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Htr2a expression responds rapidly to environmental stimuli in an Egr3-dependent manner

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Abstract

Pharmacologic and genetic findings have implicated the serotonin 2A receptor (5-HT_{2A}R) in the etiology of schizophrenia. Recent studies have shown reduced 5-HT_{2A}R levels in schizophrenia patients, yet the cause of this difference is unknown. Environmental factors, such as stress, also influence schizophrenia risk, yet little is known about how environment may affect this receptor. To determine if acute stress alters 5-HT_{2A}R expression, we examined the effect of sleep deprivation on cortical Htr2a mRNA in mice. We found that 6 hours of sleep deprivation induces a 2-fold increase in Htr2a mRNA, a more rapid effect than has been previously reported. This effect requires the immediate early gene early growth response 3 (Egr3), as sleep deprivation failed to induce Htr2a expression in Egr3—/— mice. These findings provide a functional link between two schizophrenia candidate genes and an explanation of how environment may influence a genetic predisposition for schizophrenia.

Keywords

Schizophrenia; sleep deprivation; immediate early	gene; early	growth response	3 (Egr3); Htr2a
serotonin 2A receptor (5-HT _{2A} R)			

A.M.M and A.L.G. developed the concept and designed experiments. A.K.M. bred and genotyped the mice used in this study. X.Z., D.I.E., A.K.M. and A.M.M. completed the sleep deprivation and brain dissections. X. Z. and A.M.M. performed quantitative RT-PCR. A.M.M. conducted statistical analysis. A.M.M. and A.L.G. interpreted the results and prepared the manuscript.

Conflict of Interest

The authors report no conflicts of interest.

Supporting Information

The primer sequences for the genes of interest (*Htr2a*, *Egr3*, and *Hprt1*) that were examined in this study and additional experimental procedures. This information is available free of charge via the Internet at http://pubs.acs.org/

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Introduction

Genes and environment interact to influence the risk for complex diseases such as neuropsychiatric disorders. Approximately 50% of the risk for a severe mental illness, schizophrenia, is attributed to genetic causes. Environmental factors, may account for the remaining risk.

Numerous pharmacologic, molecular, and genetic studies suggest that dysfunction in the serotonin 2A receptor may play a role in the pathogenesis and, or treatment, of schizophrenia. $^{2-10}$ While these studies address the importance of the Htr2a gene, that encodes the receptor, little is known about how environment may influence the expression of this gene.

Stress is the most well studied environmental influence on schizophrenia vulnerability. Prenatal stressors, such as exposure to infection or famine, perinatal events, such as obstetrical complications, and stressful life events have all been associated with increased risk for the illness. Little is known about whether stress may alter expression of *Htr2a*. Immediate early genes (IEG) transcription factors are activated in response to multiple types of stress, such as sleep deprivation, and in turn regulate expression of downstream target genes. As such they are intriguing candidates for linking both genetic and environmental influences on risk for mental illness. To test the hypothesis that acute stress may alter the expression of 5-HT_{2A}Rs, we examined the effect of sleep deprivation, a mild form of stress, on cortical *Htr2a* expression in mice. To further test the mechanism underlying this effect we examined the role of the IEG transcription factor, early growth response 3 (*Egr3*), a gene previously associated with risk for schizophrenia 19–21, in the regulation of the *Htr2a* gene.

Results and Discussion

To determine if *Htr2a* expression can be altered by acute environmental changes we used the intervention of sleep deprivation, which has been shown to alter 5-HT_{2A}R levels in humans.²² We measured *Htr2a* messenger ribonucleic acid (mRNA) expression in the cortex of wild type male mice after 6 hours of sleep deprivation using quantitative real time polymerase chain reaction (RT-PCR).

Wild type (WT) male mice were sleep deprived (experimental group) or allowed to sleep in the home cage (control group) for 6 hours prior to sacrifice. Cortex was isolated and processed for RNA isolation and quantitative RT-PCR. Six hours of sleep deprivation produced a greater than 2-fold increase in Htr2a expression compared to undisturbed controls (t(1,15) = 3.322, p < 0.05; Figure 1).

