Title: Sleep deprivation rapidly upregulates serotonin 2A receptor expression via the immediate early gene Egr3

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Abstract:

Serotonin 2A receptors (5-HT2A Rs) mediate the effects of hallucinogenic drugs and antipsychotic medications, and are reduced in schizophrenia patients’ brains. However, the mechanisms that regulate 5-HT2A R expression remain poorly understood. We show that an environmental stimulus, sleep deprivation, upregulates 5-HT2A Rs in the mouse frontal cortex (FC) in just 6-8 hours. 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.

One Sentence Summary: Just 6-8 hours of sleep deprivation upregulates brain levels of the receptor that mediates the response to hallucinogens.
Serotonin 2A receptors (5-HT2ARs) mediate hallucinogenic effects of drugs including psilocybin, mescaline, and LSD (reviewed in (1, 2)), and are a key target of “second-generation antipsychotic” medications (SGAs) used to treat schizophrenia and other psychotic disorders (3, 4). Numerous studies have revealed that 5-HT2AR levels are reduced in the brains of schizophrenia patients, both post-mortem and in vivo ((5-9) and citations in (10)). However, the molecular mechanisms that regulate expression of this receptor that plays a critical role in the symptoms, and treatment, of mental illnesses remain poorly understood.

The delayed effect of medications that treat psychiatric illness have led to the conclusion that the neurobiological changes that underlie their therapeutic response, occur in a timeframe of weeks. However, our results demonstrate that the environmental stimulus of sleep-deprivation, which produces rapid antidepressant effects in humans, upregulates 5-HT2AR levels in the mouse frontal cortex (FC) within just 6-8 hours (for mRNA and protein, respectively).

Our prior studies revealed that mice lacking the immediate early gene (IEG) Egr3 have reduced levels of 5-HT2AR and, like humans experiencing psychosis, are resistant to sedation by SGAs, a feature shared by 5-HT2AR knockout mice (11-14). These findings led us to hypothesize that EGR3, an activity-dependent transcription factor, may directly regulate expression of the 5-HT2AR gene (Htr2a). If this were the case, it would suggest that Htr2a, like Egr3, should be upregulated in response to environmental stimuli.

Indeed, we found that 6h of sleep deprivation (SD), which upregulates Egr3 in the cerebral cortex (15), significantly increased Htr2a mRNA in mouse cerebral cortex, and that this induction required Egr3 (16). However, it was unclear whether this upregulated expression resulted in an increase in 5-HT2AR protein and, if so, whether this was occurring throughout the cortex, or in regions consistent with antero-posterior gradient in which Htr2a is expressed (17, 18).

We first tested whether we could replicate published in situ hybridization findings showing that SD upregulates Egr3 (15), 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 the same 6h of SD can upregulate Htr2a expression in the same cortical regions, and whether this requires Egr3 (Fig.s 1E – 1G). In the AFC, SD did not significantly increase Htr2a expression in either WT or Egr3-/- mice (Fig. 1E). However, in the PFC of WT mice, SD significantly increased Htr2a mRNA compared to SDc, a result not seen in Egr3-/- mice (Fig. 1F). In the MPC, SD increased Htr2a mRNA when both WT and Egr3-/- mice were analyzed together, but the increase was not significant in either genotype alone (Fig. 1G).

RNAseq in situ hybridization shows that Htr2a expression is significantly lower in Egr3-/- than WT mouse PFC at baseline (SDc), and is not increased by 6h SD in Egr3-/- mice, as it is in WTs (Fig. 1H).
Fig. 1.

