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Htr2a Expression Responds Rapidly to Environmental Stimuli in an *Egr3*-Dependent Manner

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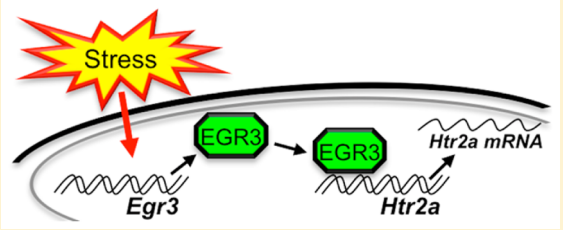
Supporting Information

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 h of sleep deprivation induces a twofold 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*

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.[1] Environmental factors may account for the remaining risk.

The serotonin system has been implicated in the etiology of schizophrenia; however, the mechanisms by which serotonin influences susceptibility to mental illness are poorly understood. The serotonin 2A receptor (5-HT_{2A}R) has been of particular interest, as agonists of this receptor cause psychosis in normal individuals and it is a major target of atypical antipsychotic medications.[2,3] Human genetic studies have identified associations between variations in *Htr2a*, the gene that encodes 5-HT_{2A}R, and risk for severe mental illnesses including schizophrenia.[4] In addition, numerous in vivo and postmortem studies have identified differences in the levels of 5-HT_{2A}Rs and *Htr2a* mRNA in the brains of patients with schizophrenia compared with normal controls.[5−8] 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.[9] 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.[10−14] However, little is known about whether stress may alter expression of *Htr2a*. A recent study in humans reported that 24 h of sleep deprivation significantly increased 5-HT_{2A}R binding in cortex of normal individuals.[15] This suggests the possibility that 5-HT_{2A}R levels are dynamic and may respond to stress.

Immediate early gene (IEG) transcription factors are activated in response to multiple types of stress, and in turn regulate expression of downstream target genes. As such they are intriguing candidates for linking both genetic and environmental influences to risk for mental illness.[16,17] 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 test further 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,[18−20] 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.[15] We measured *Htr2a* messenger ribonucleic acid (mRNA) expression in the cortex of wild type male mice after 6 h of sleep deprivation using quantitative real-time polymerase chain reaction (RT-PCR).

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Wild type (WT) male mice were sleep deprived (experimental group) or allowed to sleep in the home cage (control group) for 6 h 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 using a two-tailed unpaired *t* test (t (1,15) = 3.322, $p < 0.001$; Figure 1).

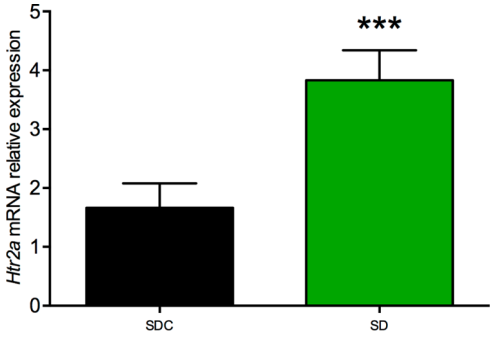

Figure 1. Sleep deprivation increases cortical *Htr2a* expression. Levels of *Htr2a* mRNA (mean ± SEM) in the cortex of WT mice, measured by quantitative RT-PCR, are significantly increased following sleep deprivation compared with nonsleep deprived, time of day controls. ***$p < 0.001$, $n = 8$. (SDC, sleep deprivation controls; SD, sleep deprivation.)

These results indicate that *Htr2a* expression can be activated by an acute physiologic stress. Next we wanted to determine one possible mechanistic factor 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.[21] In addition, our prior studies had shown that *Egr3* deficient (*Egr3*−/−) mice have an approximately 70% loss of 5-HT_{2A}R radioligand binding 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.[21] 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 using a two-tailed unpaired *t* test (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*.[21]

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 4 kb upstream of the *Htr2a* transcription 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.

