Regulation of the Serotonin 2a Receptor Encoding Gene *Htr2a* by Early Growth Response Gene 3 (*Egr3*)

by

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ABSTRACT

Schizophrenia is considered a multifactorial disorder with complex genetic variants in response to environmental stimuli. However, the specific genetic contribution to schizophrenia risk is largely unknown. The transcription factor early growth response gene 3 (EGR3) can be activated rapidly after stimuli and thus may translate environmental stimuli into gene changes that influence schizophrenia risk. However, the downstream genes that may be regulated by EGR3 are not clear. While the 5-Hydroxytryptamine receptor 2A (5HT2aR) - encoding gene Htr2a has been implicated in the etiology of schizophrenia, the mechanisms by which Htr2a influences susceptibility to this illness are poorly understood. We previously found that in addition to schizophrenia-like abnormalities, Egr3 -/- mice have approximately 70% deduction of 5HT2aR level in the prefrontal cortex, which underlines their resistant to the sedating effect of clozapine. These findings indicate that the two schizophrenia candidate genes are in the same biological pathway that integrates multiple components resulting in schizophrenia. This dissertation is aimed to identify the mechanisms by which Egr3 regulates the expression of Htr2a in response to environmental stimuli like stress.

To determine if Egr3 alters Htr2a transcription under stress, I examined messenger ribonucleic acid (mRNA) levels of these two genes in wildtype (WT) and Egr3 -/- mice after 6hrs of sleep deprivation (SD). I found both genes are increased in WT mice after SD compared with controls. In addition, Egr3 is required for Htr2a induction because SD fails to induce Htr2a expression in Egr3 -/- mice. Next, I performed chromatin immunoprecipitation (ChIP) to determine if EGR3 binds to Htr2a promoter in vivo. I found a significant increase of EGR3 binding to Htr2a distal promoter 2hrs after seizure. To determine the functionality of this binding, I co-transfected the CMV- EGR3 vector or CMV- vector alone with the Htr2a distal promoter reporter clone. I found overexpression of EGR3 activates the Htr2a distal promoter-driven luciferase gene. Although the ChIP assay shows no direct binding of EGR3 to Htr2a proximal promoter, I found EGR3 overexpression activates Htr2a proximal promoter-driven luciferase gene. These findings suggest that EGR3 regulates Htr2a probably through both direct and indirect ways.
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SIGNIFICANCE

Schizophrenia is a disabling disease that affects all major areas of life. Twin studies show concordance rates of approximately 50% in monozygotic twins, which is higher than dizygotic (17%) twins, and that schizophrenia has a heritability of about 80% (Cardno & Gottesman, 2000). This indicates that schizophrenia is not a completely genetic disease. Other factors, such as environmental stressors, may interact with genes and contribute to this disease. Better understanding of environmental factors may improve our ability to identify the schizophrenia susceptibility genes.

For the past 15 years, my thesis mentor has been investigating the hypothesis that immediate early genes (IEGs) are positioned to account for the dual genetic and environmental influences on risk for schizophrenia and other psychiatric illnesses. These genes are rapidly activated in the brain in response to environmental stimuli, such as stress. The early growth response gene3 (EGR3) is an IEG which is expressed extensively in the central nervous system and plays important roles in synaptic plasticity, neuronal development, learning and memory (Vol, Beckmann, & Wilce, 1997). Thus, dysfunction of EGR3 during development may result in pathology that gives rise to the symptoms of schizophrenia.

Studies have demonstrated that EGR3 is associated with schizophrenia in several populations and expressed at reduced levels in postmortem patients’ brains (Huentelman et al., 2015; S. H. Kim et al., 2010; Tabarés-Seisdedos & Rubenstein, 2009; Yamada et al., 2007b; Zhang et al., 2012). EGR3 was recently identified as the central gene in a network of transcription factors and miroRNAs implicated in schizophrenia susceptibility (Guo, Sun, Jia, & Zhao, 2010). Moreover, Egr3 -/- mice display behavioral abnormalities seen in mouse models of schizophrenia, which are reversed by treatment with either haloperidol or clozapine (Gallitano-Mendel, Wozniak, Pehek, & Milbrandt, 2008). In addition, our Egr3 -/- mice are resistant to the sedating effect of clozapine. This parallels the increased tolerance of schizophrenia patients to antipsychotic effects (Cutler, 2001). The EGR3 transcription factor, encoded by EGR3, contains a zinc finger DNA-
binding domain. Thus, identification of the downstream targets regulated by EGR3 will help us better understanding the regulatory mechanisms that integrate multiple genetic and environmental influences on schizophrenia risk.

Recently, 5HT$_{2A}$ receptor antagonists have received more attention because they improve schizophrenia symptoms with high efficiency, and fewer side effects, compared with typical antipsychotics (Iqbal, Asnis, Wetzler, Kay, & van Praag, 1991). Although numerous studies have shown that the 5HT$_{2A}$R-encoding gene $Htr2a$ plays an important role in schizophrenia, the influence of environment on $Htr2a$ gene expression, and how this might affect schizophrenia risk, remains unknown. We previously found that, in addition to schizophrenia-like behavioral abnormalities (Gallitano-Mendel et al., 2007), $Egr3^{-/-}$ mice have a nearly 70% decrease in prefrontal cortical 5HT$_{2A}$Rs. This underlies their resistance to sedation by clozapine, a phenomenon that parallels the increased tolerance of schizophrenia patients to antipsychotic side effects (Williams et al., 2012). These findings indicate that the two schizophrenia candidate genes function in the same biological pathway that integrates multiple components implicated in schizophrenia. This dissertation is aimed at identifying the potential mechanisms by which the immediate early gene $Egr3$ regulates expression of $Htr2a$. We accomplished this aim by examining the expression levels of genes and proteins and detecting gene regulation mechanisms using $in$ $vivo$ and $in$ $vivo$ methods.

Epidemiology of Schizophrenia

Schizophrenia affects more than 21 million people worldwide according to the current report from world health organization (WHO), and approximately 1.2% of Americans have the disorder (Nemade & Dombeck, 2009). Concerning prevalence, the median lifetime prevalence estimates for persons were 4.0 per 1,000 and for lifetime risk were 7.2 per 1,000 (John, Saha, Chant, & Welham, 2008). Concerning sex differences in the incidence of schizophrenia, evidence from a meta-analysis shows that the male:female rate ratio is 1.4 (Aleman, Kahn, & Selten, 2003). However, two recent high-quality prevalence studies confirmed the lack of sex difference in lifetime prevalence of schizophrenia, which suggests the sex difference identified in the
incidence rates is not reflected in prevalence estimates (Aleman et al., 2003; John et al., 2008). Future research is needed to explain such a paradox. In addition, migrant status is strongly associated with both an increased incidence and an increased prevalence of schizophrenia. Epidemiologic studies examining factors such as social stressors (Selten, Cantor-graae, & Ae, 2005) and lack of nutrition (Aleman et al., 2003) reveal those factors that are related to race/ethnicity play more important role in schizophrenia risk than the migration per se.

The incidence of schizophrenia has been found to be higher in urban areas than in rural regions (Pedersen & Mortensen, 2006). The factors underlying this remain unclear. However, environmental pollutants and stress related to overcrowding have been proposed as contributors (Cantor-Graae, 2007). Interestingly, Northern latitudes are also associated with the prevalence of schizophrenia, but only in males (Saha, Chant, Welham, & McGrath, 2006). It has been shown that low prenatal vitamin D levels influence schizophrenia risk (J. McGrath, 1999). Low levels of vitamin D are prevalent during winter months and at higher latitudes. Thus, people from high latitudes are expected to have a higher incidence of schizophrenia. Frequently, a decrease in social, occupational, and interpersonal functioning precedes the onset of overt symptoms of the disorder. The “prodrome” that leads to full-blown schizophrenia varies by geographic location, with acute onset of the illness has been observed in 70-80% of patients in India and Nigeria, in contrast to only approximately 50% of cases in the United States and Europe (Jablensky et al., 1992).

The outcome of schizophrenia in developed countries is worse than in developing countries, which suggests environment plays a crucial role in schizophrenia risk. It has been reported that cases with full remission after a single episode is 3% and 54% in the USA and India, respectively. In addition, the percentage of cases with continuous psychotic illness is 2% in Nigeria and 33% in Japan (Jablensky et al., 1992). The mechanism underlying this phenomenon has not been identified. One possible explanation is that people in developing counties might be more tolerant of illness, and individuals with the illness may receive greater support by family attitudes and extended family networks (Jablensky et al., 1992). Studies suggest a reduction in the incidence of schizophrenia over time, probably through approaches such as prevention of
obstetric complications, while prevalence estimates have remained stable (John et al., 2008). Still, cost caused by schizophrenia worldwide represents about 1% of the global burden of all the diseases that were estimated in 1990. This number is expected to rise to 1.25% by 2020 according to the global health estimates of WHO (Murray & Lopez, 1996). In the United States, it has been shown that the economic impact of schizophrenia is close to that of diabetes. However, considering the potential gains by reducing morbidity and mortality through treatment, higher indirect costs are needed for schizophrenia than for diabetes (Barbato, 1998). Therefore, schizophrenia is very costly for both families and society.

**Etiology of schizophrenia - genetic and environmental factors**

Over decades, researchers have made a great advance to uncover schizophrenia susceptibility genes. The recent genome-wide association studies on 36,989 cases and 113,075 controls have identified 128 independent associations spanning 108 schizophrenia-associated genetic loci (Ripke et al., 2014), 83 of which are newly determined. More than 80% of these loci are in or near known gene regions, among which includes genes that involved in encoding calcium channels subunits, glutamatergic/dopaminergic-serotoninergic transmission and synaptic plasticity. In additions, these associations converge upon genes that are expressed in certain tissues/cells instead of randomly distributed across genes of all classes and function. Compared to other organs, the risk variants are found more enriched in the brain, particularly in the cortex and the striatum. Moreover, they reported single nucleotide polymorphisms (SNPs) on chromosome 6, where the major histocompatibility complex (MHC) genes are located, and this finding suggest etiological relevance of immune genes and inflammatory pathways with schizophrenia. However, examination on gene located at these 108 loci found only six immune candidates (DPP4, HSPD1, EGR1, CLU, ESAM, NFATC3) which act on both the immune system and the brain (Pouget et al., 2016). This suggests that the immune system involves in a completely different way compared with autoimmune diseases.

As discussed above, genetic factors are clearly involved the etiology of schizophrenia, but the environment is also crucial to develop the illness. It was proposed that environmental risk
factors interact with genetic factors during early life stage in which the formation of the nervous system causing abnormalities, and thus leading to psychosis later in life. The early life environmental risk factors that have been identified include obstetric complications, prenatal and postnatal infection, and others for brain development (Dean & Murray, 2005). Several studies found that vitamin D deficiency during pregnancy (J. J. McGrath, Burne, Féron, MacKay-Sim, & Eyles, 2010), childhood abuse, season of birth (Mortensen et al., 1999), or adolescent cannabis exposure (Malone, Hill, & Rubino, 2010) show strong effects on schizophrenia risk. Moreover, an individual’s experience of prenatal or perinatal adverse events increases their risk for schizophrenia compared with healthy subjects (M. Cannon, Jones, & Murray, 2002). In addition, schizophrenic individuals also display progressive minor physical and cognitive abnormalities (Waddington, Torrey, Crow, & Hirsch, 1991). In addition, early developmental delays and abnormalities in motor activities and speech, and in social and cognitive development, are identified in schizophrenic children in British cohort studies (Jones, Rodgers, Murray, & Marmot, 1994). These findings suggest that schizophrenia patients probably already have subtle abnormalities in brain function very early in life. Environmental factors interact with genetic factors and shape individual risk to mental disorders. It has been found that fetal hypoxia reduces gray matter (GM) and increases cerebrospinal fluid (CSF) volume in schizophrenia patients compared with control subjects (T. Cannon et al., 1993).

Moreover, environmental risk factors acting later in life are also seen as potential etiological factors. Later life risk factors especially drug abuse has been show associated with increased risk of psychosis. Studies from Swedish (Andreasson S,Allebeck P, Engstrom A, 1987) and Dunedin (Arseneault et al., 2002) both reported an elevated relative risk for schizophrenia amongst drug users compared to nonusers. In addition, cannabis users who are 15 years old or later are four times more likely to be diagnosed with schizophrenia (Arseneault et al., 2002). These findings are consistent with another three large-scale longitudinal investigations in Israel (Weiser, Knobler, Noy, & Kaplan, 2002), New Zealand (Fergusson, Horwood, & Swain-Campbell, 2003), and the Netherlands (J van Os et al., 2002) which all demonstrate a link.
between cannabis use and later psychosis. Dopamine sensitization may be the reason that repeated exposure to drugs of abuse can lead to psychosis (Kapur, 2003).

Social adversity and stressful life events are thought to be another environmental factor that increases risk for psychosis. Stressful life events such as limited access to health care, lack of social support, and unemployment are found play a role in schizophrenia susceptibility (Sharpley, Hutchinson, McKenzie, & Murray, 2001). In addition, the role of marital status in schizophrenia risk is also remarkable. Unmarried individuals are four times likely to develop schizophrenia in comparison with their married counterparts (Agerbo, Byrne, Eaton, & Mortensen, 2004). This finding suggests that marriage and other close interpersonal relationships could act as a protective factor for schizophrenia.

Although both genetic and environmental factors are associated with schizophrenia risk, the two risk factors are unlikely to do so in an unrelated manner. Instead, it is proposed that environmental risk factors act on a complex set of schizophrenia candidate genes, and the interplay between genetic and environmental factors probably determine an individual’s risk for schizophrenia. Epigenetic modification is an example of gene-environment interdependence which impact gene expressions. Methylome-wide association analysis (MWAS) finds promoter methylation is significantly associated with altered gene transcription and thus contribute to abnormalities in neurotransmitter systems and neurodevelopment in patients with schizophrenia (Y. Li et al., 2015). In addition, human gene-environment interaction studies have emphasized the role of integrating genetic risks with environmental factors that leads to diseases (Jim Van Os et al., 2014).

**Symptoms of schizophrenia**

Since no biological marker has been identified yet, diagnosis of schizophrenia relies on mental status examination by clinic interview, and observation of behaviors. There are two major current classification systems, ICD-10 and DSM-IV (Barbato, 1998). The two criteria are overlapped in some extent but also remain differences. However, the latest version, DSM-V, is probably better suited for utilization (American Psychiatric Association, 2013) because it attempts
to combine research findings and various diagnostic practices allow understanding the neuropathology of the disease. However, different approach to the diagnosis of schizophrenia requires considering the boundaries between schizophrenia and other psychotic disorders are not clearly defined, so early diagnosis is difficult. No single symptom is specific to schizophrenia, so clusters of symptoms need to be considered. In clinical settings, the most observed symptoms are (Barbato, 1998):

A. Delusions: these are false beliefs that are not based in reality, for example, patients may suffer from false beliefs of being harmed or harassed. Delusions occur in most people with schizophrenia.

B. Hallucinations: sensory perceptions in the absence of external stimuli. Hallucinations can be in any senses, but auditory hallucinations, especially voices, are classic in schizophrenia.

C. Thought disorder: usually disorganized speech, such as loosening of associations, that leads to impaired effective communications.

D. Disturbances in motor behavior: such as assuming inappropriate or bizarre positions, or complete lack of response.

E. Negative symptoms: patients may have reduced, or lack of, ability to function normally, such as disabilities in work, interpersonal relations, or self-care markedly below the level previously accomplished.

**Treatment of schizophrenia**

Base on the binding affinity to dopamine receptors and serotonin 2A receptors, antipsychotics are separated into three categories (Mailman & Murthy, 2010): 1) typical or traditional/conventional antipsychotics that have high dopamine (D<sub>2</sub> receptor) antagonism; 2) atypical antipsychotics that have high 5-HT<sub>2A</sub> antagonism; 3) third generation of antipsychotics that demonstrate partial D<sub>2</sub> agonism and partial 5-HT<sub>2A</sub> antagonism.

There are three major brain dopamine pathways: the mesolimbic and meso-cortical systems, the nigrostriatal and the tuberoinfundibular (hypothalamic) system (Ungerstedt, 1971).
The common action of conventional antipsychotic drugs is they all block dopamine D<sub>2</sub> receptors in mesolimbic and nigrostriatal brain areas (Barbato, 1998). Their effect on psychotic symptoms is mediated thorough their action in the mesolimbic system. However, in the nigrostriatal pathway, they disrupt basal ganglia functioning and result in extra pyramidal symptoms (EPS), such as tardive dyskinesia, a common side effect of conventional antipsychotics. Haloperidol and fluphenazine are the most common conventional antipsychotics that can cause EPS (Note, 2009). In addition, it is evident that these dopamine D<sub>2</sub> receptors antagonists are more efficient in treating positive symptoms compared with negative symptoms (Patel, Cherian, Gohil, & Atkinson, 2014). For instance, haloperidol, one of the widely used typical antipsychotics, is effective in attenuating the positive symptoms that are associated with acute episodes (Kane, 1989). Studies show that 60-65% of D<sub>2</sub> receptors must be occupied to decrease the positive symptoms of the illness. However, over 77% of D<sub>2</sub> blockade rate is associated with EPS (Nyberg, Eriksson, Oxenstierna, Halldin, & Farde, 1999). Based on this, methods for determining the lowest effective dose are recommended in practice.