These results indicate that Htr2a expression can be activated by an acute physiologic stress. Next we wanted to determine the mechanism underlying this result. IEG transcription factors are logical candidates to investigate as they translate events in the environment into long-term gene expression in the brain. The IEG transcription factor, early growth response gene 3 (Egr3), was a particularly intriguing candidate as it has been reported to be activated in the cortex of mice in response to the identical sleep deprivation protocol.²³ In addition,

our prior studies had shown that Egr3 deficient (Egr3–/–) mice have an approximately 70% loss of 5-HT_{2A}R biding in the prefrontal cortex compared to WT littermate controls.¹⁸ These findings suggested a potential role for Egr3 in the regulation of the 5-HT_{2A}R.

We therefore chose to test whether Egr3 may be required for regulating the expression of Htr2a in response to sleep deprivation. In prior work, Thompson and colleagues used *in situ* hybridization to demonstrate activation of Egr3 expression in response to sleep deprivation.²³ Therefore, we first wanted to confirm that we were able to detect these changes using quantitative RT-PCR.

We performed quantitative RT-PCR to examine the levels of Egr3 mRNA in the cortex of sleep deprived and control male WT mice. Six hours of sleep deprivation resulted in a greater than 3-fold induction of Egr3 mRNA expression in cortex as compared to controls (t(1,17) = 4.857, p < 0.001; Figure 2). These results confirm the prior $in \ situ$ hybridization findings demonstrating that sleep deprivation induces cortical expression of $Egr3.^{23}$

The role of EGR3 as a transcription factor suggests the possibility that it may directly regulate expression of the Htr2a gene. For this to be the case, certain criteria need to be met. First EGR binding sites should be present in the Htr2a promoter region. We used the MEME suite FIMO tool to search for EGR binding sites in the proximal 3kb upstream of the Htr2a start site. This revealed two high probability EGR consensus binding sequences, one at -2791-2778 bp and the other in the proximal promoter, at -75-62 bp.

An additional criterion is that Htr2a and EGR3 should be expressed in the same cells. Since antibodies against 5-HT_{2A}R fail to produce discrete cellular labeling needed to identify colocalization, we used a transgenic mouse line expressing an Htr2a fluorescent reporter construct Tg(Htr2a-enhanced green fluorescent protein (EGFP))DQ118Gsat/Mmcd (abbreviated "Htr2a-EGFP"). ²⁴ In these mice, EGFP marks the location of Htr2a expression.

Since *Egr3* is an activity-dependent gene, levels of EGR3 are difficult to detect in unstimulated animals. We therefore used sleep deprivation to activate EGR3 expression in *Htr2a*-EGFP mice. Figure 3a shows EGR3 labeling and Figure 3b shows EGFP labeling in cortical sections from *Htr2a*-EGFP mice. Figure3c and 3d show co-localization of *Htr2a*-EGFP and EGR3 in cortical neurons, indicated by yellow immunofluorescence. These results demonstrate that EGR3 is expressed in at least a subset of *Htr2a* expressing neurons, and that the *Htr2a* promoter contains binding sites for EGR3, meeting the minimal criteria for EGR3 to regulate expression of *Htr2a*.

We next examined whether Egr3 was necessary for the induction of Htr2a in response to sleep deprivation. To test this hypothesis, we examined the effect of 6 hours of sleep deprivation, compared with undisturbed sleep, on mice lacking Egr3 (Egr3–/– mice). In contrast to WT mice (Figure 1), sleep deprivation failed to increase Htr2a expression in Egr3–/– mice (Figure 4).

In this study, we have shown that acute stress activates Htr2a expression in the mouse cortex and that this activation requires the immediate early gene Egr3. We found that a

period of 6 hours of sleep deprivation induced a greater than 2-fold increase in cortical Htr2a expression in WT mice compared to undisturbed controls. In Egr3 deficient mice, sleep deprivation had no effect on cortical Htr2a mRNA levels.

