditions). (**B**) However, SD significantly upregulates *Htr2a* expression in the PFC of WT, but not *Egr3*−/−, mice, compared to SDc (two-way ANOVA, sig. main

effect of SD ($F_{1, 53} = 12.08$, $p = 0.0010$; WT: SDc, $n = 15$; SD, $n = 15$; *Egr3*−/−: SDc, $n = 12$; SD, $n = 15$); post-hoc analyses showed a sig. increase of *Htr2a* mRNA after SD (vs. SDc) in WT mice ($p = 0.0088$), but not in the *Egr3* −/− mice ($p = 0.6777$)). (**C**) In the MPC, SD increased *Htr2a* in WT, but not *Egr3*−/−, mice, compared to SDc (two-way ANOVA, sig. main effect of SD ($F_{1,47} = 13.14$, $p = 0.0007$; WT: SDc, $n = 15$; SD, $n = 14$; *Egr3*−/−: SDc, $n = 10$; SD, $n = 12$)); post-hoc analyses showed sig. increase of *Htr2a* mRNA after SD (vs. SDc) in WT mice ($p = 0.0205$) but not in the *Egr3* −/− mice ($p = 0.2343$). (**D**) Representative images from n = 3 per group of RNAscope *in situ* hybridization demonstrating *Htr2a* expression in SDc and SD WT and *Egr3*−/− mice. Bonferroni-corrected comparisons: * $p < 0.05$, ** $p < 0.01$. Values represent means ± SEM. (Abbreviations: p - post).