Atypical (also referred to as “second generation”) antipsychotics have higher binding affinity for 5-HT<sub>2A</sub>Rs than D2 receptors and they act predominantly on mesolimbic neurons with little effect on nigrostriatal neurons (Barbato, 1998). The mechanism of action of these medications implies an effect on psychotic symptoms with a less extrapyramidal side effect. However, they are more likely to have metabolic side effects such as weight gain and diabetes mellitus and thus lead to cardiovascular diseases (Raedler, 2010). Atypical antipsychotics are more effective in treating negative symptoms compared with typical antipsychotics. This effect probably due to the blockade of both 5-HT<sub>2A</sub> and D<sub>2</sub> receptors, resulting in increased level of dopamine in the prefrontal cortex where dopaminergic receptors are found to be hypoactive in schizophrenia patients (Patel et al., 2014).

As the atypical antipsychotic, clozapine has been found effective on positive symptoms and it can improve psychotic symptoms in schizophrenia patients who are resistant to conventional antipsychotics (Buchanan, 1995). However, patients treated with clozapine may develop agranulocytosis which can be fatal if not detected. So, patients must have their white
blood cell count monitored weekly for the first 18 weeks, and then every two weeks as long as they undergo clozapine treatment (Kar, Barreto, & Chandavarkar, 2016).

Aripiprazole, as the third generation of antipsychotic, was approved in 2002 (https://www.accessdata.fda.gov). Unlike the typical and atypical antipsychotics, aripiprazole is a D<sub>2</sub> receptor partial agonist and has partial 5-HT<sub>1A</sub> receptors agonist activity and 5-HT<sub>2A</sub> receptors antagonist activity (Bandelow & Meier, 2003; Lieberman, 2004). So, in the situation of low endogenous dopamine levels, aripiprazole functions as a D<sub>2</sub> receptor agonist. On the other hand, when endogenous dopamine levels are high, aripiprazole may compete with endogenous dopamine and cause partial antagonism offering clinical benefit (Shirley & Perry, 2014). With these properties, aripiprazole can moderate dopaminergic hyperactivity in the mesolimbic system and compensate dopaminergic hypo-activity in the meso-cortical system (Bandelow & Meier, 2003; Bowles & Levin, 2003). In addition, the antagonist activity at 5-HT<sub>2A</sub> receptors may improve negative symptoms and cognition deficits, and the function of partial 5-HT<sub>1A</sub> receptors agonists may mediate anxiolytic and anti-depressant effects (Bandelow & Meier, 2003; Grunder, Kungel, Ebrecht, Gorocs, & Modell, 2006). Thus, aripiprazole could improve all three categories of schizophrenia symptoms: positive, negative and cognitive. However, aripiprazole still has some side effects include neuroleptic malignant syndrome and a movement disorder known as tardive dyskinesia (Shirley & Perry, 2014).

Although different generations of antipsychotics are generally effective at improving psychotic symptoms in schizophrenia, resistance to treatment and side effects are still major challenges of successful treatment of the disorder. A valuable animal model is important for research of complex psychiatric disorders like schizophrenia. Indeed, many of the animal models for schizophrenia have been validated based on their responsiveness to current drugs.

A new potential resource for screening novel drugs is host cell lines, especially patient-specific induced pluripotent stem cells (iPSCs). These are able to self-renew and can be reprogrammed into any cell type, create new possibilities for drug discovery. It has been reported that several iPSCs from schizophrenia patients such as disrupted in schizophrenia 1 (DISC1)-mutant neurons (Wen et al., 2014), dopaminergic neurons (Nguyen et al., 2013), serotonergic
neurons (Licinio & Wong, 2016), have been created. The ability to model different stages of schizophrenia using cultured iPSCs may help to overcome the limitations of animal models and provide the potential for personalized diagnosis and treatment.

**Animal models of schizophrenia**

Change in locomotor activity is widely used to validate rodent models of schizophrenia and provide a functional measurement of antipsychotic response. The proposed locomotor activity measures for schizophrenia are based on the dopamine hypothesis. Studies have found that drugs that cause psychosis when given at high doses to humans produce locomotor hyperactivity when administered to rodents (Ellenbroek & Cools, 1990). Schizophrenia patients also exhibit perseverative, or "stereotyped" behaviors. Thus, many studies using animal models have focused on perseveration or stereotypy induced by psychostimulants. Another behavior measured in schizophrenia animal model is the startle reflex, namely, the pre-pulse inhibition (PPI) of startle paradigm. This is based on the clinic observation that schizophrenia patients are unable to automatically filter or “gate” irrelevant thoughts and sensory stimuli they received and thus lead to cognition disorders (Braff & Geyer, 1990). Numerous studies have observed deficits in the habituation of startle responses of schizophrenia patients (Taiminen et al., 2000). Moreover, social withdrawal is one of the characteristic negative symptoms of schizophrenia. Social isolation animal model has been studied in monkeys and rats. However, considering the cross-species differences, particularly language communication in human social activities, it is difficult to directly compare social behaviors across species.

Schizophrenia patients suffer from cognitive deficits, which are the major contributor to their impaired social and vocational functioning (Green, 1996). Although some of the atypical antipsychotics, and the third generation antipsychotic aripiprazole, may improve cognitive deficits, they lack effectiveness for these symptoms in many schizophrenia patients (Keefe, Silva, Perkins, & Lieberman, 1999). Thus, a valuable schizophrenia animal model for discovery of novel treatments that target cognitive dysfunctions is crucial. However, because of the limited cognitive capacity of laboratory animals it is possible to design behavioral paradigms in rodents that can
consider as analogous with those measured in human. Currently, most animal cognitive tasks are those with working memory such as delayed matching or nonmatching to sample (Kesner, Hunt, Williams, & Long, 1996) and discrete trial delayed alternation (Aultman & Moghaddam, 2001).

Genetic contributions to schizophrenia have been clearly established. The linkage and association studies have identified several schizophrenia candidate genes such as Dysbindin 1 (DTNBP1) (Owen, Williams, & O'Donovan, 2004), neuregulin1 (NRG1) (Pitcher et al., 2011), and Disrupted-in-Schizophrenia-1 (DISC1) (Hennah, Thomson, Peltonen, & Porteous, 2006), early growth response 3 (EGR3) (Huentelman et al., 2015), 5-Hydroxytryptamine 2A receptor gene HTR2A (Sujitha et al., 2014). Conventional mutant mice of some schizophrenia gene are generated (Liu et al., 2005; Tourtellotte & Milbrandt, 1998). However, dysfunction of those genes throughout development and postnatal life may contribute to some of the behavior and physiology abnormalities. In addition, some conventional mutant mice such as Egr3 /- mice developmental abnormalities preclude the use of some behavior tests. For example, Egr3 /- mice have scoliosis and lack muscle spindles, resulting in motor abnormalities that interfere with their performance in tests requiring certain motor skills (Tourtellotte & Milbrandt, 1998).

Excitingly, conditional and inducible mutant mice of schizophrenia model have been generated, e.g. conditional mutant Disc1 mouse model using the Tet-off system (Pletnikov et al., 2008). The Tet-off system is controlled by the CAMKII promoter with inducible expression of mutant Disc1. This inducible system can limit the Disc1 mutation to forebrain regions. In addition, mutant Disc1 transgenic mice exhibit spontaneous hyperactivity in the open field and altered social interaction, and transgenic female mice show deficient spatial memory (Pletnikov et al., 2008). Moreover, conditional mutate Egr3 mice express Cre-recombinase regulated by the endogenous Egr3 gene locus was also generated. This method enables selective inactivation of Egr3 at any time during development in neurons and Schwann cells (Oliveira Fernandes & Tourtellotte, 2015; Quach, Oliveira-Fernandes, Gruner, & Tourtellotte, 2013).
Serotonin system and schizophrenia

Serotonin, or 5-hydroxytryptamine (5-HT), has been implicated in many physiologic or behavioral functions. Moreover, most antipsychotics are currently used for schizophrenia are thought to act partially through serotoninergic mechanism. One of the reasons that 5-HT is involved in so many process could be the molecular diversity and different distribution of the 5-HT receptor subtypes that are expressed in many tissues. In addition, 5-HT cell bodies clustered in the brainstem raphe nuclei but have vast projections to influence all regions in the brain. The radioligand-binding assays was developed in 1970s and deepen our understanding the subtypes of receptors for serotonin (Frazer & Hensler, 1999). Initially, serotonin binding sites that high affinity to the radioligands \([^3]H\) 5-HT are designated 5-HT\(_1\) receptors, while the ones prefer \([^3]H\) spiperone are termed 5-HT\(_2\) receptors. Using molecular cloning techniques and in situ hybridization and immunohistochemistry, many of the 5-HT receptors are identified and the organization, distribution of those receptors has been demonstrated in cellular and subcellular levels.

Generally, there are two categories of serotonin receptors extensively distributing in central and peripheral nervous systems (D Hoyer et al., 1994): 1) ligand-gated ion channel receptors (LGICs): the 5-HT\(_3\)Rs. This receptor super-family consists of five subunits, each of which possesses four transmembrane segments and a large, extracellular N-terminal region; 2) G protein-coupled receptors (GPCRs): all other subtypes of serotonin receptors, except 5-HT\(_3\)Rs, belong to this family. Members of this receptor family all have the highly conserved seven transmembrane domains, an intracellular carboxy-terminus and an extracellular amino-terminus (Frazer & Hensler, 1999).

Sustained agonist exposure could induce internalization, desensitization and down-regulation of receptors. Agonist binding to cell surface receptors leads to activation of the G-protein-coupled receptor (GPCRs), then the enzyme GPCR kinase (GRK) phosphorylates the receptor. Once phosphorylated, GPCRs are desensitized and internalized into endosomes through \(\beta\)-arrestin binding. Once internalized, GPCRs may be recycled back to the cell surface or be trafficked to lysosomes and degraded (down-regulation) (Smith, Sim-Selley, & Selley, 2010).
In addition to GRK promoted phosphorylation event, second messenger-dependent kinases and protein kinases C and A are all indicated involving the desensitization of 5-HT$_{1A}$ receptor (Raymond, Mukhin, Gettys, & Garnovskaya, 1999). Protein kinase C also mediates 5-HT$_{2A}$ receptor desensitization (Roth et al., 1995). Interestingly, 5-HT$_{2A}$ receptor antagonists such as clozapine also can induce receptor to internalize in cultured cortical pyramidal cells and cause the receptor redistribution from dendrites to cell bodies (Willins et al., 1999). However, further studies are needed to determine whether this novel antagonist property is related to therapeutic action in schizophrenia.

By interacting with G proteins, those receptors are able to modulate the activity of different effector systems to produce an excitatory or inhibitory response. Although serotonin can activate those receptors, the activation of different receptors can cause distinct signaling pathways that influence various biological and neurological processes such as aggression, anxiety, appetite, cognition, learning, memory. The serotonin receptors are also the target of a variety of pharmaceutical drugs, including many antidepressants, antipsychotics, hallucinogens. Thus, further understanding of those receptors will create more opportunities for drug discovery.

**Early growth response genes**

The early growth response genes (EGRs) are immediate early genes (IEGs). Members of this family include Egr-1 (also known as NGFI-A, krox-24, krox-24, zif268 and TIS8), Egr2 (alias krox-20), Egr-3 (alias pilot), Egr-4 (alias NGFI-C and pAT133). These genes encode neuronal activity-inducible transcription factors. They share a highly homologous (90% identical or conserved residues) zinc finger DNA-binding domain which recognizes an identical DNA response element (Beckmann & Wilce, 1997). The EGR zinc fingers belong to Cys$_2$His$_2$ class. This type of zinc fingers adopts a ββα fold and contains the amino acid sequence motif ‘X$_2$-Cys-X$_{2,4}$-Cys-X$_{12}$-His-X$_{3,4,5}$-His’ where X represents amino acids that are less well conserved. EGR proteins typically contain tandem repeats with more than two of zinc fingers comprising the DNA-binding domain and each tandem domain binding in the major groove of DNA with a 3-bp intervals (Pabo, Peisach, & Grant, 2001). All EGRS recognize and bind to the consensus motif GCG$^{3/4}$GGGCG, which is commonly referred to as the GSG motif (Christy & Nathans, 1989).
Because of the high conserved binding motif among those proteins, it is more likely that EGRs bind to DNA in a similar manner.

As IEGs, EGRs are rapidly and transiently activated in response to a wide range of stimuli. Thus, the activation of EGRs can be the biomarker to map the activation of neuronal pathways (Bahrami & Drablos, 2016). In addition, EGRs encode zinc finger transcription factors, so the induction of EGRs may also activate downstream genetic cascade and determine the program of genetic process leading to the long-term effects of the neuronal stimulation.

The basal expression level of EGRs is generally low. in situ hybridization studies have demonstrated basal expression of Egr1 is detected in the mouse cortex where the highest level is in layer IV and VI, and in the hippocampus, with the highest in the CA1, lower in CA2-4 and the dentate gyrus (Schlingensiepen, Lüno, & Brysch, 1991). In addition, it has been reported that the level of mRNA in rat hippocampus for EGR1 is increased and reaches peak levels at 0.5 – 1hr after the maximal electroconvulsive seizure (MECS) (K J O'Donovan, Wilkens, & Baraban, 1998). EGR1 protein levels closely follow its mRNA expression, with highest levels at 0.5 - 1hr and returned to basal levels by 4hrs after seizure. These findings suggest that the expression pattern of Egr1 mRNA is correspondently with its protein expression. This probably indicates that the basal expression of Egr1 is regulated at transcriptional level. Moreover, the spatial expression pattern of Egr1 mRNA and protein is also observed in the physiological excitation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors using natural sensory input. In addition, the induction of Egr1 mRNA and protein expression can be abolished by NMDA receptor antagonist tetrodotoxin (Worley et al., 1991). These findings suggest that Egr1 not only activated by artificial stimuli but also play an important role in mediating normal cortical physiology (Worley et al., 1991). In addition to NMDARs, kainate/alpha-amino-3-hydroxy-5- methyl-4-oxazolidoneprionic acid (AMPA) receptor also induce Egr1 expression in the sensorimotor cortex and dorsal striatum, and the AMPARs antagonist DNQX can block the Egr1 activation (Wang, Daunais, & Mcginty, 1994).

In situ hybridization has detected Egr2 mRNA in multiple layers of cortex and white matter tracts of the corpus callosum. It is report that EGR2 protein is extensively expressed in
neurons in central nervous system (CNS) and in glial cells in peripheral nervous system (PNS). In addition, Egr2 is necessary for the myelination of Schwann cells in PNS and the disruption of this gene leads to defects in but not affect CNS (Topilko et al., 1994). Egr3 has been found in the cerebral cortex, hippocampus, dentate gyrus, caudate putamen and amygdala (Yamagata, Kaufmann, Lanahan, Barnes, & Andreasson, 1994). Egr4 expression is highest in cortex and rarely detectable in the cerebellum (Crosby et al., 1992). In cerebral cortex, both Egr3, and Egr4 are highly expressed in layer II (Crosby et al., 1992; Yamagata et al., 1994), and IV except Egr2 mostly in layer II and III (Herdegen et al., 1993).

**Regulation of EGR proteins**

EGR proteins in the central nervous system can be induced by excitatory amino acids, mostly the glutamate. It has been reported that glutamate, mainly through the NMDA receptors and with partial involvement of AMPA receptors, induces Egr1 mRNA expression in primary cortical and striatal cultured neurons (Vaccarino, Hayward, Nestler, Duman, & Tallman, 1992). In addition, Kainic acid can induce EGR1 protein and mRNA expression in cortical and hippocampal neurons both *in vivo* (Gass, Herdegen, Bravo, & Kiessling, 1993) and *in vitro* (T. M. Murphy, Worley, & Baraban, 1991), probably via L-type voltage-sensitive calcium channel current. Kainic acid induced seizure also activates EGR2 (Gass, Herdegen, Bravo, & Kiessling, 1994) and EGR3 (L. Li, Carter, Gao, Whitehead, & Tourtellotte, 2005a) protein expression in hippocampus. In contrast, the inhibitory neurotransmitter γ-aminobutyric acid (GABA) also affect EGR1 protein expression in hippocampus. Blocking GABA$_A$ receptors by pentylenetetrazole induces Egr1 mRNA levels in hippocampus and neocortex but not in the midbrain, cerebellum, or brain stem (Saffen et al., 1988). Egr4 mRNA is also induced by pentylenetetrazole, mostly in the dentate gyrus, but last longer than Egr1 (Crosby et al., 1992).

Each of the genes that encode EGR1-4 proteins contain a single exon, which means the zinc fingers are all encoded by the same exon. Previous studies suggested that the transient signaling after IEG activation was regulated by combination of several mechanisms for rapid degradation and inactivation (Bahrami & Drablos, 2016). e.g. Some of the IEG transcripts
were degraded by microRNAs targeting 3'UTR regions of those transcripts (Arner et al., 2015). The IEG proteins particular FOS transcription factor families are usually unstable and are degraded by the proteasome without prior ubiquitination (Gomard et al., 2008).

All the EGR proteins contain an activation and repression domain outside of the zinc finger region, except EGR4 which does not contain a region of homology to the EGR1 R1 repression domain (Crosby et al., 1992). In addition, the EGR protein repressor NGFI-A binding protein 1 (NAB1) repress both EGR1 and EGR2 activity by binding to R1 region, but do not inhibit EGR3 (Russo, Sevetson, & Milbrandt, 1995). This difference is probably due to the four amino acids in the Egr-3 protein sequence which diverge from those in EGR1 and EGR2. A second repressor protein, NGFI-A binding protein 2 (NAB2), which also binds the R1 domain of EGR1 and EGR2 has been identified (Svaren et al., 1998). Interestingly, nerve growth factor (NGF) induces NAB2 but not NAB1 in PC12 cells with slightly delayed kinetics compared with EGR1, which indicate the cooperation of the two repressors in regulating EGR proteins. Moreover, it has been found that NAB1 does not interfere with DNA regulated by EGR1, but rather forms a complex with EGR1 that binds to Egr1 binding sites (A. H. Swirnoff et al., 1998). Overall, these findings provide a mechanism for the differential regulation of immediate-early transcription factors.