Sleep deprivation upregulates Htr2a in an Egr3-dependent, and region-specific, manner. (A) SD protocol. In WT mice quantitative RT-PCR shows that 6h of SD (B) does not increase Egr3 expression in AFC regions (t27 = 0.6679; p = 0.510), but significantly upregulates Egr3 mRNA in (C) PFC (t28 = 2.615, p = 0.0142) and (D) MPC (t27 = 3.5; p = 0.0016) regions, compared to SDCs. (E – G) In WT and Egr3-/- mice 6h of SD (E) does not increase Htr2a expression in AFC (ANOVA; no sig. main effect of SD (F1, 52 = 3.488, p = 0.0675) or genotype (F1, 52 = 0.1129, p = 0.7382), yet (F) significantly upregulates Htr2a expression in the PFC of WT, but not Egr3-/-, mice, compared to SDCs (ANOVA: sig. main effect of SD (F1, 54 = 5.857, p = 0.0189), and sig. main effect of genotype (F1, 54 = 11.95, p = 0.0011); post-hoc analyses showed a sig. increase of Htr2a mRNA after SD (vs. SDC) 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 (F1, 54 = 5.976, p = 0.0181)) but Htr2a mRNA increases were not sig. when comparing either genotype alone (post-hoc analyses showed no sig. differences between genotypes or SD conditions). (H) RNAseq in situ hybridization demonstrating Htr2a expression in SDC and SD WT and Egr3-/- mice (sections from PFC region). Bonferroni-corrected comparisons: * p < 0.05, ** p < 0.01, *** p < 0.001, n = 12-15. Values represent means ± SEM. (AFC: anterior frontal cortex; h: hours; PFC: posterior frontal cortex; SD: sleep deprivation; SDC: SD control; sig: significant, MPC: mid to posterior cortex; WT: wildtype).

To determine if SD also increases 5-HT2AR protein expression, we performed receptor autoradiography in brain sections with 3H-M100907, a selective 5-HT2AR antagonist, following...
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8h SD (to allow time for translation of mRNA) (Fig. 2A). In the AFC, SD does not significantly increase 5-HT$_2$AR binding in either WT or $Egr3^{-/-}$ mice (Fig. 2B). However, SD increases WT expression sufficiently to produce a significant difference in 5-HT$_2$AR levels between WT and $Egr3^{-/-}$ mice that is not present in SDc animals.

In the PFC, 8h of SD significantly increases 5-HT$_2$AR levels in WT mice but not in $Egr3^{-/-}$ mice (Fig. 2C). In addition, 5-HT$_2$AR levels are significantly greater in WT than $Egr3^{-/-}$ mice in this region both at baseline (replicating our prior radioligand binding assay findings (12)), and following SD.

In the MPC, where endogenous $Htr2a$ expression is lower than in more anterior cortical regions, SD does not increase 5-HT$_2$AR levels in WT or $Egr3^{-/-}$ mice (Fig. 2D). Figures 2E – 2G show representative autoradiographic images from WT and $Egr3^{-/-}$ mice under SDc and SD conditions.

**Fig. 2.**

**Fig. 2.** SD increases 5-HT$_2$AR 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$_2$AR levels in WT mice than
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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-HT₂AR 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-HT₂AR levels; notably, 5-HT₂ARs were lower in Egr3−/− mice than WT under both basal (SDe) and SD conditions (ANOVA: sig. main effect of genotype (F(1,62) = 38.79, p < 0.0001) but not of SD (F(1,62) = 1.371, p = 0.25)). Representative ³H-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.

In all regions, radioligand binding reveals significant differences in 5-HT₂AR levels between WT and Egr3−/− mice after SD and, in all but the most anterior (AFC) region, also at baseline (SDe). In contrast, differences in Htr2a mRNA levels between WT and Egr3−/− mice are seen only in the PFC region following SD. This difference could be due to the longer perdurance of protein than mRNA.

These data reveal the novel finding that 5-HT₂ARs can be upregulated in the PFC in a matter of hours in response to an environmental stimulus, and that this requires Egr3. These results suggest that EGR3, an activity-dependent IEG transcription factor, may directly regulate expression of Htr2a in response to environmental events.

Fig. 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). (D) ChIP-qPCR shows ECS increases binding of EGR3 to Htr2a distal promoter in frontal cortex issue (n = 11). Unpaired student t-tests, * p <0.05. Values represent means ± SEM.