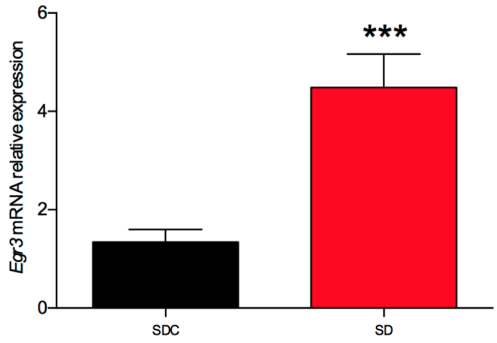

Figure 2. Sleep deprivation increases cortical *Egr3* expression. Levels of *Egr3* mRNA (mean ± SEM) in the cortex of WT mice, measured by quantitative RT-PCR, are significantly increased following sleep deprivation compared with nonsleep deprived time of day controls. ***$p < 0.001$ as compared to control, $n = 8$. (SDC, sleep deprivation controls; SD, sleep deprivation.)

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 the 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").[22] 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

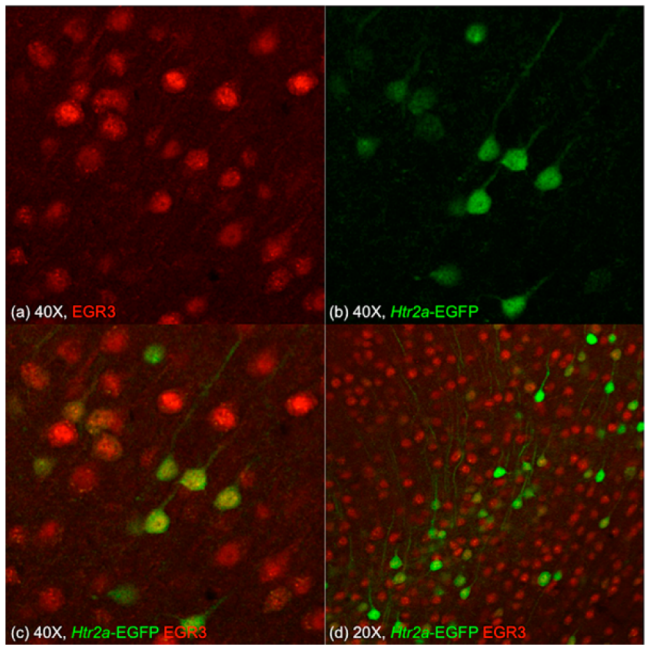

Figure 3. *Htr2a*-EGFP is expressed in EGR3 containing cells. Co-localization of EGR3 (red) and *Htr2a*-EGFP (green) in male adult mice after sleep deprivation. (a) Anti-EGR3 Ab. (b) Anti-EGFP Ab. (c) Overlay of anti-EGR3 Ab and anti-EGFP positive cells (yellow). (d) Representative image at 20×.

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EGFP labeling in cortical sections from *Htr2a*-EGFP mice. Figure 3c and d shows colocalization 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 h 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 using a two-tailed unpaired *t* test ($t(1,12) = 0.7647$, $p = 0.4592$; Figure 4).

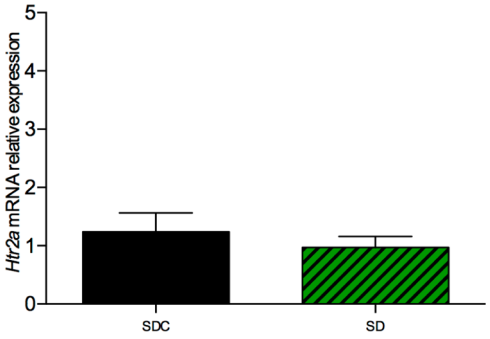

Figure 4. Sleep deprivation fails to induce *Htr2a* expression in *Egr3*−/− mice. Levels of *Htr2a* mRNA (mean ± SEM) are not increased in the cortex of *Egr3*−/− mice following sleep deprivation, compared with non-sleep-deprived control *Egr3*−/− mice, $n = 7$. (SDC, sleep deprivation controls; SD, sleep deprivation.)