All the zinc fingers are required and sufficient for nuclear translocation of the EGR1 transcription factor (Matheny, Day, & Milbrandt, 1994). Mutate the cysteine residues which coordinate the zinc ion cause cytoplasmic EGR1 staining, which indicates the secondary structure of the zinc fingers may also involves in the nuclear targeting process. In addition, The zinc fingers are also necessary and sufficient for EGR2 and EGR4 nuclear translocation (Vesque & Charnay, 1992). There is no evidence about the nuclear translocation of EGR3 to date.

Considering the high homology of the zinc fingers between EGR3 and other EGRs, the nuclear localization domain of EGR3 would be expected to be similar to that of the other EGRs.

Posttranslational regulation has been observed for EGR1. It has been reported that EGR1 proteins are phosphorylated on a serine residue (Lemaire et al., 1990), and this is induced by serum treatment in BALB/c3T3 cells (X Cao et al., 1990). The phosphorylation of EGRs could
enhance their DNA-binding activity (Xinmin Cao, Mahendran, Guy, & Tan, 1993). However, most of the studies are for EGR1. Further investigation is needed for other EGRs.

**EGR proteins are induced by physiological stimulation and seizures**

EGRs can be induced by wide range of stimuli. EGR1 protein are activated by tactile stimulation of whiskers predominantly in layer IV rat somatosensory cortex (Mack & Mack, 1991). EGR1 protein levels are increased in the suprachiasmatic nucleus of Syrian hamsters following exposure to a 1hr light pulse after being adapted to the dark (Holford, 2012). Restraint stress for only 10 minutes has been shown induced *Egr1* mRNA expression in cerebral cortex and this induction can be blocked by administration of β-adrenergic receptor antagonist, propranolol (Bing, Filer, Miller, & Stone, 1991). However, water maze training is not able to affect the expression of *Egr1* mRNA in rat brain (Wisden et al., 1990a).

Electroconvulsive seizures (ECS) can induce *Egr1* mRNA expression in hippocampus and neocortex independently of the NMDA receptor but requires sodium channel activation (Minatohara, Akiyoshi, & Okuno, 2016). ECS also induces EGR3 protein but this expression is delayed compared to that of EGR1. For example, EGR1 protein starts to increase 1hr and highest level is 2hrs after seizure while EGR3 reach peak level at 4hrs but last longer (Kevin J. O'Donovan, Tourtellotte, Milbrandt, & Baraban, 1999).

**Developmental expression of EGR proteins**

*Egr1* expression is rarely detectable in the embryonic nervous system (McMahon, Champion, McMahon, & Sukhatme, 1990) but increases throughout postnatal development to adult levels. In the neocortex, *Egr1* starts to increase in the frontal and occipital cortex at PND 14. Hippocampal *Egr1* expression is low at birth but increases in all subfields of the hippocampus by PND 14. In addition, *Egr1* expression parallels the time of synaptic formation in the developing rat brain (Herms, Zurmohle, Schlingensiepen, Brysch, & Schlingensiepen, 1994). *Egr2* is expressed in the developing mouse hindbrain at embryonic day 9.5. According to the progress of the development, *Egr2* is also seen in early neural crest cells, neural-crest derived boundary caps (P.
Murphy, Davidson, & Hill, 1989). In addition, both of the transcription factors, EGR2 and Hox-2.9, are thought to regulating hindbrain segmentation and development. Egr3 expression is detected in the caudate putamen, the hippocampus on PND 1 (Yamagata et al., 1994). Yamagata et al. also reported Egr3 expression increases in the neocortex and dentate gyrus on PND 5 and reaches adult levels by PND 12. Egr3 has not yet been reported in the embryo. The expression of Egr4 in brain is similar to Egr1 and only detected postnatally. Egr4 is first detectable in the cerebral cortex, midbrain and cerebellum on PND 7. Then increases to adult levels by day 17 (Crosby et al., 1992).

**EGR3 and schizophrenia**

EGR3 is a transcription factor and the EGR3 gene belongs to immediate early genes (IEGs) that are expressed in response to neuronal activity (Beckmann & Wilce, 1997). As an effector molecule of mitogen activated protein kinase (MAPK) signaling pathways (Gregg & Fraizer, 2011; To, Knower, & Clyne, 2013), EGR2 regulate target gene expressions required for long-term structural or physiologic synaptic changes associated with learning and memory, functions that are impaired in schizophrenia patients. It has been shown that Egr1 and Egr3 directly regulate the plasticity-associated activity-regulated cytoskeletal-related gene (Arc, also known as Arg3.1), a synaptic activity-induced effector molecule that is also required for L-LTP (L. Li, Carter, Gao, Whitehead, & Tourtellotte, 2005b). Similarly, another plasticity-associated gene encoding the GABA receptor subunit 4 gene, GABRA4 has been shown to be regulated by Egr3 in response to seizure in mice (D. Roberts et al., 2005). Both ARC and GABA have been shown are associate with schizophrenia. Studies showing that reduced GABA transmission in prefrontal cortex of postmortem schizophrenia patients’ brains (Tse, Piantadosi, & Floresco, 2015). An ARC SNP is also associated with schizophrenia risk (Huentelman et al., 2015).

While Egr3 −/− mice appear to have normally developed brains, they have profound impairments in context and cue-associated learning and memory, and they have profoundly impaired short-term and long-term object recognition memory (L. Li et al., 2007). Our previous study also found Egr3−/− mice display short-term memory impairment, and abnormal responses to
novelty and handling stress (Gallitano-Mendel et al., 2007). Therefore, we believe Egr3 has an essential role in learning and memory presumably by regulating genes required for memory acquisition, consolidation and/or retrieval.

In addition to affecting synaptic plasticity and memory, Egr3 plays a role in promoting the immune response to pathogens (S. Li et al., 2012). Studies on patients with schizophrenia (Drexlage et al., 2010; Padmos et al., 2008) revealed increased EGR3 protein expression, which is also correlated to the increased gene expression of important pro-inflammatory cytokines such as IL-1B, TNF and IL-6 in monocytes, suggesting the potential role of transcription factor EGR3 in the inflammatory state of schizophrenia patient monocytes (Weigelt et al., 2011). Similarly, studies on salivary samples of patients with first-episode psychosis demonstrated that reduced BDNF mRNA levels are associated with stress and inflammation (Mondelli et al., 2011). These findings suggest altered BDNF and EGR3 gene expressions contribute to psychosis are partly induced by stress through a biological pathway that may involve increased inflammation.

In particular, EGR3, which resides at the chromosomal location 8p21.3, was suggested to be a potential susceptibility gene in schizophrenia because chromosome 8p is considered as a potential hub for developmental neuropsychiatric disorders (Tabarés-Seisdedos & Rubenstein, 2009; Yamada et al., 2007b). Studies on different populations have reported that genetic variation in EGR3 plays an important role in schizophrenia susceptibility (S. H. Kim et al., 2010; Zhang et al., 2012). In addition, decreased prefrontal hemodynamic response during a verbal fluency task is associated with an EGR3 polymorphism in schizophrenia patients, indicating genetic variation in EGR3 may affect prefrontal cortex function (Nishimura et al., 2014). Animal studies also support a role for Egr3 in schizophrenia pathogenesis. We have previously reported that Egr3-deficient (Egr3−/−) mice display locomotor hyperactivity, a phenotype associated with schizophrenia (Gainetdinov, Mohn, & Caron, 2001), which is reversed by treatment with either haloperidol or clozapine (Gallitano-Mendel et al., 2008). Moreover, EGR3 was recently identified as the central gene in a network of transcription factors and miroRNAs implicated in schizophrenia susceptibility (Guo et al., 2010).
In summary, EGR3 plays an important role in neuron development, synaptic plasticity, learning, memory, and in promoting the immune response to pathogens. However, dysfunction of EGR3 could affect its downstream target genes and thus may impair signaling transductions which are crucial for those functions. Thus, as an IEG, dysfunction in EGR3 can account for both genetic and environmental risk factors for schizophrenia.

Aim of the research

The goal of this project is to determine the potential mechanism by which Egr3 regulates Htr2a in response to stimulation. Identification of the mechanism could provide information for the proposed regulatory network which integrates genetic and environmental factors influences on schizophrenia and could provide future therapeutic targets to diagnose and treat schizophrenia. To determine whether Egr3 alters Htr2a transcription under stress, we will examine mRNA level of Egr3 and Htr2a in WT and Egr3-/- mice after 6hrs of sleep deprivation and compared with non-disturbed controls. To determine whether EGR3 regulates Htr2a by directly binding to Htr2a promoter, we will perform chromatin immunoprecipitation using cortical tissues from mice 2hrs after seizure. To further identify the functionality of EGR3 binding to Htr2a promoter, we will measure luciferase signals after co-transfecting an Htr2a promoter -drive luciferase vector with CMV-EGR3 vector or CMV-vector alone in cultured neuro2a cells.
CHAPTER 2
EARLY GROWTH RESPONSE 3 (EGR3) IS ESSENTIAL FOR THE SEROTONIN 2A RECEPTOR ENCODING GENE (HTR2A) EXPRESSION IN RESPONSE TO STRESS IN MOUSE CORTEX

Abstract

Studies have shown that the 5-Hydroxytryptamine receptor 2A (5HT$_{2A}$Rs) encoding gene - _Htr2a_ is associated with schizophrenia and expressed at reduced levels in postmortem patients’ brains. However, how environmental factors like stress affect _Htr2a_ expression and influence schizophrenia risk is not clear. Immediate early genes (IEGs) are the first to be expressed in response to environment stimuli. The IEG transcription factor early growth response 3 (_Egr3_) has been associate with schizophrenia. We previously reported that _Egr3_-/- mice have a 70% decrease of 5HT$_{2A}$Rs in prefrontal cortex which underlies their resistance to sedation by clozapine. These findings suggest a potential link between the two schizophrenia candidate genes, _Egr3_ and _Htr2a_. Particularly, it suggests that _Egr3_, a transcription factor, may regulate expression of _Htr2a_.

To determine if environmental stimuli, such as stress could alter the expression levels of these two schizophrenia candidate genes, we examined messenger ribonucleic acid (mRNA) levels of _Egr3_ and _Htr2a_ in the cortex of sleep deprived wildtype (WT) mice and found a increase of both _Egr3_ and _Htr2a_ after 6hrs of sleep deprivation (SD) compared with controls. Next, to determine if _Egr3_ is required for _Htr2a_ expression under stress, we measured _Htr2a_ mRNA level following SD in _Egr3_-/- mice. SD fails to induce _Htr2a_ expression in the cortex of the _Egr3_-/- mice, suggesting the increase of _Htr2a_ is dependent on _Egr3_. _Htr2a_ has been shown display an anteroposterior gradient pattern in cortex. To further identify the specific cortical regions that underline these changes, we examined _Egr3_ and _Htr2a_ mRNA levels in anterior cortex, prefrontal cortex (PFC) and posterior cortex of the same animals used for initial quantification in whole cortex. We found that _Htr2a_ is only significantly induced in PFC in an _Egr3_ dependent manner. In addition, _Egr3_ is activated by SD in PFC and posterior cortex but not in anterior cortex. Our findings indicate that _Egr3_ alters _Htr2a_ transcription in PFC under SD. Moreover, _Egr3_ and _Htr2a_
has different distribution in the cortex, with *Egr3* is activated to a great degree in the posterior cortex and *Htr2a* display an anteroposterior gradient pattern.

**Introduction**

Schizophrenia patients have cognitive deficits, and disturbances in perception and behavior, that impair their ability to work, interact with family, and fulfill other major life functions. Antipsychotics can reduce the symptoms and allow the patient to function better, but these treatments do not cure the illness. A better understanding of the molecular mechanisms of schizophrenia will not only increase our knowledge but may also lead to better treatment to patients suffering from this mental disorder.

Both genetic and environmental factors play significant roles in the etiology of schizophrenia (Moran, Stokes, Marr, Bock, & Desbonnet, 2016). The serotonin 2A receptor -encoding gene *Htr2a* is one of the most studied schizophrenia candidate genes. Several lines of evidence suggest a potential role for dysfunction of this receptor in psychotic illness. 1) The serotonin 2A receptor agonists such as lysergic acid diethylamide (LSD), can cause hallucinations which is one of the characteristic symptoms of schizophrenia; 2) Almost all atypical antipsychotic drugs currently used in therapy, have high affinities for 5-HT$_{2A}$ receptors. 3) The levels of *Htr2a* mRNA and 5-HT$_{2A}$ receptors are found decreased in the frontal cortex of postmortem brains of schizophrenic patients (Chalovich & Eisenberg, 2005; Hernandez & Sokolov, 2000); 3 4) *Htr2a* polymorphisms (e.g. rs6311 and rs6313) are also associated with schizophrenia (Sujitha et al., 2014).

In addition, environmental factors such as prenatal/postnatal infection, head injury, drug abuse, and social adversity have been reported to influence schizophrenia susceptibility (Dean &

* The *Egr3* and *Htr2a* expression of the right whole right cortex in this chapter have been published (Maple et al., 2015).
Murray, 2005). It has been found that individual's prenatal exposure to infection or famine, and perinatal adverse events, increases the risk to develop schizophrenia (M. Cannon et al., 2002). Social adversity and stressful life events are environmental factor that increase risk of psychosis. Factors such as limited access to health care, lack of social support, and unemployment, are associated with greater rates of schizophrenia (Sharpley et al., 2001). However, little is known about how environment stimuli affect Htr2a gene expression and thus might influence schizophrenia risk.

Immediate early genes (IEGs) are activated rapidly in response to a wide variety of environmental stimuli such as stress (Senba & Ueyama, 1997). Early growth response gene 3 (EGR3), a member of the EGR family of IEGs, resides at the human chromosomal locus 8p21.3. Chromosome region 8p is considered as a potential hub for developmental neuropsychiatric disorders (Tabarés-Seisdedos & Rubenstein, 2009; Yamada et al., 2007b). Studies in several ethnic populations have reported that genetic variation in EGR3 plays an important role in schizophrenia susceptibility (Huentelman et al., 2015; S. H. Kim et al., 2010; Yamada et al., 2007a; Zhang et al., 2012). In addition, decreased prefrontal hemodynamic response during a verbal fluency task is associated with an EGR3 polymorphism in schizophrenia patients, indicating genetic variation in EGR3 may affect prefrontal cortex function (Nishimura et al., 2014). Moreover, EGR3 was recently identified as the central gene in a network of transcription factors and micro ribonucleic acids (miroRNAs) implicated in schizophrenia susceptibility (Guo et al., 2010). In addition, the zinc finger transcription factor EGR3, that is encoded by the Egr3, mediates the transcription of various genes underlying neuronal plasticity and development (Gallitano-Mendel et al., 2007; L. Li et al., 2005a). Thus, the activation of Egr3 not only reflects local neuronal activity in response to various stimuli, but also induces an internal cascade of genetic expression that controls neuronal modifications. However, little is known about the genes that are regulated by EGR3 and that contribute to schizophrenia.

Our previous studies using Egr3 -/- mice revealed abnormalities in behavior and synaptic plasticity that resemble those associated with schizophrenia (Gallitano-Mendel et al., 2007). In addition, Egr3 -/- mice have a nearly 70% decrease of prefrontal cortical 5HT2ARs, which
underlies their resistance to sedation by clozapine, a phenomenon that parallels the increased tolerance of schizophrenia patients to antipsychotic side effect (Williams et al., 2012). These findings suggest that the 5HT$_{2A}$R-encoding gene $Htr2a$ may be regulated downstream of $Egr3$.

Since $Egr3$ is activated by environmental stimuli, then, if $Egr3$ regulates $Htr2a$, we should see changes of $Htr2a$ expression in response to stimulation that activates $Egr3$. To test this hypothesis, we first determined whether SD, a physiologic stimulus, which has previously been shown to activate Egr3 expression in WT mice (Thompson et al., 2010), could induce $Htr2a$ gene. Then, we used qRT-PCR to compare $Htr2a$ mRNA levels in WT and $Egr3$-/- mice during normal sleep and after SD. To further identified the specific cortical regions where $Egr3$ could alter the $Htr2a$ gene expression after SD, we examined $Egr3$ and $Htr2a$ levels in dissected cortical brain regions (anterior cortex, prefrontal cortex and posterior cortex).

Methods

Animals

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

Sleep deprivation (SD)

Animals were single-housed for 5 days prior to the experimental procedure for habituation. Animals were assigned to two groups (Fig.1): 6 hrs sleep deprivation (SD) group, time - matched controls allowed to sleep in their home cages (control group). SD started at the beginning of the light period (8:00 a.m. mountain standard time, MST). Mice were kept awake by “gentle handling” as previously described (Maple, Zhao, Elizalde, McBride, & Gallitano, 2015; Thompson et al., 2010). Briefly, animals were disturbed by a combination of cage tapping,
introduction of novel objects (e.g., balled paper towels), cage rotation, and stroking of vibrissae and fur with an artist’s paintbrush (Terao, Steininger, et al., 2003; Thompson et al., 2010). The number and type of stimulation events required to keep each animal awake during the SD procedure was recorded by the experimenters and was compared between the WT and Egr3-/- mice.

**Fig.1. Experimental design for sleep-deprivation (SD) and cortical tissue isolation for quantitative reverse transcription polymerase chain reaction (qRT-PCR).** Mice were assigned to two groups: 6 hrs sleep deprivation (SD) group, time-matched controls for the SD group (control group). SD started at the beginning of the light period (8:00 a.m.) and ended at 2:00 p.m. All control mice with normal sleep were in their home cages during the same period as the SD procedure. All sleep deprived mice and their time-matched control were sacrificed the same time immediate after SD. Cortical tissues were removed and processed for qRT-PCR to measure the Egr3 and Htr2a expression.