We have previously shown that
EGR3 is expressed in Htr2a-expressing neurons in the mouse FC, an essential criterion for EGR3 to potentially directly regulate Htr2a expression (16). Bioinformatic analyses identified two high probability EGR binding sites in the Htr2a promoter (Fig. 3A).

To determine whether EGR3 protein binds to these binding sites in mouse cortex we conducted chromatin immunoprecipitation (ChIP). We used electroconvulsive seizure (ECS) to induce neuronal activity and maximally express EGR3 in the PFC (Fig.s 3B and 3C). Compared to the positive control region, the promoter of activity-regulated cytoskeleton associated protein (Arc) (a validated EGR3 target gene (19)), binding of EGR3 to the distal Htr2a promoter was significantly increased following ECS, compared to non-stimulated controls (Fig. 3D).

Fig. 4.

**Fig. 4.** EGR3 drives gene expression via binding sites in the Htr2a promoter. (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, (B) Htr2aD distal promoter (t4 = 21.17, p < 0.0001), and (C) Htr2aP proximal promoter (t4 = 8.977, p < 0.001) 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 t-tests, ***p < 0.001, ****p < 0.0001, n = 3. (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 (*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 (19), 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). We found that both regions of the Htr2a promoter containing high-probability EGR3 binding sites (Fig. 3A) drive expression of luciferase in response to EGR3 expression. EGR3 expression induces a 4.9 fold increase in the positive control Arc promoter-driven luciferase (Fig. 4A) and 3.9-fold increase in the Htr2a distal promoter-driven luciferase (Fig. 4B). In addition, although the proximal Htr2a promoter did not show a statistically significant increase in EGR3 binding in the ChIP assay, in vitro expression of EGR3 induced a 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-HT2ARs.

These results reveal a novel mechanism of 5-HT2AR regulation, rapid environmentally-induced expression of Htr2a via the activity dependent IEG transcription factor EGR3. Prior studies have shown that 5-HT2AR levels can be altered in response to ligand binding; for example, 5-HT2AR agonists and antagonists trigger receptor internalization and recycling (20, 21). However, little is known about transcriptional regulation of Htr2a, or that environmental stimuli rapidly alter 5-HT2AR levels. Interestingly, our findings are supported by a recent human study reporting that 24 h sleep deprivation causes a significant increase in brain 5-HT2AR levels detectably by Positron Emission Tomography (PET) scan (22).

5-HT2ARs are abundantly expressed in the neocortex and play important roles in cognition and mood. They also mediate hallucinogenic effects of numerous drugs including LSD, psilocybin, and mescaline (1, 2). Investigation of these drugs has recently undergone a resurgence in the search for treatments for severe psychiatric symptoms including depression (23) and anxiety disorders including PTSD (24-26). 5-HT2ARs are also a key target of SGAs, which treat the symptoms of psychosis (4).

As far back as 1976 numerous post-mortem and in vivo studies have revealed that 5-HT2AR (or 5-HT2R) levels are reduced in schizophrenia patients’ brains (5-10). Such findings may lead to the assumption that brain neurotransmitter receptor levels are a relatively stable characteristic. Our findings suggest, in contrast, that levels of this critical receptor are dynamically, and rapidly, regulated at the transcriptional level in response to neuronal activity and in response to environmental events. This suggests the intriguing possibility that the reduced 5-HT2AR levels reported in schizophrenia patients may be a consequence of disrupted neural activity, an
hypothesis supported by numerous findings of abnormal IEG expression in patients’ brains (Fig. 4E, (27, 28).

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. Notably, 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 (29). Although EGR3 itself is not within a GWAS locus, it interacts in co-regulatory feedback loops with the EGRs and NAB2 (30, 31), and EGR3 expression is reduced in the brains of schizophrenia patients, including in our analyses of data from the NCBI GEO database, showing reduced levels of both EGR3 and HTR2A mRNA in the prefrontal cortex of schizophrenia patients, compared with controls (Fig. 4E and 4F) (27).

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-HT2AR expression in schizophrenia patient brains. 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.

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