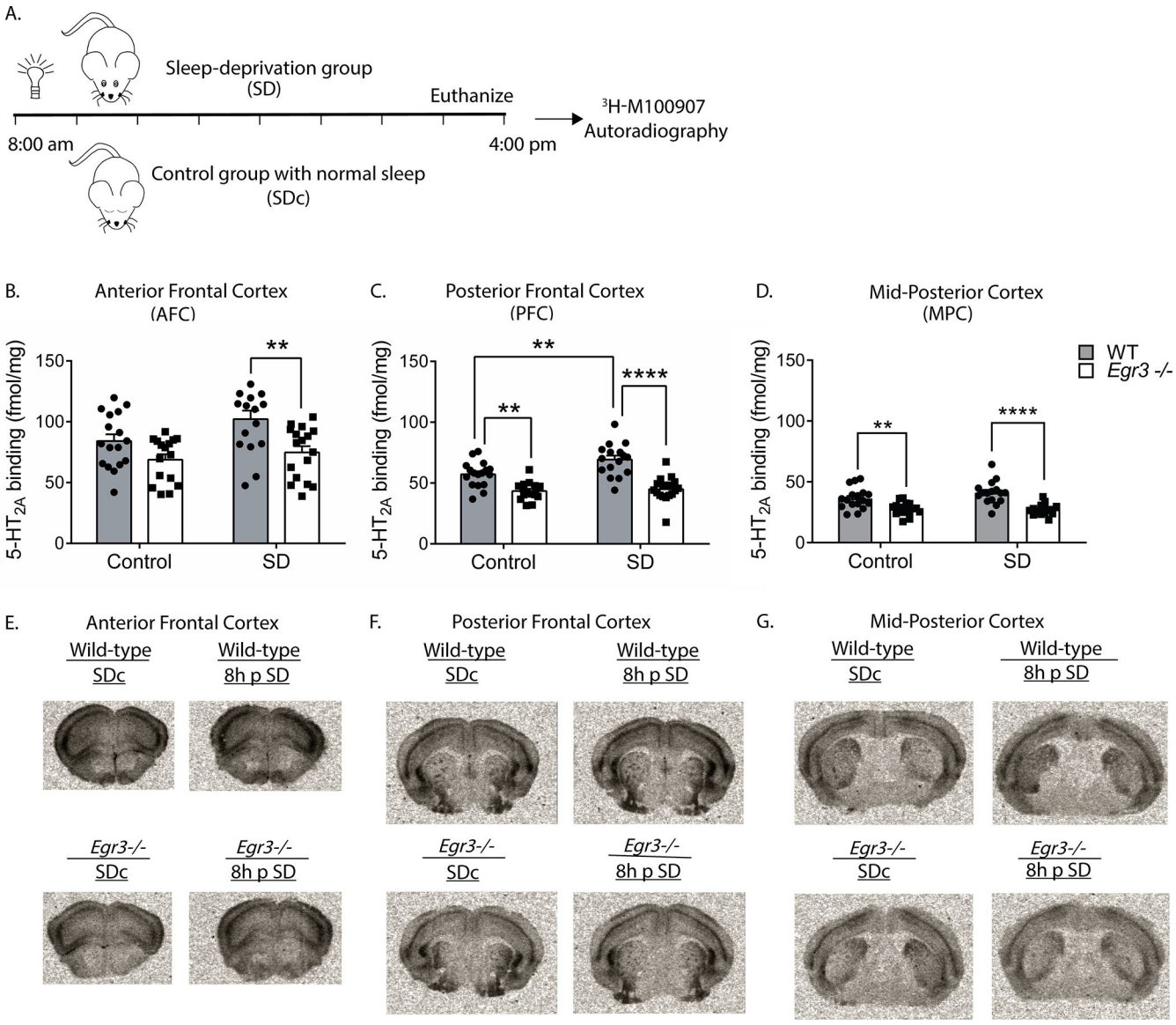

Figure 3. SD increases 5-HT$_{2A}$R levels in the PFC of WT mice in an *Egr3*-dependent manner.

(**A**) 8h SD protocol. Quantification of ^{3}H-M100907 binding autoradiography shows that SD, compared with SDc: (**B**) in AFC results in significantly greater 5-HT$_{2A}$R levels in WT mice than *Egr3*−/− mice after SD (two-way ANOVA, sig. main effects of SD ($F_{1, 62} = 4.61$, $p = 0.0358$) 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 (two-way ANOVA, sig. interaction between SD and genotype ($F_{1, 62} = 4.18$, $p = 0.0451$)). (**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 (two-way ANOVA, sig. main effect of genotype ($F_{1, 62} = 38.79$, $p < 0.0001$)) but not of SD ($F_{1, 62} = 1.371$, $p = 0.2461$). Representative ^{3}H-M100907 autoradiography images of brain tissue sections from (**E**) AFC, (**F**) PFC, and (**G**) MPC. For experiments in (**B**-**D**) WT: SDc, $n = 17$; SD, $n = 16$; *Egr3*−/−:

SDc, $n = 16$; SD, $n = 17$. Bonferroni-corrected comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Values represent means ± SEM.