Schizophrenia risk is caused by genetic and environmental factors. Numerous lines of evidence suggest dysfunction of *HTR2A* may play a role in schizophrenia. Serotonin 2A agonists, such as lysergic acid diethylamide (LSD), psilocybin, and mescaline, cause psychosis in normal individuals.^{25, 26} In addition, antagonist binding to 5-HT_{2A}Rs is one of the key features of second-generation antipsychotics. In human genetic association studies, *HTR2A* has been one of the most well replicated schizophrenia candidate genes.^{4, 27} Multiple studies have identified deficits in 5-HT_{2A}R binding in schizophrenia patients, both *in vivo* and post-mortem, including in medication-naïve individuals.^{6, 28–30} And epigenetic studies demonstrate down-regulation of *HTR2A* in schizophrenia patients that is associated with early age of disease onset.³¹ These findings provide substantial support for dysfunction in the *HTR2A* gene in schizophrenia. However, little is known about how environment may affect this gene.

Numerous types of environmental stressors have been implicated in schizophrenia risk, such as *in utero* exposure to infection, perinatal trauma and stressful life events. ^{11, 12, 15, 16} Stress is associated with first episode psychosis as well as exacerbation of symptoms. ^{13, 14} Sleep deprivation, in particular, is physiological stress that may influence psychosis. ³² However, few studies have examined how environmental stress influences genes associated with schizophrenia. Determining the underlying mechanism by which stress alters gene expression may provide insight into the etiology of schizophrenia as well as other psychiatric illnesses.

Review of the literature revealed only two studies examining the effects of environmental stress on 5-HT_{2A}Rs in the brain. One study, in mice, reported that a repeated stress of two inescapable foot shock exposures was found to increase cortical *Htr2a* mRNA levels when measured 24 hours after an escape test.³³ The other study, in humans, used positron emission tomography (PET) imaging to reveal an increase in 5-HT_{2A}R binding in the cortex after 24 hours of sleep deprivation.²² Our study adds to this prior literature by using a physiological stress in mice to identify an *acute* induction of cortical *Htr2a* mRNA after only 6 hours of sleep deprivation. These results demonstrate that *Htr2a*, a gene associated with schizophrenia risk, can be rapidly activated in response to an environmental stimulus. Further, these results suggest the possibility that the altered levels of 5-HT_{2A}Rs found in patients with schizophrenia may not be chronic, long-standing characteristics, but could be influenced by the patients' life events.

How might the acute stress of sleep deprivation influence genes associated with schizophrenia, such as Htr2a? IEG transcription factors translate environmental stimuli into long-lasting changes in the brain. Several lines of evidence suggest that the IEG Egr3 would be an ideal candidate for regulating the induction of Htr2a by sleep deprivation. Previous studies have shown that sleep deprivation activates expression of Egr3. In the current study, induction of Egr3 by sleep deprivation was detected using quantitative RT-PCR, thus confirming these prior in situ hybridization findings. We have previously shown that Egr3

deficient mice display a nearly 70% decrease in 5-HT_{2A}R levels in the cortex compared to their WT littermates. ¹⁸ The current results, support our prior findings suggesting that the decrease in 5-HT_{2A}R levels in Egr3 deficient mice may be due to decreased gene expression in these animals.

One of the limitations of conventional knock out animals is that dysfunction of the gene throughout development and postnatal life may result in neuroanatomical abnormalities. However, extensive studies on these animals have revealed no neuroanatomical abnormalities and normal cortical neuron gene expression.^{34, 35}

We have hypothesized that EGR3 is a critical transcription factor in a biological pathway of proteins involved in schizophrenia risk. $^{17, 18}$ Bioinformatics studies have supported this hypothesis, finding that EGR3 may be a critical gene in a putative network of transcription factors and microRNAs implicated in schizophrenia susceptibility. 36 Human genetic studies further support a role for EGR3 in this mental illness, as variations in the EGR3 gene have been associated with schizophrenia in three populations. $^{19-21}$ Post-mortem studies have also shown decreased levels of EGR3 expression in the brains of schizophrenia patients. $^{20, 37}$ Animal studies show that mice lacking Egr3 display schizophrenia-like abnormal behaviors, such as increased locomotion, aggression and deficits in memory and synaptic plasticity. $^{17, 38}$ In the current study, we have used sleep deprivation as a mild form of stress to determine if Htr2a could be acutely activated in response to an environmental stimulus and to determine whether this activation required Egr3. We observed that Egr3 is necessary for the rapid induction of Htr2a after sleep deprivation. This result suggests that Htr2a may be a downstream target of Egr3 in this hypothesized pathway for schizophrenia risk.