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

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

*Egr3* Forward primer: 5’-CTGGAGGCAGTTCTCCTTG-3’; *Egr3* Reverse primer: 5’-TGGATCAAGGCATCCTAAC-3’; *Htr2a* Forward primer: 5’-TCACCATTGCGGGAAACAT-3’; *Htr2a* Reverse primer: 5’-ATCAGCTATGGCAAGTGACAT-3’; *Pgk1* Forward primer: 5’-TGTTAGCAGCAATCCAGCTA-3’; *Pgk1* Reverse primer: 5’-CAGACAAATCTGATGAGTA-3’.
**Statistical analysis**

For all the qRT-PCR data, the value of cycle threshold (Ct) of gene of interest (\textit{Egr3} and \textit{Htr2a}) were normalized to the housekeep gene phosphoglycerate kinase 1 (\textit{Pgk1}) to be the first ΔCt. Relative expression was determined using the comparative $2^{\Delta\Delta\text{Ct}}$ method (Livak & Schmittgen, 2001). In addition, the SD project was performed over 4 years (from 2013 to 2016) using over more than 5 cohorts of animals. Considering potential environmental variations between each study and the rapid activation and degradation feature of \textit{Egr3}, instead of regular comparative $2^{\Delta\Delta\text{Ct}}$ method that combine all first ΔCt of the experiment group and then normalize to the combination of all first ΔCt of the control group without considering different cohorts, we normalized the first ΔCt of experiment group to the control group of that day to decrease variation between each cohort. The gene fold change was calculated by the equation $(2^{\Delta\Delta\text{Ct}}_{\text{SD group}})/(2^{\Delta\Delta\text{Ct}}_{\text{control group}})$.

Statistical analysis was performed using Prism 7.0 (GraphPad Software, Inc, La Jolla, CA). The amount of the stimulus during SD was analyzed in Graphpad using repeated measures two-way ANOVA (RM – two way ANOVA). Unpaired Student’s t-tests with a two-tailed $p$-value were used for conditions in which there were two groups. To determine significant effect in which there were two factors, we performed a two-way ANOVA with post-hoc Tukey tests, with gene expression levels as the dependent variable, and genotype and experiment conditions as independent variables. All values included in the figure legends represent means ± SEM. Statistical significance was determined by $p < 0.05$. 
Fig. 2. Diagram of cortical regions dissected immediately following SD. Colors (a) indicate different cortical regions were collected, right whole cortex (yellow), left anterior cortex (green), left prefrontal cortex (pink), left posterior cortex (blue). To collect left anterior cortex, we did a 1\textsuperscript{st} cut at 2.10mm Bregma showed in (b), then we dissected along the arrow and kept the neocortex region above the arrows as left anterior cortex. To remove the prefrontal cortex, we did a 2\textsuperscript{nd} cut at 0.14mm Bregma showed in (c), then we dissected along the arrow and kept the neocortex region between the arrows as left prefrontal cortex. All the remaining neocortex was collected as left posterior cortex. Create figure using the Mouse Brain Atlas as reference (Rosen et al, 2000).
Results

**Fig. 3. WT and Egr3 -/- mice require same amount of stimulus during SD.** No significant effect of interaction between time and genotype is observed ($p = 0.8411$). A significant time effect ($p < 0.001$) but no significant genotype effect on the amount of stimulus ($p = 0.1461$) is detected. Two-way ANOVA with repeat-measurement (RM). $n = 12$. All values included in the figure legends represent means ± SEM.

SD induces *Htr2a* expression in the cerebral cortex in an *Egr3* - dependent manner

Our first question is whether SD, a stimulus that activates expression of *Egr3* (Thompson et al., 2010), could affect *Htr2a* expression. However, prior to testing this we had to demonstrate that we can replicate the previous finding that this stimulus causes and increase in expression of *Egr3*, which had been detected using *in situ* hybridization to label mRNA. To address this, we first examined the levels of *Egr3* mRNA using qRT-PCR in the samples from whole cortex after SD in
WT mice. We found that 6 hours of SD induces a roughly 3.5-fold increase of Egr3 expression compared to non-SD controls (Fig. S4a; t_{17} = 4.857, p < 0.0001). Next, we measured Htr2a mRNA levels from the same samples from WT animals following SD compared with non-SD controls. This demonstrated that SD induces a greater than 2-fold increase in Htr2a expression compared to non-SD controls. ANOVA revealed a significant genotype by intervention (SD) interaction on Htr2a gene expression level (F_{1, 29} = 5.948, p =0.0211). There is also a main effect of genotype on the Htr2a gene expression (F_{1, 29} = 9.752, p =0.0040) but the main effect of experiment conditions on Htr2a expression level is not significant (F_{1, 29} = 3.33, p = 0.0784). Tukey's multiple comparisons test shows that Htr2a mRNA expression level is significantly increased in WT mice following SD compared with the non-sleep deprived control group (Fig. 4b; p <0.05), but not in the Egr3 -/- mice groups (Fig. 4b; not significant).

There is also a significant effect of SD on Htr2a mRNA level between the WT and the Egr3 -/- mice in the sleep deprived group (Fig. 4b; p <0.01). These findings suggest that the

$$ Egr3 $$

mRNA level after SD

$$ Htr2a $$

mRNA level after SD

![Graphs showing Egr3 and Htr2a mRNA levels](image)

**Fig. 4.** In the right cortex, SD induces Htr2a expression and this induction is dependent on Egr3. (a) SD induces Egr3 mRNA expression in WT mice. (b) SD increases Htr2a mRNA expression in WT mice but not in Egr3 -/- mice. * p < 0.05, ** p < 0.001, *** p < 0.0001, n = 8-11. All values included in the figure legends represent means ± SEM.

Immediate early gene Egr3 can be activated by SD. Htr2a gene is also induced by SD in the WT
mice but this increase is dependent on the *Egr3*, since the level of *Htr2a* gene is not changed in the *Egr3* -/- mice.

**In the anterior cortex, *Egr3* and *Htr2a* expression level does not change following SD**

a. *Egr3* mRNA level after SD  
b. *Htr2a* mRNA level after SD

![Graphs showing *Egr3* and *Htr2a* mRNA levels before and after SD](image)

**Fig. 5. In the anterior cortex, there is no significant change of *Egr3* and *Htr2a* mRNA expression after SD.** (a) SD fails to induce *Egr3* mRNA expression in WT mice. (b) The level of *Htr2a* mRNA is not significantly increased following SD. n = 13 -15. All values included in the figure legends represent means ± SEM.

Next, we wanted to identify the specific cortical regions in which SD increases expression of *Htr2a*. It has been reported that *Htr2a* has an anterior-posterior gradient of expression in the mouse cortex (Mengod, Pompeiano, Martínez-Mir, & Palacios, 1990; Pompeiano, Palacios, & Mengod, 1994). We wanted to see if SD was increasing *Htr2a* levels in regions in which it is already expressed, or activating its expression in regions in which there is little to no detectable expression at baseline.

First, we examined the expression of *Egr3* and *Htr2a* in anterior cortex after SD. We found there is no significant difference in *Egr3* gene expression in the anterior cortex of WT mice following SD compared with the non-SD control group (Fig. 5a; t$_{27}$ = 0.6679; p = 0.5098). In addition, there is no significant interaction effect between SD and genotype on *Htr2a* gene
expression (Fig. 5b; $F_{1, 52} = 0.4406, \ p = 0.5098$). We also did not see the main effect of experimental conditions (Fig. 5b; $F_{1, 52} = 3.488, \ p = 0.0675$) as well as the main effect of genotype (Fig. 5b; $F_{1, 52} = 0.1129, \ p = 0.7382$) on $Htr2$ expression. These results suggest that the expression of $Egr3$ and $Htr2a$ is not significantly changed after SD in the anterior cortex.

In the prefrontal cortex, SD induces both $Egr3$ and $Htr2a$ expression and $Egr3$ is required for $Htr2a$ induction

In the prefrontal cortex, we found that the $Egr3$ is significantly increased 2.0-fold in WT mice following 6 hours of SD compared with non-sleep deprived controls (Fig. 6a; $t_{28} = 2.615, \ p = 0.0142$). Moreover, there is a significant interaction effect between SD and genotype on the $Htr2a$ expression level (Fig. 6b; $F_{1, 54} = 6.413, \ p = 0.143$). There is also a main effect of genotype ($F_{1, 54} = 11.95, \ p = 0.0011$) and the experiment conditions ($F_{1, 54} = 5.857, \ p = 0.0189$) on the $Htr2a$ expression level. Tukey's multiple comparisons test shows a significantly increase of $Htr2a$ mRNA level (1.7-fold) after SD compared with SD controls in WT mice (Fig. 6b; $p < 0.01$) but not in the $Egr3^{-/-}$ mice (Fig. 6b; $p = 0.9998$). There is also a significant difference of $Htr2a$ expression between the WT and the $Egr3^{-/-}$ mice after SD (Fig. 6b; $p < 0.001$).
In the posterior cortex, Egr3, but not Htr2a, is significantly increased following SD

In the posterior cortex, Egr3, but not Htr2a, is significantly increased following SD. In the posterior cortex, SD significantly induces Egr3 gene expression with 2.6-fold in the WT mice (Fig. 7a; t_{27} = 3.5; p = 0.0016). However, there is no significant effect of the interaction between SD and genotype on the Htr2a mRNA expression level (Fig. 7b; F_{1,49} = 0.06356, p = 0.8020). There is a main effect of experimental conditions on Htr2a expression levels (Fig. 7b; F_{1,49} = 5.976, p = 0.0181) but genotype show no main effect (Fig. 7b; F_{1,49} = 0.4822, p = 0.4907). These results indicate that SD increased Egr3 expression in the posterior cortex but the Htr2a expression is not affected by SD.
Schizophrenia is a complex mental disorder influenced by many factors including genetic variations at over 100 genomic loci, as well a range of environmental stressors. Numerous studies have emphasized the important role of Htr2a dysregulation in schizophrenia. However, how environment factors affect Htr2a expression remains unclear. The schizophrenia susceptibility gene EGR3, as an IEG transcription factor, is rapidly activated by various stimuli. Thus, activation of EGR3 not only reflects local neuronal activity after stimulation but also orchestrates a downstream genetic program that presumably modifies neuronal function. Thus, identification of the potential targets that are regulated by EGR3 will help us to answer how the interaction between genes and environment influence schizophrenia risk.

Previously, we found a decrease of 5HT2A Rs level in prefrontal cortex of Egr3 -/- mice (Williams et al., 2012), which suggests Htr2a is potential downstream regulated by Egr3. If Htr2a is, indeed, regulated by Egr3, then we would expect to see an alternation of Htr2a expression by an environmental stimulus which activates Egr3. In the present study, we first found that Htr2a expression is increased by SD, a stimulus that activates Egr3 (Thompson et al., 2010). Then we
demonstrated that the increase of $Htr2a$ requires the activation of the immediate early gene $Egr3$ because SD fails to induce $Htr2a$ in the $Egr3$-/− mice. These findings are exciting because they provide evidence for the regulation of $Egr3$ to its potential downstream target $Htr2a$. Moreover, this is the first report that the level of $Htr2a$ can be rapidly altered, in just six hours, in response to a physiologic environmental stimulus. This is particularly important because the 5HT2AR mediates the perceptual disturbance caused by hallucinogenic drugs, and the action of antipsychotic medications, and is decreased in the brains of schizophrenia patients.

In the present study, we adopted a ‘gentle-handling’ method, one of the most-frequently used SD techniques (L. A. Toth & Bhargava, 2013), which has been applied to rodents and causes loss of both non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS). In this approach, animals are under continuous observation and disturbed by combination of tapping on the cage, providing novel objects, or gentle prodding (e.g., with paintbrush). The primary rationale for using this method is that this is one of the few physiologic interventions that had been shown to activate expression of $Egr3$. Besides the gentle-handling method, other SD protocols called automated methods (e.g. ‘disk-over-water’ approach) also are widely used for animal SD research. In these approaches, the animals are forced to walk to keep pace with the disk, otherwise they will be carried into a pool of water (L. A. Toth & Bhargava, 2013). However, our $Egr3$-/− mice display hyper-locomotor activity (Gallitano-Mendel et al., 2007). So, the ‘gentle-handling’ was determined to have fewer potentially confounding effects automated methods for these animals. Furthermore, our analysis of the amount of stimulus during SD reveals that there is no significant difference of intervention intensity between the WT and $Egr3$-/− mice during SD procedure (Fig.3). However, we do observe a significant time effect on the amount of stimulus (Fig.3), which indicates that as the duration of SD increases, the increased intensity of arousal intervention is required to keep the mice awake.

Stress, such as sleep deprivation is able to rapidly activate the expression of immediate early genes (IEGs) in the brain, and labeling of IEGs has enabled the visualization of activated neurocircuitry after stress (Senba & Ueyama, 1997; Terao, Greco, Davis, Heller, & Kilduff, 2003).
In order to determine if we could induce $Egr3$ after 6hrs of SD, we measured the $Egr3$ mRNA level in samples of whole cortex using qRT-PCR. We found that there is a significant increase of $Egr3$ mRNA after 6hrs of SD compared with the non-sleep deprived WT control group (Fig.4a). It has previously been reported that 6hrs of SD significantly activates the immediate early growth gene $Egr3$ (Terao, Greco, et al., 2003; Thompson et al., 2010) in the mouse cortex using in situ hybridization. Our finding of qRT-PCR analysis is consistent with the prior work.

Previously we found there is a nearly 70% reduction of 5HT$_2A$ receptor in the prefrontal cortex of $Egr3$ -/- mice compared with WT controls (Williams et al., 2012), which suggests that $Egr3$ is required for the expression of $Htr2a$ gene. Next, we want to determine if 6hrs of SD could induce the $Htr2a$ expression in mouse, and whether $Egr3$ is required for the $Htr2a$ gene expression in response to the acute physiology stress - SD. We measured the cortical expression levels of the $Egr3$ and $Htr2a$ mRNA after SD. We found that there is a significant increase of $Htr2a$ mRNA expression in the right whole cortex of the WT mice following SD compared with the non-sleep deprived control group (Fig.4b). However, the induction of $Htr2a$ is not observed in the $Egr3$ -/- mice after SD compared with the control group (Fig.4b). Interestingly, our results in animals are supported by a recent study in humans that showed a 24hrs of total SD causes significant increases of cerebral serotonin 2A receptor binding in human (Elmenhorst, Kroll, Matusch, & Bauer, 2012). In addition, it has been reported that $Htr2a$ has an anterior-posterior gradient of expression cortex and $Htr2a$ mRNA level is highest in frontal cortex (Mengod et al., 1990; Pompeiano et al., 1994). Next, we wanted to see if SD was increasing $Htr2a$ levels in regions in which it is already expressed, or activating its expression in regions in which there is little to no detectable expression at baseline.

To further identify specific cortical regions in which $Htr2a$ expression is affected by $Egr3$ following SD, we removed the left whole cortex from the same mice as the right whole cortex study and dissected it into 3 parts (Fig.2): left anterior cortex, left prefrontal cortex and the left posterior cortex. We examined both $Egr3$ and $Htrr2a$ mRNA expression levels in those 3 cortical regions using qRT-PCR. Our results indicate that only the prefrontal cortex that displays an increase of $Htr2a$ gene expression following SD and is dependent on $Egr3$ (Fig.6b). There is no
significant change of *Egr3* and *Htr2a* in the anterior cortex after SD (Fig.5). In addition, we only found the *Egr3* induction but not the *Htr2a* in the posterior cortex of sleep deprived WT mice compared with the undisturbed wildtype (WT) littermate controls (Fig.7).

*Egr3*, as an IEG, is the molecular switch to activate targets in response to changes in environment, and is expressed at very low level at baseline. However, it can be induced following 6hrs of SD in the mouse neocortex, particularly in the posterior regions include visual and caudal somatosensory cortex using *in situ* hybridization method (Thompson et al., 2010). Our qRT-PCR results showed that the *Egr3* mRNA level was increased at 2.0-fold (Fig.6a) and 2.6-fold (Fig.7a) in the prefrontal cortex and the posterior cortex respectively, which are greater than the 1.2-fold (Fig.5a) of *Egr3* mRNA in the anterior cortex. These data suggested that the *Egr3* gene was activated by 6hrs of SD and the activation mostly was in the posterior cortical area, and this is consistent with the prior findings that *Egr3* was mostly induced in visual and caudal somatosensory cortex (Thompson et al., 2010).

Previously, localization studies of the *Htr2a* gene by *in situ* hybridization have reported that the *Htr2a* mRNA is expressed in a strong anteroposterior gradient in the rodent cerebral cortex, with the highest level is observed in the frontal cortex (Mengod et al., 1990; Pompeiano et al., 1994). Furthermore, the study on human show that 24hrs of SD causes significant increases of cerebral serotonin 2A receptor binding in the ventrolateral prefrontal cortex (Elmenhorst et al., 2012). We found that the *Htr2a* mRNA levels in the anterior and prefrontal cortex of WT mice is increased 1.5-fold (Fig.5b) and 1.7-fold (Fig.6b) respectively. In addition, the *Htr2a* level in the posterior cortex was 1.3-fold (Fig.7b). Overall, our qRT-PCR data support the anteroposterior gradient distribution of *Htr2a* mRNA expression in rodent cortex, and the frontal cortex are more enriched of *Htr2a* gene compared with the posterior cortex. Although the cortical region that *Egr3* could alter *Htr2a* transcription in response to stress was identified, we were unable to determine the location in a cellular or subcellular level due to the limitation of qRT-PCR technique. Other methods like *in situ* hybridization would reveal the location or/and organization of the *Egr3* and *Htr2a* genes, and immunohistochemistry and autoradiographic assay would allow us to identify the distribution and localization of the EGR3 and HTR2A proteins.
Both 5HT$_{2A}$R and its encoding gene HTR2A are found decreased in the frontal cortex of postmortem brains of schizophrenic patients (Chalovich & Eisenberg, 2005; Hernandez & Sokolov, 2000). In the present study, we show for the first time that a brief intervention, only 6hrs of a physiologic environmental change, can drastically alter the level of the Htr2a gene in the mouse brain. This is very exciting because SD could be a means to normalize the deficit in schizophrenia patients. Although the exact mechanism is not clear, SD has been one of an effective treatments for major depressive disorders (Hines, Schmitt, Hines, Moss, & Haydon, 2013). To our knowledge, SD has not been reported as a schizophrenia treatment to date. However, our findings in the animal studies provide potential therapeutic role of SD in this mental disorder. SD induced HTR2A expression probably is an adaptation for normal people. However, schizophrenia patients lack of HTR2A in response to stimuli because of the deficient of EGR3. Our findings are not just limited the laboratory mouse. In fact, similar findings have been reported that 24hrs of total SD shows increased binding of cerebral 5HT$_{2A}$Rs in humans using positron emission tomography (PET) (Elmenhorst et al., 2012).