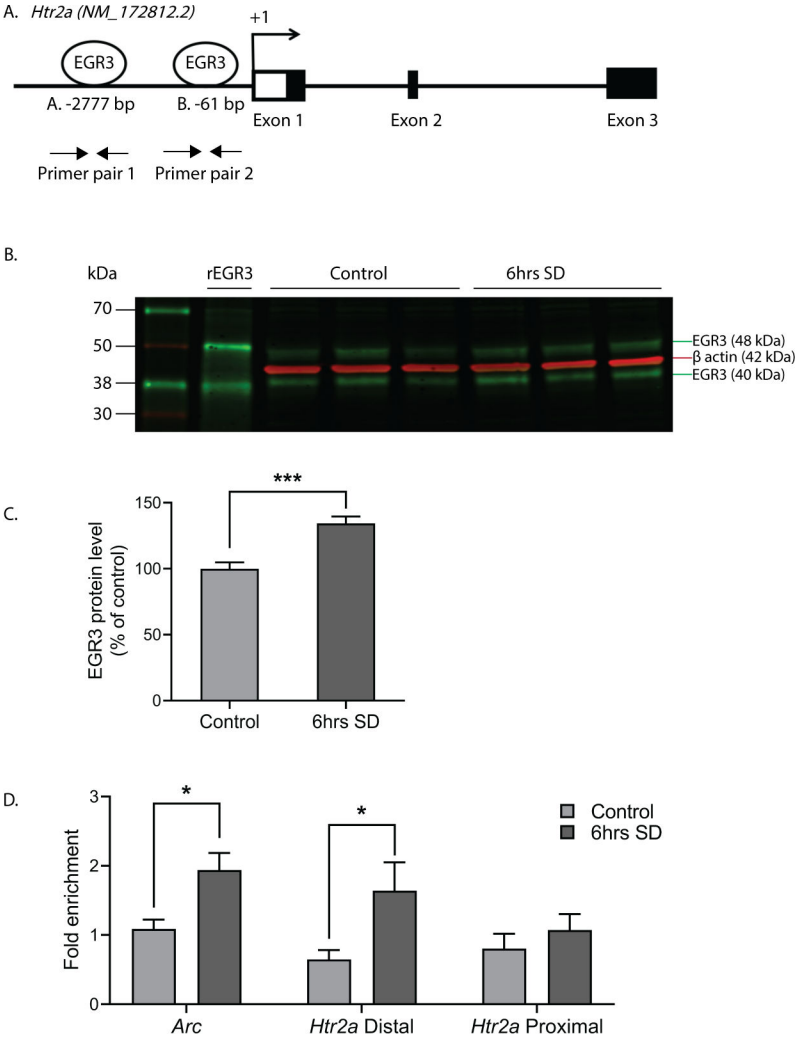

Figure 4. EGR3 binds to the *Htr2a* promoter in frontal cortex.

(**A**) Schematic showing high probability EGR3 consensus binding sites in the *Htr2a* promoter. **(B)** Representative Western blot image ($n = 3$ shown of $n = 11$ per group, Chameleon Duo Pre-Stained Protein Ladder (Li-COR Biosciences) and (**C**) Western blot average protein levels, show significant upregulation of activity dependent EGR3 protein following 6h of SD in WT frontal cortex ($t_{20} = 4.778$; $p = 0.0001$; $n = 11$ per group). **(D)** ChIP-qPCR shows SD increases binding of EGR3 to *Arc* promotor (positive control; SDc, $n = 8$; SD, $n = 10$) and *Htr2a* distal promoter (SDc, $n = 9$; SD, $n = 9$), but not *Htr2a* proximal promoter (SDc, $n = 9$; SD, $n = 11$), in frontal cortex tissue (Respectively, $t_{16} = 2.802$; $p = 0.0128$, $t_{16} = 2.297$; $p = 0.0354$, and $t_{18} = 0.8463$; $p = 0.4085$). Unpaired student's *t*-test, * $p < 0.05$, *** $p < 0.001$. Values represent means ± SEM. (Abbreviations: rEGR3 - recombinant EGR3 protein).