Our findings suggest that stress-induced *Htr2a* expression may be a normal adaptive function. This expression may be disrupted in patients with schizophrenia, possibly due to dysfunction of *EGR3*. However, this presents a conundrum; if stress induced *Htr2a* expression is beneficial, then why do antipsychotics block 5-HT_{2A}Rs? This highlights a paradox in the field regarding the role of 5-HT_{2A}Rs in psychosis and its treatment.

The paradox lies in the fact that 5-HT_{2A}R agonists, such as lysergic acid diethylamide (LSD), mescaline, and psilocybin, exert their effects via stimulation of 5-HT_{2A}Rs^{25, 26}, and the high affinity of atypical antipsychotics to 5-HT_{2A}Rs is hypothesized to contribute to their antipsychotic effects.³⁹ This would suggest that a decrease in 5-HT_{2A}Rs would be beneficial. However, the majority of studies in patients with schizophrenia have revealed decreased 5-HT_{2A}Rs and mRNA levels^{5-10, 40}, and epigenetic down-regulation of *HTR2A* activity³¹, suggesting this deficit is pathologic. Although this paradox remains to be deciphered, several observations may be relevant. One, not all 5-HT_{2A}R agonists induce psychosis.²⁶ Two, selective 5-HT_{2A}R antagonists are not effective as stand-alone antipsychotic treatment. Three, all effective antipsychotics block dopamine D2 receptors and the interplay between D2 and 5-HT_{2A}R function may be a critical feature of antipsychotic efficacy. Finally, an increase in receptor density is not the same as an agonist action at the receptor, and vice versa. So, although both the 5-HT_{2A}R and *EGR3* have been associated with schizophrenia risk, their exact functions in the illness remain to be determined.

In summary, we have shown that acute stress activates 5-HT_{2A}R expression in the mouse cortex and that this activation requires the immediate early gene Egr3. These findings imply that Egr3 may directly regulate 5-HT_{2A}R expression after an environmental stimulus. Finally, these results provide a functional link between two schizophrenia candidate genes suggesting a mechanism to explain how both genetic and environmental factors influence risk for schizophrenia.

Methods

Animals

Male adult mice were housed on a 14/10hr light/dark schedule with *ad libitum* access to food and water. Previously generated male Egr3 mice⁴¹ were backcrossed to C57BL/6 mice for more than 20 generations. Egr3 –/– and WT littermate mice were generated from breedings of heterozygous (Egr3 +/–) mice and assigned as "matched pairs" at the time of weaning. Matched pairs were exposed to identical conditions for all studies

The transgenic mouse line Tg(*Htr2a*- EGFP EGFP)DQ118Gsat/Mmcd (subsequently abbreviated "Htr2a-EGFP"), containing a bacterial artificial chromosome (BAC) which expresses EGFP under control of the *Htr2a* promoter was originally obtained from the NIH's Mutant Mouse Regional Resource Center at the University of California, Davis and were obtained as a generous gift from Dr. Rodrigo Andrade.²⁴

Experimental Procedures

Animals were assigned to two treatment groups (n = 8 per group): 6 hours of sleep deprivation and time of day matched controls for the sleep deprivation group. Mice were individually housed 5 days prior to the experimental procedure. Sleep deprivation started at the beginning of the light period (6:00 a.m. - 8:00 p.m.) and mice were kept awake by "gentle handling" including a combination of cage tapping, introduction of foreign objects (e.g., balled paper towels), cage rotation, and stroking of vibrissae and fur with an artist's paintbrush. Time of day matched control animals were left undisturbed in their home cages in the animal colony during the same period as the sleep deprivation procedure. "Gentle handling" procedures have been used extensively to induce sleep deprivation. 42,43