However, people may argue if it is beneficial to increase HTR2A then why many atypical antipsychotics are 5HT$_{2A}$R antagonists? This question brings us to the paradox that 5HT$_{2A}$R and HTR2A are found decreased in postmortem brains of schizophrenic patients (Chalovich & Eisenberg, 2005; Hernandez & Sokolov, 2000), whereas most atypical antipsychotics have 5HT$_{2A}$R antagonism (Moran et al., 2016) and some of the 5HT$_{2A}$R agonists can cause hallucinations. There is no clear answer for this now. However, it should be noted that the atypical antipsychotics clozapine, a 5-HT$_{2A}$Rs antagonist, also acts as an agonist at 5-HT$_{2A}$Rs to counter dizocilpine-induced behaviors by activation of Akt/ Protein kinase B (PKB) (Schmid, Streicher, Meltzer, & Bohn, 2014). In addition, clozapine also can induce serotonin 2A receptor to internalize in cultured cortical pyramidal cells and cause the receptor redistribution from dendrites to cell bodies (Willins et al., 1999). So, the antagonist acts on receptors through a very complex manner rather than just simply block a receptor. Moreover, all effective antipsychotics currently in use bind to D$_2$ receptors. It is also reported that 5-HT$_{2A}$ and D$_2$ receptor antagonists increase dopamine release in the prefrontal cortex by via 5-HT$_{1A}$ receptor activation (Ichikawa et al., 2001).
Thus, the interaction between dopamine and serotonin systems may determine the efficacy of antipsychotics.

In conclusion, we employed an acute physiology stress, sleep deprivation and successfully activate two schizophrenia susceptibility genes, \textit{Egr3} and \textit{Htr2a}. Most importantly, we validated that \textit{Egr3} is required for the expression of \textit{Htr2a} in response to stress. Given the transcriptional regulation function of EGR3, these results provide potential regulation effect of \textit{Egr3} on the \textit{Htr2a} under stressful conditions.
CHAPTER 3
EARLY GROWTH RESPONSE 3 (EGR3) DIRECTLY BINDS TO THE SEROTONIN 2A RECEPTOR GENE (HTR2A) PROMOTER AND ACTIVATES GENE EXPRESSION

Abstract

To date, no gene has been definitively demonstrated to cause schizophrenia. Through its action as an immediate early gene transcription factor, EGR3 translates environmental stimuli into gene expression changes in the brain that affect numerous cellular and molecular processes that are dysfunctional in schizophrenia. However, few of the downstream genes regulated by EGR3 have been identified.

While the 5HT₂A-encoding gene Htr2a gene has been implicated in the etiology of schizophrenia, the mechanisms by which Htr2a influences susceptibility to this severe mental illness are poorly understood. In chapter 2 we describe that sleep deprivation (SD) activates Htr2a in an Egr3 dependent manner, particularly in mouse prefrontal cortex (PFC). These findings are consistent with our previous finding that the Egr3 -/- mice display approximately 70% of decrease in 5HT₂A levels in PFC. We believe that the decrease of Htr2a mRNA and 5HT₂A levels in PFC of Egr3 -/- mice underlines the fact that Egr3 -/- mice are resistant to the sedating effect of clozapine. Thus, we expected that EGR3, a transcription factor, alters the Htr2a gene expression by directly binding to the Htr2a promoter under stress condition.

In this study, we first identified two putative EGR3 binding sites in the Htr2a promoter by bioinformatics analysis. To determine EGR3 protein expression pattern, we performed western blot using prefrontal cortical tissues. We found that EGR3 protein levels increased 2 hrs after electroconvulsive seizure (ECS). Based on these findings, we performed chromatin immunoprecipitation (ChIP) to determine whether EGR3 binds to Htr2a promoter in vivo. We found that ECS produces a significant increase in EGR3 binding to the distal EGR consensus binding site of the Htr2a promoter. To further determine the functionality of this binding, we tested the ability of EGR3 to regulate expression of Htr2a promoter-driven luciferase reporter constructs in vitro. Results show that overexpression of EGR3 significantly activates the Htr2a distal
promoter-driven luciferase gene. Interestingly, we found that EGR3 also activates expression of a luciferase reporter driven by the proximal EGR binding site in the Htr2a promoter, although this region was not significantly bound by EGR3 in our ChIP assay.

**Introduction**

Over the past decade, researchers have made great advances in identifying schizophrenia susceptibility genes. Genome-wide association studies have revealed large number of schizophrenia risk genes from 108 schizophrenia-associated genetic loci (Ripke et al., 2014). Among these are genes that encode calcium channels subunits, and genes involved in glutamatergic, dopaminergic, and serotoninergic neural transmission and in synaptic plasticity. However, genes cannot fully account for schizophrenia susceptibility. Studies show concordance rates of approximately 50% in monozygotic twins, which is higher than dizygotic (17%) twins. Research has led to the consensus that schizophrenia has a heritability of about 80% (Cardno & Gottesman, 2000). This indicates that schizophrenia is not a completely genetic disease. Other factors such as the environmental events may interact with genes and contribute to this disease. Better understanding of the role of environmental factors in schizophrenia risk may help us to identify the schizophrenia susceptibility genes.

The Immediate early genes (IEGs) are rapidly activated within minutes after various stimuli, without the need for de novo protein synthesis, thus they are considered as markers for neuronal activation (Bahrami & Drablos, 2016). Egr3 is a member of the Early Growth Response family of IEGs, which function as transcription factors. Studies on humans and animals support the role for Egr3 in schizophrenia pathogenesis (Cheng et al., 2012; Amelia Gallitano-Mendel, Wozniak, Pehek, & Milbrandt, 2008; S. H. Kim et al., 2010; Williams et al., 2012; Zhang et al., 2012). Previously, we found that Egr3 /-/- mice display hyper-locomotor phenotype, which has been associate with schizophrenia (Gainetdinov et al., 2001). In addition, Egr3 /-/- mice are resistant to the sedating effect of clozapine which is reversed by treatment with either haloperidol or clozapine(Gallitano-Mendel et al., 2008). This parallels the increased tolerance that schizophrenia patients have antipsychotic side effects, compared to healthy controls (Cutler,
Moreover, a 5HT₂AR selective agent MDL11939 can replicate the effect of clozapine in Egr3/−/− mice (Williams et al., 2012). This pharmacologic response may be explained by our finding that 5HT₂AR binding is nearly 70% reduced in the prefrontal cortex of Egr3/−/− mice (Williams et al., 2012). This is also supported by findings of studies showing that 5HT₂AR−/− mice were cataleptic following haloperidol and risperidone treatment, but did not respond to clozapine’s locomotor - suppressing effects (McOmish, Lira, Hanks, & Gingrich, 2012).

Many studies have shown that the 5HT₂AR-encoding gene Htr2a plays an important role in schizophrenia. In postmortem studies, it has been reported that expression levels of the 5HT₂AR and the 5HT₂AR-encoding gene, Htr2a, are reduced in postmortem schizophrenia patients’ brains (Chalovich & Eisenberg, 2005; Hernandez & Sokolov, 2000). Clozapine is one of the atypical antipsychotics and is currently one of the most efficacious antipsychotics (McEvoy et al, 2006; Meltzer et al, 2003). The most popular explanation for clozapine improving efficacy in treating some of the schizophrenia symptoms is the targeting of 5HT₂AR. However, even numerous studies evident that the dysfunction of Htr2a influence schizophrenia risk, little is known about the mechanism by which environment factors alter Htr2a gene expression. Stress, such as prenatal/postnatal infection and social adversity, have been reported to influence schizophrenia susceptibility (Dean & Murray, 2005). In chapter 2, we employed the method of sleep deprivation to expose wild type (WT) and Egr3−/− mice to a physiologic stress that has been shown to induce expression of Egr3 in the WT mouse cortex. We found that 6hrs of SD induces both Egr3 and Htr2a expression, particularly in the mouse prefrontal cortex. In addition, SD fails to increase Htr2a mRNA level in the Egr3−/− mice, which indicates Egr3 is required for the Htr2a induction in response to stress. Our findings provide further evidence that these two schizophrenia candidate genes are in the same biological pathway that integrates genetic and environmental risk for schizophrenia.

Based on those findings, we hypothesized that EGR3 directly regulates Htr2a gene expression by binding to EGR response elements in the Htr2a promoter. To determine whether EGR3 directly binds to Htr2a promoter in vivo, we performed chromatin immunoprecipitation (ChIP) using frontal cortical tissues from WT mice 2hrs after electroconvulsive seizure (ECS). To
further investigate whether the binding of EGR3 to Htr2a promoter is functional, we co-transfected individual Htr2a promoter reporter clones with CMV EGR3 vector or with CMV vector alone. After 24hrs of transfection in neuro2a cells, we measured luciferase signals which are driven by each gene promoter. We hope this study will identify part of the regulation mechanisms between the two schizophrenia candidate genes and provide knowledge of how genetic and environmental interactions affect schizophrenia risk.

**Methods**

**Animals**

The Egr3 \text{-/-} mice to be used in the study were generated by the deletion of sequences that encode the zinc fingers, the DNA-binding domain of the protein (Tourtellotte & Milbrandt, 1998). The mice were back-crossed to a C57BI/6 background for more than 30 generations. Study animals were generated from heterozygote \times heterozygote mates. Animals were housed on a 14/10 h light/dark cycle (lights on 8:00 a.m.) with \textit{ad libitum} access to food and water. All animals used for ECS studies were adult wildtype (WT) mice. WT mice from the same litters were used as controls.

**Electroconvulsive seizure (ECS)**

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

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

2 hours after ECS, prefrontal cortex was removed and homogenized in the 1% Nonidet P-40 lysis buffer containing 0.5% sodium deoxycholate with proteinase inhibitor (1:10,000; Sigma-Aldrich, St. Louis, MO) using the Wheaton Tenbroeck style tissue grinder (ThermoFisher Scientific, Waltham, MA). Cell debris was pelleted by centrifugation at 17,000 rpm for 5 min, 4 °C. The supernatant was saved and protein concentration was quantified using Pierce BCA protein assay kit (ThermoFisher Scientific, Waltham, MA). Protein samples were denatured for 5 min, 100 °C on block heater (VWR, Radnor, PA). 25ug of protein was loaded into homemade 4-8% gradient polyacrylamide gels. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-rad Laboratories, Hercules, CA) for 1hr using a semi-dry transfer apparatus (Bio-rad Laboratories, Hercules, CA) which constantly delivers a voltage of 15 V. Then the membranes were blocked for 1hr with 3% (w/v) non-fat dry milk (LabScientific Inc., Highlands, NJ). After washing with phosphate buffered saline plus 0.1% of Tween-20 (PBST), the membranes were incubated overnight at 4°C with the rabbit anti-EGR3 antibody (1:200, Santa Cruz Biotechnology, Dallas, TX) and the mouse anti-beta actin antibody (1:5000, Sigma-Aldrich, St. Louis, MO). The next day, membranes were washed with PBST and incubated with IRDye800CW secondary antibodies (1:10,000 for EGR3, Li-COR Biosciences, Lincoln, NE) and the IRDye680 secondary antibodies (1:20,000 for beta actin, Li-COR Biosciences) dissolved in 3% non-fat dry milk for 1hr at room temperature with gentle shaking. After washing for 5 times, each time for 5 minutes, the membranes were visualized on an Odyssey instrument (Li-COR Biosciences). Protein expression levels were determined as the ratio of EGR3 to the internal control beta-actin and were reported as percentage of control group.
Bioinformatics analysis to identify putative EGR3 binding sites on the Htr2a gene promoter

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

Chromatin immunoprecipitation (ChIP)

ChIP protocol is modified from previously reported (H.-D. Kim et al., 2016). Briefly, 2hrs after ECS, frontal cortex was removed and was cut into 1mm pieces using razor blades. Next, we crosslinked the protein-DNA complexes by incubating the frontal cortical tissues in 1% formaldehyde (Sigma-Aldrich, St. Louis, MO) on a rotator for 12 minutes. Then we quenched the formaldehyde with 125 mM glycine (Sigma-Aldrich, St. Louis, MO) for 5 minutes. Next, we homogenized samples using a Q125 sonicator (Qsonic, LLC., Newtown, CT) with a power of amplitude 40%, sonication duration 7, number of cycles 2. Cool samples on ice between each cycle. After that, we sheared chromatin into 200bp-1000bp using a Bioruptor XL (Diagenode Inc., Denville, NJ) at 4°C with the high sonication intensity for 30s on/30s off for 35 cycles. Fragment size was verified with an Agilent bioanalyzer (Agilent Technologies, Santa Clara, CA). 50ul of sheared chromatin were removed as input control with an input fraction 5%. The magnetic beads-antibody complex was prepared by incubating 7.5ug of the anti-EGR3 antibody (Santa Cruz Biotechnology, Dallas, TX) with the magnetic sheep anti-rabbit beads (Invitrogen Corp., Carlsbad, CA) at 4°C overnight on a rotator. After washing the bead-antibody complex with 0.5% BSA blocking solution, add 70ul of the beads-antibody complex in to each ChIP sample, incubated for 16hrs at 4°C on a rotator. To control for nonspecific binding, a normal IgG IP was performed in
parallel. Next beads were collected by placing sample tubes in a magnetic rack (ThermoFisher Scientific, Waltham, MA), then washed with the following buffer: low salt wash buffer (0.1% SDS, 1% TritonX100, 2mM EDTA, 150mM NaCl, 20mM Tris-HCl), high salt wash buffer (the same as low salt wash buffer, except using 200mM NaCl instead), LiCl wash buffer (150mM LiCl, 1% NP40, 1% NaDOC, 1mM EDTA, 10mM Tris-HCl). Then cross-linked protein and DNA complex were reversed at 65°C overnight and RNA were removed by 1hr incubation with 2ul of RNase A (Roche Applied Science, Indianapolis, IN) at 37°C. Next proteins were digested with 2ul of proteinase K (20 mg/mL, Invitrogen Corp., Carlsbad, CA). Clean up DNA using a DNA purification kit (QIAGEN Inc., Germantown, MD). qPCR was performed using FastStart SYBR green master mix (Roche applied science) in a 7500 Fast real-time PCR machine (Applied Biosystem, Foster City, CA). The total reaction volume per well was 25ul reaction (2ul DNA, 9.5 µL nuclease-free water, 12.5 µL SYBR-Green Master Mix 2X, 0.5ul µL of each forward and reverse primers with 10uM stock). The thermal cycling parameters are: initial denaturation at 94°C for 10 minutes, 50 cycles of (denature at 94°C for 20 seconds, anneal and extension at 60°C for 1 minute). The primers used were as below:

Arc forward: 5’-TCGCTGCCCAGGACTAGGTA-3’;
Arc reverse: 5’-TTCACAGCCCCGAGTAGTA-3’;
Htr2a proximal forward: 5’-CTTGGATAGAAGTGCTGGATGCT-3’;
Htr2a proximal reverse: 5’-GGGTACATGGCAGTCATATTTTAGG-3’;
Htr2a distal forward: 5’-CTGGGCTCTAAAGGCAACTGA-3’;
Htr2a distal reverse: 5’-TGCGCACGTGTATACAGAGTAGGT-3’

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

1. The *Htr2a* proximal promoter luciferase reporter (Fig. 13) – the dual-reporter system contains a Gaussia luciferase gene (GLuc) which is driven by a *Htr2a* proximal promoter insert which corresponds to *Htr2a* promoter sequence located approximately 1061bp upstream and 200 bp downstream of the transcription start site (TSS) of the *Htr2a* gene. In the same vector, a secreted alkaline phosphatase (SEAP) is driven by a cytomegalovirus (CMV) promoter and serves as the internal control for signal normalization, and this internal control SEAP exists in all the other promoter reporter clones in the present study. This *Htr2a* proximal promoter luciferase reporter contains the EGR3 putative binding site GCGCGGGGGAGGGG (Fig. 9).

2. The *Htr2a* distal promoter luciferase reporter (Fig. 12) – this dual luciferase promoter reporter clone contains an GLuc gene and is driven by the insert which is -2727bp to -2841bp upstream of the *Htr2a* TSS and this fragment contains the EGR3 putative binding site AGGAGGGGGAGTCT (Fig. 9).

3. The *Arc* promoter luciferase reporter (Fig. 11) – is a positive control for the illuminometer and the functionality of our CMV-Egr3 vector. We used the *Arc* promoter reporter clone containing an insert which is 1049bp upstream and 200bp downstream of the TSS of the *Arc* gene. This *Arc* promoter luciferase reporter contains the EGR3 binding site ‘GCCGCCCACGGGCC’ (Fig. 9) which was confirmed previously (L. Li et al., 2005a).