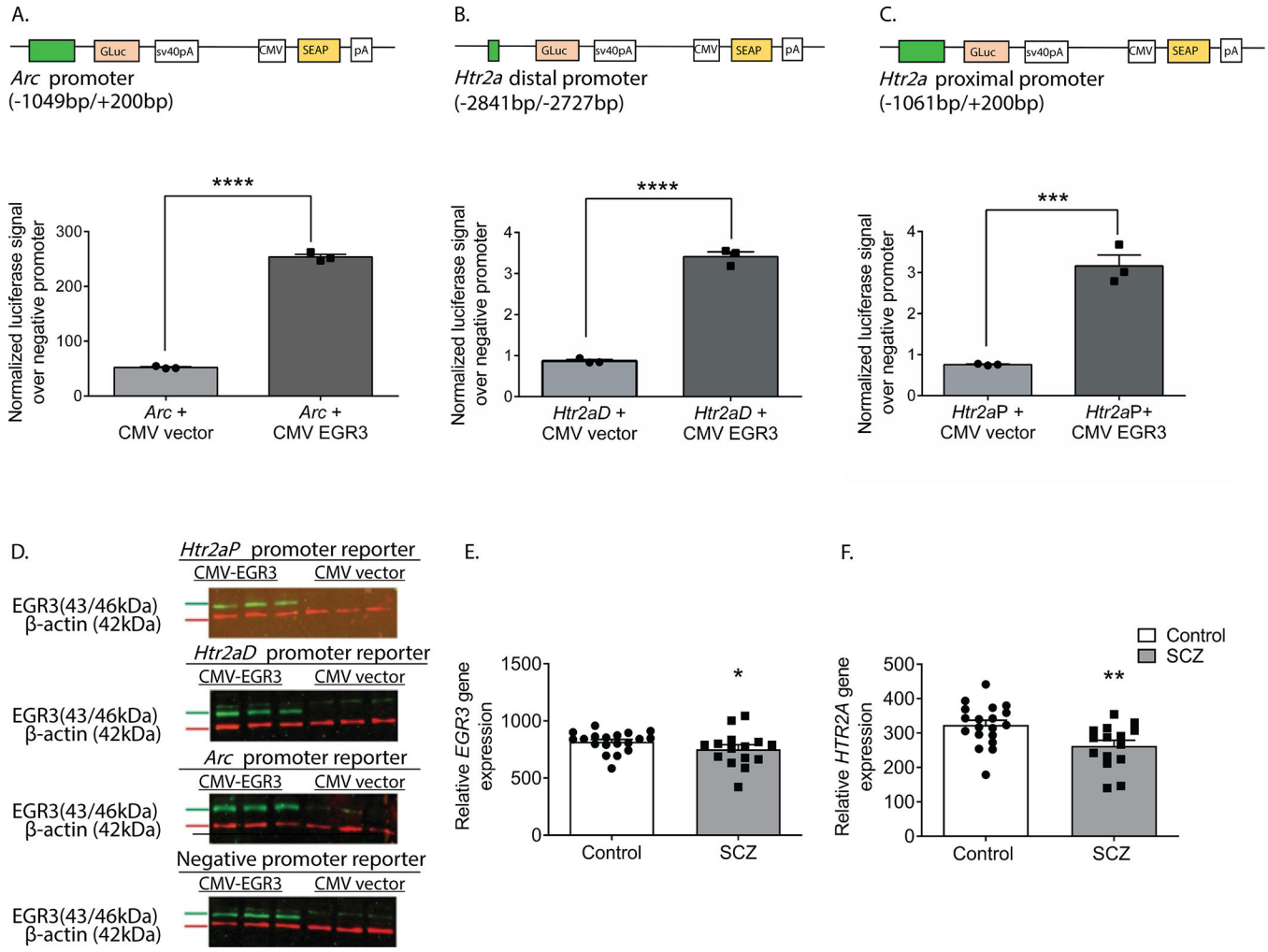

Figure 5. EGR3 drives gene expression via binding sites in the *Htr2a* promoter and EGR3 and HTR2A expression is reduced in schizophrenia brains.

(**A-C**) Schematics of dual luciferase/SEAP reporter constructs containing EGR consensus binding sites and results of *in vitro* assays in neuro2a cells. CMV-driven EGR3 overexpression significantly upregulates expression of luciferase reporters driven by (**A**) *Arc* promoter ($t_4 = 42.28$; $p < 0.0001$), (**B**) *Htr2aD* distal promoter ($t_4 = 21.17$; $p < 0.0001$), and (**C**) *Htr2aP* proximal promoter ($t_4 = 8.977$, $p = 0.0009$) regions. (**D**) Western blot validation of EGR3 expression following transfection with CMV-EGR3 versus CVM empty vector, from cultures expressing reporter constructs driven by promoters from *Arc*, *Htr2aD*, *Htr2aP*, or negative promoter control vector. Unpaired student's *t*-test, (**A-D**) $n = 3$ per group. (**E, F**) *EGR3* and *HTR2A* mRNA levels are significantly decreased in human 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* ($U = 81$, $p = 0.0331$) expression and (**F**) *HTR2A* ($U = 63$, $p = 0.0050$) expression, in control ($n = 19$) vs. schizophrenia ($n = 15$) patients. Mann-Whitney U test, * $p < 0.05$, ** $p < 0.01$,

*** $p < 0.001$, **** $p < 0.0001$. Values represent means ± SEM. (Abbreviations: CMV - cytomegalovirus, GLuc - *Gaussia* luciferase, SEAP - secreted alkaline phosphatase).