Quantitative real-time polymerase chain reaction (RT- PCR)

Animals were killed immediately after sleep deprivation via isoflurane overdose, brains were immediately removed and the cortex was dissected from the right hemisphere spanning from Bregma: 3.20 mm, Interaural: 7.00 mm to Bregma: 3.80 mm, Interaural: 0.00 mm, using the Coronal C57BL/6J Atlas from the Mouse Brain Atlas. ⁴⁴ Collected tissue was snapfrozen on dry ice and stored at -80° C until quantitative RT-PCR studies were performed. RNA was isolated and reverse transcribed into complementary DNA, which was used for quantitative RT-PCR analysis of *Egr3*, *Htr2a*, and *Hprt1*, a housekeeping gene. Quantitative RT-PCR details and primer sequences are available in Supporting Information.

Immunohistochemistry

Immediately after sleep deprivation, mice were sacrificed by overdose with isoflurane euthanasia and perfused with 4% buffered paraformaldehyde. Brain tissue was sliced at 40 µm thick coronal and immunohistochemistry was performed to detect EGR3 and EGFP. See Supporting Information for details.

Statistical Analysis

Quantitative RT-PCR results of *Egr3* and *Htr2a* mRNA expression in WT and *Egr3* –/– mice were analyzed using a Student's T-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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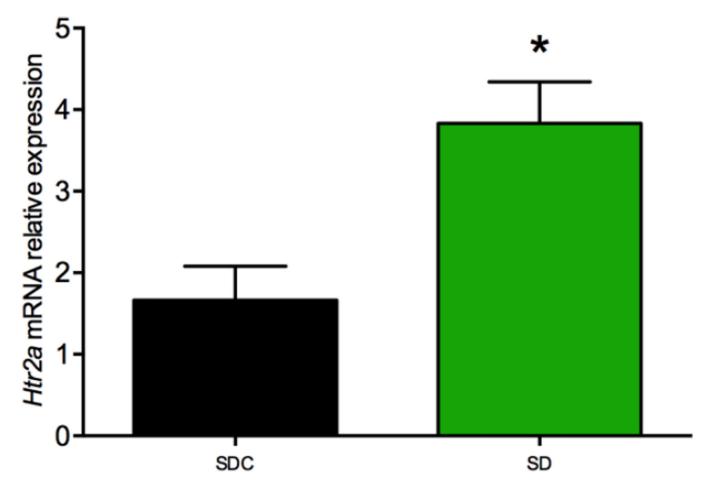


Figure 1. Sleep deprivation increases cortical Htr2a expression Levels of Htr2a mRNA (mean \pm SEM) in the cortex of WT mice, measured by quantitative RT-PCR, are significantly increased following sleep deprivation compared with non-sleep deprived, time of day controls. * p < 0.01, n = 8. (SDC, sleep deprivation controls; SD, sleep deprivation).

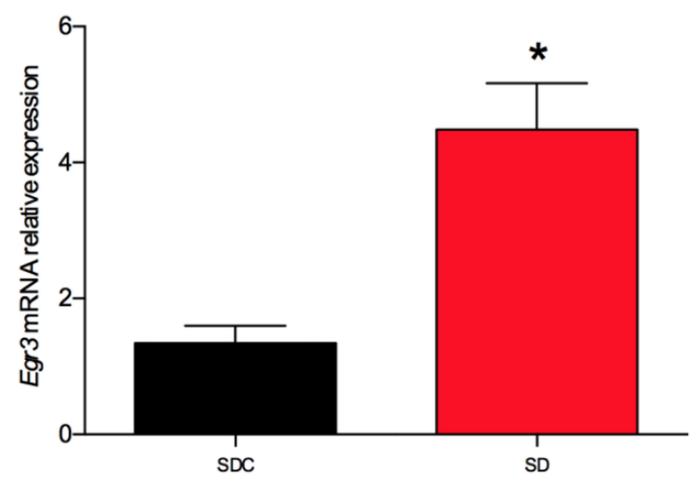


Figure 2. Sleep deprivation increases cortical Egr3 expression Levels of Egr3 mRNA (mean \pm SEM) in the cortex of WT mice, measured by quantitative RT-PCR, are significantly increased following sleep deprivation compared with non-sleep deprived time of day controls. * p < 0.01 as compared to control, n = 8. (SDC, sleep deprivation controls; SD, sleep deprivation).