4. The non-promoter luciferase reporter – as a negative control would allow us to detect the basic activity of the dual-reporter vector. This luciferase reporter contains an insert which is a non-promoter sequence (TGCAGATATCCTCGCCC).

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

Promoter reporter plasmid growth

We transformed 100 ng of each promoter reporter vector into 50 ul of GCI-5a competent cells (Genecopeia Inc., Rockville, MD). After incubation on ice for 30 minutes, we performed heat-shock on a block heater at 42 °C for 42 seconds, and then put on ice for 5 minutes. After that, 750 ul of room temperature super optimal broth with catabolite repression (SOC, ThermoFisher Scientific) medium was added to the DNA - competent cell mixtures. Incubated the mixture at
37°C for 1 h with shaking at 225 rpm. Next, plated 10 ul and 100 ul of mixture on Lennox Luria-Bertani broth agar (LB, ThermoFisher Scientific) plates containing 50 ug/ml kanamycin (ThermoFisher Scientific). Incubated the plates at 37°C, overnight. The next day, picked 5 clones and grew in 5 ml of Miller’s LB broth medium (ThermoFisher Scientific) containing 50ug/ml kanamycin. After overnight growth, plasmid DNA was purified and was sent for sequencing. The sequencing primers for all vectors used: forward: 5’-AGTTACTTAAGCTCGGGCCC-3’; reverse: 5’-TTGTTCGCAGTCGCTCAGGC-3’. This pairs of primers targeted the linker sequence between the promoters and the GLuc genes.

To validated the Htr2a proximal and distal promoter clones, we also used primer3 (Koressaar & Remm, 2007) to design sequencing primers specific to the Htr2a proximal promoter clone: forward: 5’-CTTGGATAGAAGTGCTGGATGCT-3’; reverse: 5’-GGGTACATGGCAGTCATATTTTTAGG-3’. Sequencing primers specific to the Htr2a distal promoter clone: forward: 5’-TGACCTGTTCGTTGCAACAAA-3’; reverse: 5’-GGCTGTCAGTGACCTCCTTA-3’.

**CMV-EGR3 vector, CMV-vector sub-cloning and plasmids growth**

The CMV-EGR3 vector is obtained from Dr. Jeffrey Milbrandt lab (Washington University). 150 ng of CMV-EGR3 vector was added into 50ul of Dh5alpha competent cells (New England Biolabs, Ipswich, MA), incubated on ice for 30 minutes. Next, we performed heat-shock of the DNA-competent cell mixture at 42°C for 45 seconds and put on ice for 2 minutes. After adding 450 ul of room temperature Miller’s LB broth media (ThermoFisher scientific), the mixture was incubated at 37°C for 1 h with shaking at 225 rpm. Then plated 10 ul and 100 ul of mixture on Lennox LB agar plates which contains 100 ug/ml of ampicillin (ThermoFisher Scientific). Incubate the plates overnight at 37°C. The next day, we picked 5 clones and grew in 5 ml of Miller’s LB media (ThermoFisher Scientific) containing 100 ug/ml ampicillin (ThermoFisher Scientific) with shaking at 180rpm overnight. Then DNA was purified from each LB growth and was digested by the restriction enzyme BglII and BamHI (ThermoFisher Scientific) at 37°C for 2 hrs. The clones contain the Egr3 insertion were selected for a maximal prep (QIAGEN).
To generate the CMV vector, 2 ug of CMV-Egr3 vector was digested by the restriction enzyme BglII and BamHI (ThermoFisher Scientific) at 37°C, overnight. After confirming the digested DNA that Egr3 has already been deleted by enzyme digestion on a 0.8% agarose gel, the CMV vector fragment was cut from the gel and was purified using a DNA gel purification kit (QIAGEN). Then the purified CMV vector DNA was ligated using T4 DNA ligase reaction (New England Biolabs). The ligation was performed with incubation at 16°C for 2 hrs and then heat-inactivated at 65°C for 10 minutes. Next, 1 ul of ligation mixture was transformed into 50 ul of DH5alpha competent cells (New England Biolabs). Transformation was performed the same as the CMV-EGR3 vector above.

Cell culture and lipofectamine transfection

Neuro2a cells (mouse neuroblastoma cells; ATCC, Manassas, VA) were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific) which contains 10% Fetal Bovine Serum (FBS, Gibco) and 1% Penicillin-Streptomycin (PS, Gibco), at 37°C, under a humidified atmosphere of 5% carbon dioxide (CO₂) : 95% air. Seeded cells into 6-well plate (Corning Inc., Corning, NY) with 2 ml of cell culture medium and waited to be 70-90% confluent at the time for transfection. 3 hrs ahead of transfection, completed removed original culture medium and add 3ml of fresh medium for each well. 1.5 ug of CMV-Egr3 vector or CMV vector was co-transfected with 1 ug of each promoter reporter vector per well using lipofectamine 3000 reagent (ThermoFisher Scientific). Transfection was performed with 3.75 ul of Lipofectamine 3000 reagent, 5 ul of P3000 reagent and total of 250 ul of Opti-MEM per well. After transfection, cells were incubated at 37°C with 5% CO₂ : 95% air until further processing.

Three separate transfections were performed and each transfection was repeated in triplicate.

Luciferase signal measurement

24 hrs after transfection, 0.2 ml of medium from each cell culture was collected and
placed at room temperature. The duo luciferase activities were measured using the secrete-pair dual luminescence assay kit (Genecopoeia). Each sample was run in duplicate.

To detect the Gaussia luciferase (GLuc) signal, first diluted the 10X Gaussia luciferase stable buffer (GLuc-S) with distilled water to make 1X GLuc-S (1:10). Second, prepared the GLuc assay working solution by diluting the Gaussia luciferase substrate with the 1X GLuc-S buffer (1:10) and incubated at room temperature for 25 minutes, avoid light. Next, mixed 100 ul of GLuc assay working solution with 10 ul of cell culture medium for each well. The mixture was incubated at room temperature for 1 minute, avoid light. Then read the signal using a Tecan Safire2 instrument (Tecan Group Ltd., Morrisville, NC).

To measure the Secreted Alkaline Phosphatase (SEAP) level, first aliquoted 50 μl of each culture medium and heated at 65°C for 15 min, and then placed on ice. Next, prepared 1X SEAP buffer by dilute the 10X SEAP buffer with distilled water (1:10). Then prepared the SEAP assay working solution by diluting the SEAP substrate with 1X SEAP assay working solution (1:10) and incubate at room temperature for 10 minutes, protected from light. After that, 100 ul of SEAP assay working solution was mixed with 10 ul of each heated medium sample. Incubated the mixture at room temperature for 10 minutes, avoid light. Then read the signal using the Tecan Safire2 instrument (Tecan Group Ltd.).

For all measurements, the GLuc value was first normalized to the intern control SEAP luciferase value (GLuc / SEAP ratio) and then to the non-promoter luciferase reporter.

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

After collecting the cell culture medium for luciferase measurement, we discarded the remaining medium in each well. Then the cells were washed 2 times with cold 1X PBS 300 ul per well and were lysed with 300 ul per well of 1% Nonidet P-40 lysis buffer containing proteinase inhibitor (1:10,000; Sigma-Aldrich). Samples were further homogenized using a Q125 sonicator (Qsonica) with a power of amplitude 40%, 1 time for 5 seconds on ice. Then protein concentration was quantified using Nanodrop 1000 spectrophotometer (Thermo Scientific). Next, protein
samples were denatured and 25µg of proteins for each sample were loaded on gels. Western blot was performed as the same as those described in western blot for ECS protein samples above.

**Statistical analysis,**

Statistical analysis was performed using Prism 7.0 (GraphPad). Unpaired Student’s *t* tests with a two-tailed *p*-value were used for conditions in which there were two groups. All values included in the figure legends represent means ± SEM. Statistical significances were determined by *p* < 0.05.

**Results**

**EGR3 protein expression level is significantly increased 2hrs post electroconvulsive stimulus**

Egr3, as an IEG, is the molecular switch to activate targets in response to changes in environment, and is expressed at very low level at baseline. However, ChIP-qPCR, a method to investigate the interaction between proteins and DNA in the cell, includes many steps such as sonication of chromatin, selectively immunoprecipitated protein-DNA complex using an appropriate protein-specific antibody and use purified genomic DNA as template for qPCR. Thus, first we need to maximize the EGR3 protein levels in animal tissues for performing ChIP. To determine the expression timeline of EGR3 protein in the prefrontal cortex, we performed ECS on two separate animal cohorts. Protein samples were isolated and were quantified using the western blot, we found that 2hrs post ECS, the EGR3 protein level is significantly increased compared with the No ECS control group (**Fig.1; t**<sub>10</sub> = 2.28, *p* <0.05). Those results suggested that 2hrs post ECS induced robust activation of EGR3 protein expression in mouse prefrontal cortex.
Bioinformatics analysis identified two putative EGR3 binding sites on the Htr2a gene promoter

As one of the EGR family transcription factors, EGR3 contains a highly-conserved DNA-binding domain composed of three zinc-finger motifs. In addition, it has been reported that EGR3 directly binds to its downstream target gene promoters and regulate the expression of those genes (L. Li et al., 2005a; Mittelstadt & Ashwell, 1998; Salotti, Sakchaisri, Tourtellotte, & Johnson, 2015). To investigate whether EGR3 could directly bind to the Htr2a promoter and thus might be able to activate it, we searched potential EGR3 binding sites on the Htr2a gene promoter using FIMO software (Grant et al., 2011). We found two motif occurrences with a $p$-value < 0.0001. The two putative EGR3 binding sites (site A and B) are indicated in the Fig. 9.

Fig. 8. EGR3 protein expression is significantly increased in the prefrontal cortex 2hrs post electroconvulsive stimulus (ECS) compared with No ECS controls. (a) We performed ECS using two separate experiment cohorts. The representative blots were displayed in the figure. (b) EGR3 protein level of each sample is normalized to the internal control beta actin, and further normalized to the No ECS group. *$p$ < 0.05, n = 6 per group. All values included in the figure legends represent means ± SEM.
Fig. 9. Diagrams of putative EGR3 binding sites in the mouse Htr2a and Arc promoters. (a) Two EGR3 binding sites were located -2777bp (site A, AGGAGGGGGAGTC) and -61bp (site B, GCGCGGGGGAGGGG) upstream of the Htr2a gene transcription start site. (b) Confirmed EGR3 consensus binding site ‘GCCGCCCACGGGCC’ which is -37bp upstream of the Arc promoter served as a positive control (L. Li et al., 2005b). Black boxes indicate coding sequences (CDS), transparent boxes indicate non-coding exons.
Chromatin immunoprecipitation (ChIP) demonstrates EGR3 directly binds to Htr2a distal promoter in vivo

Fig. 10. ChIP-qPCR reveals increased binding of EGR3 to the distal promoter of the Htr2a gene in the mouse frontal cortical tissues 2 hrs post ECS. Genomic DNA was purified from the ChIP and was used as a template for qPCR. The cycle threshold (CT) value of each gene promoter was normalized to that of the input and was further normalized to the CT value of the negative control antibody IgG IP. Unpaired student t-tests were used to compare the statistical significance between the values of the ChIPs from No ECS group vs 2hrs ECS group for each gene. (* p <0.05. n = 11).

To further determine whether EGR3 directly binds to the Htr2a promoter in vivo, we performed ChIP. Previously, our western blot results suggested a significant increase of the EGR3 protein expression 2hrs post ECS (Fig.8). Therefore, we used mouse frontal cortical tissues which were dissected 2hrs post ECS. Genomic DNA bound to EGR3 was isolated by ChIP, and was used as template for a quantitative polymerase chain reaction (qPCR) using primers specific to the two putative EGR3 binding sites in the Htr2a promoter (Fig.9a). Primers specific to the EGR3 binding site in the Arc promoter were used as positive control (Fig.9b). We found there is a 4.8- fold increase of EGR3 protein binds to the Htr2a distal promoter (Fig. 9a, site A) 2hrs post ECS compared with that of the No ECS control group (Fig.10; t_{20} = 2.2117, p < 0.05).
However, there is no significant difference in EGR3 binding to the \textit{Htr2a} proximal promoter (Fig. 9a, site A) between the 2hrs post ECS group and No ECS group (Fig.10; \( t_{20} = 1.33073, p = 0.19825 \)). Our positive control group shows a trend of increase of EGR3 binding to the \textit{Arc} promoter after ECS but the difference is still not significant compared with the No ECS group (Fig.10; \( t_{20} = 1.89413, p = 0.072762 \)). This is not consistent with previous findings that EGR3 directly binds to \textit{Arc} promoter in hippocampus (L. Li et al., 2005a). However, as discussed below, we used different brain tissues and only examine the frontal cortical tissues 2hrs after ECS, we probably will detect EGR3 binds to \textit{Arc} promoter if we examine more time points following ECS. Overall, the ChIP-qPCR results suggested that EGR3 directly binds to the \textit{Htr2a} distal promoter (Fig. 9a, site A) and thus might be able to activate the gene.

\textbf{EGR3 activates the \textit{Arc} proximal promoter}

It has been reported that EGR3 directly binds to \textit{Arc} promoter and actives \textit{Arc} (L. Li et al., 2005a). To validate our dual luciferase reporter assay, first we co-transfected a dual luciferase reporter clone (Fig. 11a) containing portion of the \textit{Arc} promoter (-1049bp / +200bp) with a CMV-EGR3 vector or CMV-vector alone. We found there is an approximately 4.88-fold luciferase signal increase of the \textit{Arc} promoter reporter clone when overexpress EGR3 compared with the CMV-vector alone (Fig.11b). Our results are consistent with previous findings that EGR3 significantly activated \textit{Arc} promoter - driven luciferase (L. Li et al., 2005a). This finding also suggests that CMV- EGR3 vector overexpresses a functional EGR3 protein in cultured neuro2a cell, and our transfection method and luciferase assay working in our laboratory.

\textbf{EGR3 activates the \textit{Htr2a} distal promoter by directly binding}

Our ChIP-qPCR results suggest that EGR3 binds to the \textit{Htr2a} distal promoter in the mouse frontal cortical tissues 2hrs post ECS (Fig. 10). To examine whether this binding is functional \textit{in vitro}, we co-transfected the CMV-EGR3 vector with the dual luciferase construct containing an insert from -2727 bp to -2841bp of the \textit{Htr2a} promoter (Fig. 12a.) into neuro2a
cell. A CMV vector which served as a negative control for the EGR3 overexpression was co-
transfected with the promoter reporter clone.

![Diagram](image)

**Fig. 11. Arc promoter luciferase activity.** Dual luciferase reporter constructs containing portion of the Arc promoter (a) - 1049bp upstream and +200bp downstream of the transcription start site were significantly activated by EGR3 relative to the CMV expression vector alone (b). The GLuc value was normalized to the intern control SEAP luciferase value (GLuc/SEAP ratio) and then to the non-promoter luciferase reporter. Student’s t-test, **** p<0.0001. n = 3. All values included in the figure legends represent means ± SEM.

We found that 24hrs after transfection there is an approximately 3.9-fold increase in the Htr2a distal promoter driven-luciferase signal when EGR3 is overexpressed, relative to the expression of the CMV vector alone (Fig. 12b; t₄ = 21.17, p < 0.0001). Our ChIP and luciferase results suggest that EGR3 directly binds to the Htr2a distal promoter and activates Htr2a.
EGR3 activates the *Htr2a* proximal promoter

Our ChIP-qPCR results show that there is no significant increase of EGR3 binding to the *Htr2a* proximal promoter in the mouse frontal cortical tissues 2 hrs post ECS (Fig. 10). However, we still want to know whether EGR3 could affect *Htr2a* proximal promoter activity, probably through an indirectly way. Thus, we co-transfected a promoter reporter clone containing portion of the *Htr2a* promoter which is 1061bp upstream and 200bp downstream of the *Htr2a* transcription start site (Fig.13a) with CM-EGR3 vector or CMV vector alone.

Interestingly, we found there is about 4.2-fold increase of the *Htr2a* proximal promoter luciferase signal when overexpress EGR3 protein compared with the CMV vector alone (Fig.13b;
Although our ChIP results indicate that there is no significant increase of EGR3 binding to the Htr2a proximal promoter after seizure, the luciferase results suggest the overexpression of EGR3 also significantly activates Htr2a proximal promoter.

**Figure 13. Htr2a proximal promoter luciferase activity.** Dual luciferase reporter constructs containing the Htr2a proximal promoter region (a) - 2841bp upstream and +200bp downstream of the transcription start site were significantly activated by EGR3 relative to the CMV expression vector alone (b). The GLuc value was normalized to the intern control SEAP luciferase value (GLuc/SEAP ratio) and then to the non-promoter luciferase reporter. ***p < 0.001. n = 3. All values included in the figure legends represent means ± SEM.