In this study, we have shown that acute stress activates *Htr2a* expression in mouse cortex and this activation requires the immediate early gene *Egr3*. We found that a period of 6 h of sleep deprivation induced a greater than twofold 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 associated with genetic and environmental factors. Numerous lines of evidence suggest that 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.[23,24] 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.[4,25] Multiple studies have identified deficits in 5-HT_{2A}R binding in schizophrenia patients, both in vivo and postmortem, including in medication-naïve individuals.[5−8] Moreover, epigenetic studies demonstrate down-regulation of *HTR2A* in schizophrenia patients associated with early age of disease onset.[26] 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.[9,10,13,14] Stress is associated with first episode psychosis, as well as exacerbation of symptoms.[11,12] Sleep deprivation, in particular, is physiological stress that may influence psychosis.[27] 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 h after an escape test.[28] 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 h of sleep deprivation.[15] 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 h of sleep deprivation. These results demonstrate that *Htr2a*, a gene associated with schizophrenia risk, can be rapidly activated in response to an environmental stressor. 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 ongoing life events.

How might the acute stress of sleep deprivation influence genes associated with schizophrenia, such as *Htr2a*? Immediate early gene transcription factors translate environmental stimuli into long-lasting changes in the brain. Several lines of evidence suggest that the IEG *Egr3* is one candidate for regulating the induction of *Htr2a* by sleep deprivation. Previous studies have shown that sleep deprivation activates expression of *Egr3*.[21] In the current study, induction of *Egr3* by sleep deprivation was detected using quantitative RT-PCR, thus confirming these prior in situ hybridization findings.[21] We have previously shown that *Egr3* deficient mice display a nearly 70% decrease in 5-HT_{2A}R radioligand binding in the cortex compared to their WT littermates.[17] The current results, indicating that an environmental activation of *Htr2a* requires *Egr3*, suggests that our prior findings showing decreased 5-HT_{2A}R levels in *Egr3* deficient mice may be due to decreased *Htr2a* gene expression in these animals.

One of the limitations of conventional knock out animals, like the *Egr3* deficient mice used here, is that loss of gene expression 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.[29,30]

We have hypothesized that EGR3 is a critical transcription factor in a biological pathway of proteins involved in schizophrenia risk.[16,17] 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.[31] 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.[18−20] Post-mortem studies have also shown decreased levels of *EGR3* expression in the brains of schizophrenia patients.[19,32] Animal studies show that mice lacking *Egr3* display schizophrenia-like abnormal behaviors, such as increased locomotion, aggression and deficits in memory and synaptic plasticity.[16,33] In the current study, we have used sleep deprivation as a mild form of stress to determine if *Htr2a* is acutely activated in response to an environmental stimulus and to determine whether this

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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 are antipsychotics (that block 5-HT_{2A}Rs) effective in treating schizophrenia patients, who generally show reduced levels of 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,[23,24] and the high affinity of atypical antipsychotics to 5-HT_{2A}Rs is hypothesized to contribute to their antipsychotic effects.[34] 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,[5,35−40] and epigenetic down-regulation of *HTR2A* activity,[26] 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.[24] 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 5-HT_{2A}R and *EGR3* have been associated with schizophrenia risk, their exact functions in this illness remain to be determined.

In summary, we have shown that acute stress activates 5-HT_{2A}R expression in the mouse cortex and 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 14/10 h light/dark schedule with ad libitum access to food and water. Previously generated male *Egr3* mice[41] 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 was obtained as a generous gift from Dr. Rodrigo Andrade.[22]

Experimental Procedures. Animals were assigned to two treatment groups (n = 8 per group): 6 h 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. to 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 snap-frozen 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 the 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 the 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 two-tailed unpaired Student's t test.

ASSOCIATED CONTENT

Supporting Information

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

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Author Contributions

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.

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Notes

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center For Research Resources of the National Institute of Health.

The authors declare no competing financial interest.

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