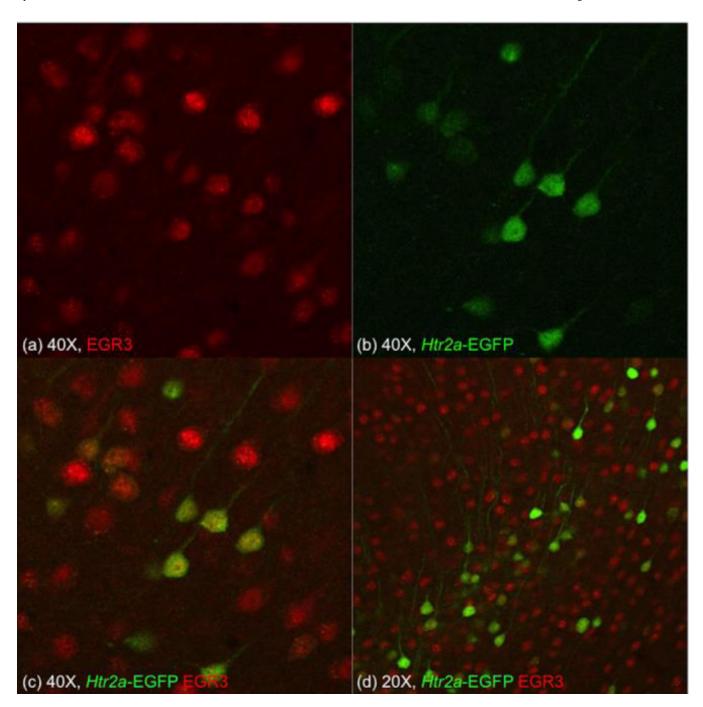


Figure 3. *Htr2a*-EGFP is expressed in EGR3 containing cells Co-localization of EGR3 (red) and *Htr2a*-EGFP (green) in male adult mice after sleep deprivation. (a) Anti-EGR3 Ab. (b) anti-EGFP Ab. (c) Overlay of anti-EGR3 Ab and anti-EGFP positive cells (yellow). d) Representative image at 20×.

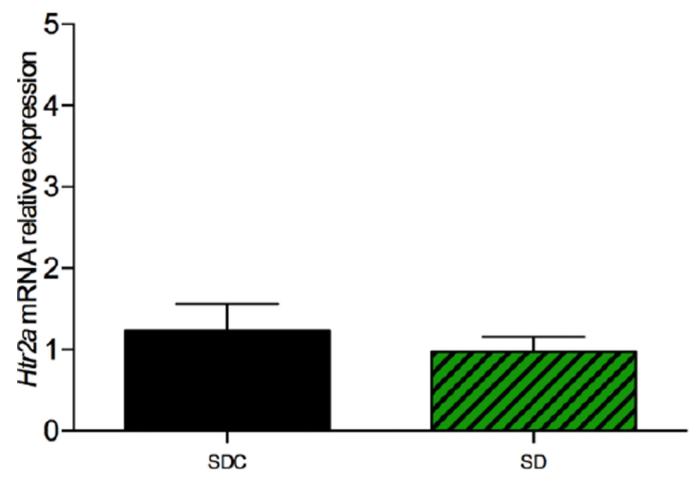


Figure 4. Sleep deprivation fails to induce Htr2a expression in Egr3-/- mice Levels of Htr2a mRNA (mean \pm SEM) are not increased in the cortex of Egr3-/- mice following sleep deprivation, compared with non-sleep deprived control Egr3-/- mice, n =8. (SDC, sleep deprivation controls; SD, sleep deprivation).