**Western blot to confirm overexpression of EGR3 protein 24hrs after transfection**

Our dual luciferase assay results show a significant luciferase signal increase 24hrs after co-transfection of CMV -EGR3 vector with promoter reporter clones of our target genes. To further validate that we indeed overexpressed EGR3 by CMV-EGR3 transfection, we performed western blot using extracted proteins from cultured neuro2a cells after collecting culture medium.
for luciferase signal measurement. We found that EGR3 expresses at barely detectable level 24hrs of after CMV vector transfection (Fig. 14). However, the level of EGR3 protein is enriched 24hrs after CMV-EGR3 vector transfection (Fig. 14). Overall, these results indicate that we have successfully overexpressed EGR3 proteins using CMV-EGR3 vector for our promoter reporter luciferase assay studies. Moreover, our luciferase signal is significantly increased due to the EGR3 transactivation, and the immediate early gene transcription factor EGR3, expressed at rarely detectable level in neuro2a cells at baseline.
Fig. 14. The expression of EGR3 proteins 24hrs after co-transfect of CMV-EGR3 vector or CMV- vector alone with each promoter reporter clone in neuro2a cells. EGR3 expressed at barely detectable level in neuro2a cells 24hrs after transfected with CMV vector. The transfection of CMV- EGR3 vector enriched EGR3 protein levels in the neuro2a cells.
Discussion

In the present study, we examined the regulations of Htr2a by EGR3 using both \textit{in vivo} and \textit{in vitro} methods. We hypothesized that EGR3 regulates Htr2a by directly binding to the Htr2a. This hypothesis was based on our previous findings that 1) 5HT2A receptors are decreased nearly 70\% in the prefrontal cortex of Egr3-/- mice, which underlines their resistance to the sedating effect of clozapine (Williams et al., 2012); 2) in Chapter 2 we found that Egr3 is required for Htr2a expression in response to SD, which suggests a potential regulatory relationship between these two schizophrenia candidate genes; 3) we identified two putative EGR3 binding sites on the Htr2a promoter using bioinformatics analysis. To test this hypothesis, we first performed ECS to induce tonic-clonic seizures on WT mice to maximize the EGR3 protein expression. To capture potentially transient binding of EGR3 to the Htr2a promoters, we crosslinked the protein-DNA complex using formaldehyde. By performing ChIP-qPCR, we found EGR3 directly binding to Htr2a distal promoter and thus might regulate Htr2a gene \textit{in vivo}. We also confirmed the functionality of this binding \textit{in vitro} by measuring luciferase activity after co-transfection with a CMV EGR3 vector which overexpressed EGR3 protein together with a Ht2a distal promoter driven luciferase reporter construct.

Prior studies examining the pattern and timeline of IEG expression have predominantly focused on mRNA, using \textit{in situ} hybridization methodology (Cullinan, Herman, Battaglia, Akil, & Watson, 1995; Sukhatme et al., 1988; Wisden et al., 1990b). However, gene expression does not always correlate with changes in protein expression. For example, it has been reported that the levels of mRNA in rat hippocampus for EGR1 and EGR3 are increased in parallel and reached peak levels at 0.5 – 1hr after the maximal electroconvulsive seizure (MECS) (K J O’Donovan et al., 1998). EGR1 protein level closely follows its mRNA expression, with levels peaking at 0.5 - 1hr and returning to basal levels by 4hrs after seizure. In contrast, EGR3 protein levels start to rise at 2hrs with the highest level at 4-6hrs after seizure.

In chapter 2, we found that 6hrs of SD significantly upregulated Egr3 mRNA expression in the prefrontal cortex. In that study, we did not examine the expression pattern of EGR3 protein in the prefrontal cortex. To address this question, we performed ECS, which have been shown
robustly induced Egr3 compared with other stimuli (Vol et al., 1997), and examined EGR3 protein levels of prefrontal cortical tissues using western blot. We found there is a significant increase of EGR3 protein levels 2hrs after ECS. However, we detected two bands of EGR3 protein both in vivo and in vitro (Fig.8 and Fig.14). We used the polyclonal anti-EGR3 antibody from Santa Cruz (catalog number sc-191) which is known to identify two bands (43/46KDa). The reason for two bands could be the polyclonal antibody is not specific for detecting EGR3 protein because the EGRs are high homologues. Particularly, the antigen which used for generating the antibody is C-terminus of the EGR3 protein. The zinc fingers are in this region and are conserved among EGRs. Another possibility is both bands are specific for EGR3 but one band is phosphorylated EGR3 protein. The posttranslational regulation has been investigated for EGR1. It has been reported EGR1 proteins are phosphorylated on a serine residue (Lemaire et al., 1990), and this is induce by serum treatment in BALB/c3T3 cells (X Cao et al., 1990). The phosphorylation of EGRs could enhance their DNA-binding activity (Xinmin Cao et al., 1993). However, further investigation is needed for EGR3 phosphorylation. Overall, these finding is consistent with the prior work that the expression of EGR3 protein level rises 2hrs after ECS. In addition, it provided the basis for us to perform ChIP assay on brain tissues 2hrs after ECS.

The Htr2a promoter has been extensively studied and found to contain multiple regulatory domains, each contributing to the overall expression pattern. A repressor domain located between -2.3kb and -4.2kb of the Htr2a promoter acts as the primary determinant for neuronal cell-specific transcription (M Toth, Ding, & Shenk, 1994). Moreover, Toth and colleagues also found that Mianserin, one of the noradrenergic and specific serotonergic antidepressants (NaSSAs), elicits a decrease in 5HT2A receptor density and this is mediated by a repressor DNA domain embedded in the basal promoter. They also found that upstream of the repressor there is a domain can reactivate the silenced promoter in some non-neuronal cells such as C6 glioma cells (Miklos Toth, 1996).

Interestingly, the putative EGR3 binding site we have identified 2777bp upstream of the Htr2a transcription start site also located in the non-neuronal repressor region of Htr2a promoter. For those non-neuronal cells thatdo not express endogenous Htr2a probably contain regulation
factors that bind within the repressor regions to inhibit Htr2a transcription. In contrast, the neuronal cells might express additional regulators to modify the repressor regions. Some of the EGR family members have been reported to serve as a transcriptional repressors in certain cells. For example, EGR1 served as a repressor for MEF2 transcription activity in both non-cardiac and cardiac cells (Zipfel, Decker, Holst, & Skerka, 1997). EGR4 functions as a repressor and binds to IL-2 and c-fos promoter to inhibit the gene expression (Zipfel et al., 1997). However, the role of EGR2 and EGR3 as transcriptional repressors has not been determined. The EGR transcription factors share a highly homologous zinc finger domain which recognizes an identical DNA response element (Vol et al., 1997). So, it would be interesting to see if EGR3 is still able to activate Htr2a in non-neuronal cells by directly binding to the Htr2a distal promoter which is located in the repressor domain.

Our studies investigating the proximal EGR3 binding site in the Htr2a promoter revealed two results that appeared to be inconsistent. The ChIP qPCR suggests no direct binding of EGR3 to Htr2a proximal promoter in vivo. However, the in vitro luciferase studies show that EGR3 overexpression activates the Htr2a proximal promoter-driven luciferase reporter. One possible explanation for this paradox is that we are unable to detect the binding in our ChIP assay due to the low binding affinity of EGR3 for the Htr2a proximal promoter. It's possible that the EGR3 binding site in the Htr2a proximal promoter is shared with another transcription factor specificity protein 1 (SP1). Sp1 belongs to the Sp/XKLF (Specificity protein/Krüppel-like factor) family transcription factors. The DNA binding domain of Sp1 consists of three contiguous Cys2His2 Zinc (Zn) fingers which are very similar with those that of EGRs transcription factors (Vol et al., 1997). Both the EGR1 binding motif (GCG/GGGCG) and SP1 motif (O/GGGCGGGGC) are usually present in mammalian (G+C)-rich promoters that lack the TATA and CCAAT box motifs (Huang, Fan, Ni, Mercola, & Adamson, 1997).

It has been reported that SP1 and EGR1 have overlapped binding motif in some gene promoters, e.g. murine adenosine deaminase (ADA) and double-sex and Mab-3 related transcription factor 1 (Dmrt1) (Ackerman, Minden, Williams, Bobonis, & Yeung, 1991; Lei & Heckert, 2002). Moreover, SP1 competes with EGR1 and binds to EGR1 binding motif to activate
luciferase gene in cultured *Drosophila* cells (Kutoh & Schwander, 1993). The two EGR1 binding sites in the *Htr2a* basal promoter overlap with SP1 binding motifs (Garlow & Ciaranello, 1995). However, the functionality of this overlap between SP1 and EGR1 in the *Htr2a* promoter has not yet been determined. It would be interesting to determine how the interplay of the two transcription factors affects *Htr2a* gene expression. Nevertheless, all EGR family transcription factors contain the same DNA-binding zinc finger domain, and thus recognize the same DNA consensus binding sequences. So, it is possible that SP1, or the other EGR family proteins (all of which are upregulated in by ECS), competed for the binding site in the *Htr2a* proximal promoter, resulting in lower levels of EGR3 binding that we were unable to pull down in our ChIP study.

EGR3 activates *Arc* gene expression by directly binding to the *Arc* gene promoter (L. Li et al., 2005a). We used *Arc* promoter reporter clone as a positive control for the *in vitro* luciferase and found that EGR3 activates *Arc* promoter-driven luciferase gene. However, we did not see significantly increase of EGR3 binding to *Arc* promoter 2 hrs post ECS in the ChIP assay, which is not consistent with prior work that EGR3 directly binding to *Arc* promoter (L. Li et al., 2005a). We performed ChIP based on the finding that EGR3 proteins significantly increase 2hrs post ECS using western blot. However, MECS induced EGR3 levels in mouse hippocampus have been shown to rise at 2hrs after seizure and reach peak level at 4-6 hrs (Kevin J. O’Donovan et al., 1999). Moreover, ECS induced biphasic expression of *Arc* mRNA in mouse hippocampal CA1 region, with a first wave 30 minutes after ECS and returned to baseline by 4 - 6 hrs, and a second one at 8 hrs post stimuli (Penke, Chagneau, & Laroche, 2011). In addition, EGR3 was shown to be required for *Arc* protein expression in the dentate gyrus 4 hrs after kainic acid-induced seizures in an immunohistochemistry study (L. Li et al., 2005a). Those studies were performed on hippocampal tissues. It has not determined whether the expression patterns of *Egr3* and *Arc* in the frontal cortex resemble those in the hippocampus. So, it is possible that we would detect that EGR3 directly binds to *Arc* promoter if we perform ChIP-qPCR using frontal cortical tissues from several timepoints ranging between 1- 6hrs after ECS.

In conclusion, we found that EGR3 alters the *Htr2a* gene expression by directly binding to the *Htr2a* distal promoter. Excitingly, we also found EGR3 activates *Htr2a* proximal promoter-
driven luciferase even though the ChIP-qPCR data does not support direct binding between them.

As we discussed above, the activation of EGR3 to Htr2a proximal promoter probably through some intermediates such as c-fos, or through directly binding. However, to further validate whether both or either of the putative binding sites are required for the directly regulation of Htr2a by EGR3, we still need to mutate the individual EGR3 binding site of the Htr2a promoter using site-directed mutagenesis.
CHAPTER 4
GENERAL DISCUSSION

Summary of Major Results

Both \textit{Egr3} and \textit{Htr2a} are associated with schizophrenia (Huentelman et al., 2015; Nishimura et al., 2014; Sujitha et al., 2014; Yamada et al., 2007b). Our previous findings that 5HT\textsubscript{2A} receptors are decreased in prefrontal cortex (PFC) of \textit{Egr3}-/- mice suggest a potential regulatory relationship between these two schizophrenia susceptibility genes (Williams et al., 2012). The aim of this dissertation is to identify the mechanism by which the immediate early gene \textit{Egr3} regulates the expression of \textit{Htr2a}, under basal conditions and in response to stress.

In chapter 2, we found that 6hrs of sleep deprivation (SD) activates expression of both \textit{Egr3} and \textit{Htr2a}, particularly in the prefrontal cortex. These findings are consistent with prior work reporting that SD increase the expression of \textit{Egr3} (Thompson et al., 2010) in mice and 5HT\textsubscript{2A}R levels in (Elmenhorst et al., 2012) in humans. Furthermore, the increase of \textit{Htr2a} is dependent on \textit{Egr3} because SD fails to induce \textit{Htr2a} in \textit{Egr3}-/- mice, which supports our previous findings that 5HT\textsubscript{2A}R protein is decreased in the PFC of \textit{Egr3}-/- mice (Williams et al., 2012) with findings at the mRNA level. Our examination in sub-regions of the cortex revealed that SD activates \textit{Egr3} mostly in posterior cortex (2.6-fold) and \textit{Htr2a} mostly in PFC (1.7-fold), which indicates that gene expression pattern in our study resembles those in prior work (Mengod et al., 1990; Pompeiano et al., 1994; Thompson et al., 2010). In addition, even \textit{Egr3}-/- mice display a hyper-locomotor phenotype (Gallitano-Mendel et al., 2007), but our analysis of the amount of stimulus show that WT and \textit{Egr3}-/- mice need the same amount of intervention to maintain wakefulness.

In chapter 3, our chromatin immunoprecipitation (ChIP) show that ECS induces significant binding of EGR3 to \textit{Htr2a} distal, but not proximal, promoter. We further confirmed the functionality of EGR3 binding to \textit{Htr2a} distal by co-transfecting \textit{Htr2a} distal promoter-driven luciferase vector with CMV Egr3 vector or CMV vector alone. Interestingly, even though ChIP shows no significant binding of EGR3 to \textit{Htr2a} proximal promoter, we found that overexpression of EGR3 activates the \textit{Htr2a} proximal promoter-driven luciferase gene. The paradox could be explained by 1) EGR3 activates Htr2a proximal promoter through other transcriptional regulators.
We believe *c-fos* is one of the intermediates because *Egr3* is required for *c-fos* expression (Fig.15) and the ENCODE project has identified EGR1 binding site in *c-fos* promoter (https://www.encodeproject.org). In addition, C-FOS heterodimerizes with JUN to form AP-1 and activates luciferase gene driven by a Htr2a proximal promoter (Garlow & Ciaranello, 1995); 2) SP1 competes with EGR3 and results in low binding affinity of EGR3 binding to *Htr2a* proximal promoter.

**Concluding Remarks**

The findings in chapter 2 and 3 provide potential mechanisms by which the immediate early gene *Egr3* regulates the expression of *Htr2a*. Numerous studies have emphasized that dysregulation of *Htr2a* is associated with schizophrenia (Gejman, Sanders, & Kendler, 2011; Sujitha et al., 2014). However, little is known about how environmental factors affects *Htr2a* expression and influence schizophrenia risk. For years, we have been focusing on the *Egr3*, a IEG that activated rapidly after stimulation. *EGR3* has been identified as the central gene in a network of transcription factors and microRNAs implicated in schizophrenia susceptibility (Guo et al., 2010). In the present study, we found that *Egr3* regulates *Htr2a* transcription in response to stress, and this regulation is by direct binding of EGR3 to a *Htr2a* distal promoter. Our findings suggest that the induction of *HTR2A* in response to stress likely represents a physiological response. These findings suggest the possibility that the reduced levels of 5HT2ARs repeatedly identified in schizophrenia patients may result from deficient induction of *HTR2A*, due to insufficient activation of EGR3 protein. Although the increased *HTR2A* expression may appear beneficial, it is rather paradoxical that many antipsychotics block 5-HT2ARs (Meltzer & Huang, 2008) but 5-HT2AR agonists such as lysergic acid diethylamide (LSD) are hallucinogens (González-Maeso et al., 2003). The paradox is difficult to be deciphered partly because of complicated biological context. For instance, the atypical antipsychotics clozapine, a 5-HT2AR antagonist, also acts as an agonist at 5-HT2ARs to counter dizocilpine-induced behaviors by activation of Akt/Protein kinase B (PKB) (Schmid et al., 2014).
To our knowledge, this is first report that identifies directly regulation mechanisms between the two schizophrenia susceptibility genes, \textit{EGR3} and \textit{HTR2A}, under stress conditions. In addition, these exciting findings supporting our proposed regulatory network in which environmental factors act on a complex set of schizophrenia candidate genes including \textit{Htr2a} via activation of the immediate early gene \textit{EGR3}, and the interplay between genetic and environmental factors probably determine an individual’s risk for schizophrenia.

\textbf{Potential regulatory networks integrate genetic and environmental influences on schizophrenia risk}

Genetic inheritance is one of the factors that contribute to the cause of schizophrenia (Cardno & Gottesman, 2000). Environmental stimuli such as stress may interact with a complex set of schizophrenia candidate genes and influence the susceptibility to the disease. \textit{EGR3} is an IEG that activated by various stimuli and is associated with schizophrenia (Huentelman et al., 2015; S. H. Kim et al., 2010; Mexal et al., 2005; Yamada et al., 2007b; Zhang et al., 2012).

Numerous studies show that \textit{EGR3} is a downstream gene of many schizophrenia risk associated proteins. For instance, \textit{EGR3} can be induced by N-methyl-D-aspartate (NMDARs). Pretreating rats with NMDARs antagonist failed to induce \textit{Egr3} mRNA compared with no treatment group (Yamagata et al., 1994). Moreover, Hypofunction of the NMDAR pathway contributes to etiology of schizophrenia (Olney, Newcomer, & Farber, 1999). \textit{EGR3} is also regulated by the calcium-responsive protein phosphatase calcineurin (CN) (Mittelstadt & Ashwell, 1998), which probably is induced by a calcium influx through NMDARs (Sheng & Kim, 2011). Human genetic studies show that CN signaling is associated with schizophrenia susceptibility (Gerber et al., 2003). Coincidentally, the conditional calcineurin knockout mice exhibit multiple
abnormal behaviors such as increased locomotor activity and decreased social interaction (Miyakawa et al., 2003) are very similar to our Egr3-/- mice (Gallitano-Mendel et al., 2007, 2008). Another schizophrenia candidate protein brain-derived neurotrophic factor (BDNF) also regulates EGR3 via a PKC/MAPK-dependent pathway (Daniel S Roberts, Hu, Lund, Brooks-Kayal, & Russek, 2006) and the induced EGR3 thus activates GABRA4 promoter and leads to upregulation of GABA(A) receptor alpha4 subunit expression (D.S. Roberts et al., 2005). Egr3 is also regulated by the neuregulin1 (NRG1) by stimulating the transcriptional activity of cAMP response element binding protein (CREB) and serum response factor (SRF) (Herndon,
Ankenbruck, Lester, Bailey, & Fromm, 2013). In addition, NRG1 and its receptor ErbB4, a receptor tyrosine-protein kinase, participate in cognitive dysfunction in schizophrenia by suppressing proto-oncogene tyrosine-protein kinase Src-mediated upregulation of synaptic NMDAR function (Pitcher et al., 2011).

In addition to be downstream of susceptibility genes, *EGR3* also directly regulates genes that have been associated with schizophrenia. As discussed above, *Egr3* stimulates the expression of *GABRA4* by binding its promoter (D.S. Roberts et al., 2005) and thus upregulates of GABA(A) receptor alpha4 subunit expression. Studies showing that reduced GABA transmission in prefrontal cortex of postmortem schizophrenia patients' brains (Tse et al., 2015). Moreover, EGR3 directly binds to *ARC* promoter and activates this gene (L. Li et al., 2005a). *ARC* SNPs is also associated with schizophrenia risk (Huentelman et al., 2015). Our present study also shows that EGR3 activates *Htr2a* by directly binding to *Htr2a* promoter. Overall, those findings led us to propose a regulatory network (Fig. 15) that integrates genetic and environmental factors influences on schizophrenia risk.

**Limitation of the current study**

In the study, we used the *Egr3* -/- mice which are generated by the deletion of sequences that encode the zinc fingers, the DNA-binding domain of the protein (Tourtellotte & Milbrandt, 1998). Although studies on these animals revealed no detectable differences in the distribution and density of neurons compared with WT mice (L. Li et al., 2007), it is still unclear the potential function of the truncated EGR3. In addition, deleting regions outside the zinc fingers increases the DNA-binding activity of EGR proteins such as EGR1 and EGR2 (Carman & Monroe, 1995; Vesque & Charnay, 1992). In contrast, intact EGR1 protein displays higher DNA-binding affinity compared with the zinc finger domain alone (Vesque & Charnay, 1992). Moreover, the sequences outside the zinc finger region of EGR4 are responsible for its faster dissociation rate from target DNA relative to other EGR proteins (a H. Swirnoff & Milbrandt, 1995). However, the functions of the sequences outside the zinc finger region of EGR3 are not determined. We cannot
exclude the possibility that the truncated EGR3 protein has a dominant inhibitory effect in the 
*Egr3* -/- mice which we are unable to detect in the present study.

It is also possible that other transcription factors, particularly EGRs, may functionally compensate for the loss of EGR3. EGRs are highly homologous and bind to the same DNA consensus binding motif (Vol et al., 1997). It has been shown that EGR3, rather than EGR1, is required for *Arc* expression in the dentate gyrus following seizure. However, the full induction of *Arc* after novel environment exposure is depend upon both EGR1 and EGR3 (L. Li et al., 2005a; Penke et al., 2011). These findings suggest that the *Arc* expression is controlled by both EGR1 and EGR3. Thus, it is not determined whether other EGRs are compensating for the lack of functional EGR3. In addition, dysfunction of *Egr3* throughout development and postnatal life may contribute to some of the behavior and physiologic abnormalities identified in *Egr3*-/- mice. With the *Egr3* conditional knockout mouse, we would be able to selectively inactivate *Egr3* at specific stages of development and in selected organs or cell types. In addition, we would be able to perform specific behavioral tests that require intact motor skills, which is precluded now because the muscle spindle morphogenesis is impaired and thus leads to abnormal proprioception in the current *Egr3* -/- mice (Tourtellotte & Milbrandt, 1998).

**Fig. 16.** *Egr3*-dependent expression of mouse *c-fos* gene. Illumina expression microarray performed on mouse hippocampal RNA at baseline (No ECS) versus 1hr post electroconvulsive stimulation (ECS) showed that *c-fos* mRNA expression is increased in WT but not *Egr3* -/- mice in response to ECS. Two-way ANOVA. ****p <0.0001. n = 4. All values included in the figure legends represent means ± SEM.
Future directions

*Characterize the role of EGR3 in regulating Htr2a proximal promoter*

The ChIP-qPCR suggests no direct binding of EGR3 to *Htr2a* proximal promoter *in vivo*. However, our *in vitro* luciferase studies show that EGR3 overexpression activates the *Htr2a* proximal promoter-driven luciferase reporter. One possible explanation for this paradox is that the activation of the *Htr2a* proximal promoter-driven luciferase construct is not through direct binding of EGR3, but occurs via other intermediating transcriptional regulators. One intermediate gene between EGR3 and *Htr2a* is *c-fos*, an IEG which encodes the transcription factor C-FOS. It can heterodimerize with the JUN family transcription factors to form the transcription factor complex activator protein -1 (AP-1) which binds DNA promoters and enhancer regions of target genes (Chiu et al., 1988). We have previously found that *Egr3* is required for *c-Fos* gene expression in hippocampal tissues 1hr post electroconvulsive seizure (ECS) (Fig. 16). Furthermore, the ENCODE project has produced genome-wide data for investigating different aspects of genomic regulation, including DNA-binding of transcription factors (https://www.encodeproject.org/). The ENCODE TFBS ChIP-sequencing analyses demonstrate a EGR1 binding site in the *c-fos* promoter in three cell lines (GM12878, H1-hESC and K562, Lab PI: Richard Myers, Hudson Alpha Institute for Biotechnology). The ENCODE TFBS ChIP also identified two C-FOS binding sites in the *Htr2a* promoter (in MCF10A-Er-Src cell line; Lab PI: Kevin Struhl, Harvard Medical School). In addition, it has been shown that in cultured neuro2a cells, AP-1 activates luciferase promoter reporter clone contains an insert which is 1000bp upstream and 300bp downstream of *Htr2a* transcription start site (Garlow & Ciaranello, 1995). Therefore, it is possible that the overexpression of EGR3 induces *c-fos* expression and results in increased AP-1 directly binding to the *Htr2a* proximal promoter and thus activates the luciferase gene.

Thus, to determine whether EGR3 regulates *Htr2a* proximal promoter through the intermediate *c-fos*, it would be necessary to mutate the AP-1 binding motif (5'-TGA<sup>G</sup>/CT<sup>C</sup>/AA-3') (Garlow & Ciaranello, 1995) in the mouse *Htr2a* proximal promoter. If this mutation could decrease or abrogate the *Htr2a* proximal promoter driven-luciferase activity which is induced by...
EGR3 expression, then this would provide direct evidence that c-fos is an intermediate transcription factor functioning between EGR3 and activation of the Htr2a proximal promoter.

It has been reported that SP1 and EGR1 have overlapped binding sites on the Htr2a proximal promoter (Garlow & Ciaranello, 1995). So, it is possible that SP1 competes with EGR3 for the overlapping binding motif in Htr2a proximal promoter results in low binding affinity of EGR3 to Htr2a and thus we did not catch the binding in ChIP. However, EGR3 probably still activates the Htr2a promoter by weak binding or through interacting with SP1. In this case, the presence of SP1 and the role of SP1 need to be determined. Incubate an anti-SP1 specific antibody (Ab) and anti-EGR3 Ab with nuclear extracts in the super-shift electrophoretic mobility shift assay (EMSA) would allow to us to detect the binding of both transcription factors to the Htr2a proximal promoter. However, to confirm the regulation of SP1 and EGR3 by direct binding to the promoter, site-direct mutagenesis would be required to mutate their binding motif in in vitro luciferase assays.

Moreover, three overlapping SP1 and EGR1 binding sites has been found in the promoter of tissue factor (TF), a gene involved in blood coagulation, with SP1 binding preferentially to site 1 and 3 while site 2 has high affinity to EGR1 (Cui et al., 1996). Sp1 is constitutively expressed and mediates basal gene activity by binding to all three sites. EGR1 binding to site 2 accounts for the strong upregulation of TF in response to phorbol12-myristate 13-acetate (PMA) or serum stimulation. Thus, the output of the interaction between SP1 and EGR3 on certain gene could be dependent on the cellular environment.

**How the promoter context of Htr2a affects the actions of EGR3**

In chapter 3, we show that EGR3 activates Htr2a expression by directly binding to the Htr2a distal promoter. We also found that EGR3 activates Htr2a proximal promoter-driven luciferase gene expression through an unclear mechanism. However, in vitro luciferase reporter system which contains the synthetic promoter constructs provides information concerning EGR3 potential action on the promoter, but does not necessarily provide information about the specific contribution of EGR3 and other co-regulators to regulation of the natural, full Htr2a promoter. The transcriptional regulation actions of EGRs may be controlled by the promoter context of the target
gene. It has been reported that cell types affect the activation or repression action of EGR1 on a gene. For example, EGR1 activates the platelet-derived A chain gene in human embryonic kidney cell 293 (HEK293) but represses transcription in murine NIH 3T3 fibroblasts (Wilms et al., 1999). Thus, the ability of EGR1 to function as an activator or repressor of transcription is cell-type specific.

As discussed in chapter 3, the distal EGR3 binding site is located in the repressor domain of Htr2a gene (see also Fig.17). The repressor domain acts as the primary determinant for neuronal cell-specific transcription (M Toth et al., 1994). In addition, upstream of the repressor there is a domain that can reactivate the silenced promoter in some non-neuronal cells such as C6 glioma cells (Miklos Toth, 1996). A potential molecular mechanism for limiting Htr2a expression to specific cortical regions is the known repression domain of the Htr2a promoter. I would hypothesize that the non-neuronal cells that do not express endogenous Htr2a likely express cellular factors that bind to, or modify the structure of, the Htr2a promoter repressor domains.

**Fig. 17. EGR3 binds to a repressor domain of the mouse Htr2a promoter.** A non-neuronal cell repressor domain exists between -1.2kb and -3.1kb upstream of Htr2a transcriptional start site (TSS, +1). The promoter region up to -4kb is sufficient for brain specific expression. The EGR3 distal binding site (A, AGGAGGGGGAGTC) is in the repressor region while the proximal binding site (B, GCGCGGGGGAGGGG) is in the base promoter region. * Figure is modified from Dr. Miklos Toth’s paper (Miklos Toth, 1996).
In addition to EGR1 serving as a repressor for MEF2 transcription activity in both non-cardiac and cardiac cells (Zipfel et al., 1997), EGR4 also has been found to function as a repressor by binding to *IL-2* and *c-fos* promoters to inhibit the gene expression (Zipfel et al., 1997). However, the role of EGR2 and EGR3 as transcription repressors is not determined. It would be interesting to test the transcriptional activity of EGR3 on the *Htr2a* distal promoter in non-neuronal cells.

Moreover, it has been found that SNPs in the HTR2A promoter could affect EGR3 binding to this gene in humans. For instance, using bioinformatics analysis, the minor allele for rs6311 impacts the alignment of consensus binding site for EGR3 at the rs6311 region of the HTR2A gene (Ruble et al., 2016). In the same study, Ruble et al. identified other transcription factors that potentially bind at the rs6311 region such as the nuclear factor 1 family (NFIA/B/C). However, further analysis and experiments are necessary to test the effect of SNPs in EGR3 on binding to the *HTR2A* promoter.

**Are Egr3 /- mice deficient in serotoninergic excitation of callosal/commissural (COM) projection neurons in PFC?**

The findings of sleep deprivation (SD) in chapter 2 reveal that *Egr3* alters *Htr2a* transcription in the mouse prefrontal cortex (PFC). The PFC is highly interconnected with other regions of the brain, including projections to the contralateral cortex and to the pontine nuclei (Avesar & Gulledge, 2012; Barbas, Hilgetag, Saha, Dermon, & Suski, 2005). Previous electrophysiological studies have shown that 5-HT selectively excites layer 5 pyramidal neurons (L5PNs) of callosal/commissural (COM) projection in PFC that innervate the contralateral cortex by acting on 5HT2ARs (Avesar & Gulledge, 2012). In contrast, 5-HT elicits inhibition in L5PNs of cortico-pontine (CPn) projection, which is mediated by 5HT1ARs. We have found that *Egr3 /- mice* have approximately 70% reduction of 5HT2ARs in PFC (Williams et al., 2012). In addition, this dissertation’s findings suggest *Egr3* is required for *Htr2a* induction after SD because SD failed to induce *Htr2a* in *Egr3 /- mice*. These findings lead us to ask the question of whether *Egr3*
mice are deficient in serotonergic excitation of COM projection neurons in PFC due to lack of Htr2a?

To answer this question, we already started collaboration with Dr. Allan Gulledge’s laboratory from neuroscience center at Dartmouth College. We performed electrophysiology recording using the protocol previously reported (Stephens, Avesar, & Gulledge, 2014). Briefly, we first performed stereotaxic injections of fluorescent beads (Retrobeads, Lumafluor Inc.) unilaterally into the contralateral medial prefrontal cortex (mPFC) to label COM projections, or into the pons to label CPn projections. After surgery, animals were in their home cages over 72 hrs for recovery. Electrophysiology recordings were performed in the coronal brain slices (250 μm thick) of the mPFC in artificial cerebral spinal fluid (aACSF). 100 μM of 5-HT dissolved in aACSF was puffed by a patch pipette placed ~50 μm from the targeted soma during recording. Based on their initial response to 5-HT, neurons are defined as “COM-excited” or “COM-biphasic”. The inhibition response to 5-HT was quantified as the duration of cessation of the action potential (AP). The excitation response was quantified as the peak increase in AP, relative to the average baseline firing frequency. The biphasic response was defined as an inhibition duration which is over 10

![Fig. 18. 5-HT focal application induces different responses in layer 5 pyramidal neurons (L5PNs) of callosal/commissural (COM) projection in Egr3-/- mice (a) and WT mice (b) with Taconic and Jackson laboratory C57BL/6 mice background, respectively. Figure 18b is from Avesar & Gulledge study (Avesar & Gulledge, 2012)
times the average baseline interspike interval, followed by an increase of AP over 1 Hz. To confirm if a specific neuronal response to 5-HT is mediated by 5HT$_{1A}$Rs or 5HT$_{2A}$Rs, antagonists for 5HT$_{1A}$Rs (WAY 100635, 30 nM; Sigma–Aldrich) and/or 5HT$_{2A}$Rs (MDL 11939, 500 nM; Tocris Bioscience) were applied to some experiments.

To date, we recorded $Egr3^{-/-}$ mice, with 28 cells of COM projections (n=3 mice) and 33 cells of CPn projections (n=3 mice). We found 4 different responses to 5-HT in COM L5PNs of $Egr3^{-/-}$ mice (Fig.18a): 5HT$_{2A}$R-mediated excitation (39% of L5PNs), 5HT$_{1A}$R-mediated inhibition (25% of L5PNs), biphasic responses in which 5HT$_{2A}$R-mediated excitation followed 5HT$_{1A}$R-mediated inhibition (11%), and no response neurons (25% NR). In contrast, previous studies (Avesar & Gulledge, 2012) found that 5-HT induces 58% excitation, 35% biphasic response, and 7% NR of COM L5PNs in WT mice (Fig.18b). These findings suggest that the decreased excitation and increased inhibition in $Egr3^{-/-}$ mice are due to decreased 5HT$_{2A}$R level in COM L5PNs. Among the 33 cells we recorded in CPn projections in our $Egr3^{-/-}$ mice, 94% are inhibited by 5-HT application, while 3% are excited and 3% are biphasic (Fig.19a). However, Avesar & Gulledge (Avesar & Gulledge, 2012) found that CPn L5PNs are 100% inhibited by 5-HT.

**Fig. 19.** 5-HT focal application induces different responses in layer 5 pyramidal neurons (L5PNs) of cortico-pontine (CPn) projection in $Egr3^{-/-}$ mice (a) and WT mice (b) with Taconic and Jackson laboratory C57BL/6 mice background, respectively. Figure 19b is from Avesar & Gulledge study (Avesar & Gulledge, 2012) and is used with permission.
in WT mice (Fig. 19b). Since the L5PNs inhibition response is mediated by 5-HT$_{1A}$Rs in CPn projection, our findings suggest a decrease of 5-HT$_{1A}$Rs CPn L5PNs in the $Egr3^{-/-}$ mice.

However, it should note that our $Egr3^{-/-}$ mice are from Taconic (C57BL/6NTac), while the WT mice from Avesar & Gulledge study are from Jackson Laboratory (C57BL/6J). Even both of the mice are C57BL/6, it has been reported that there are genetic and behavior difference among the C57BL/6 substrains (Bothe, Bolivar, Vedder, & Geistfeld, 2004; MEKADA et al., 2009; Palmer & McRoberts, 2013). For instance, 12 of 342 simple sequence length polymorphism markers (3.5%) were found different between C57BL/6J and C57BL/6NTac mice (Bothe et al., 2004). Genotyping for single nucleotide polymorphisms (SNPs) loci shows genetic differences between C57BL/6J and other C57BL/6 substrains including C57BL/6NTac (MEKADA et al., 2009). In addition, the C57BL/6J demonstrates approximately a 2-fold latency to fall off than the C57BL/6NTac in the rotarod performance (Bothe et al., 2004). Compared with other C57BL/6 substrains such as C57BL/6NTac and C57BL/6NCrl (Charles River), C57BL/6J mice also display enhanced motor coordination and reduced level of conditional fear (Palmer & McRoberts, 2013). So, we cannot exclude the possibility that the different responses to the 5-HT application of our $Egr3^{-/-}$ mice compared with the WT mice from Avesar & Gulledge study could due to the differences among C57BL/6 substrains.

To answer this question, we started to record WT mice from our lab which has the C57BL/6NTac background. So far, we recorded 16 cells of COM projections (n=1 mouse) and 5 cells of CPn projections (n=1 mouse). Unfortunately, we observed L5PNs of both COM and CPn projections in those WT mice from our lab demonstrate the same response to 5-HT as our $Egr3^{-/-}$ mice. However, we still cannot make any final conclusion yet because we only recorded one WT mouse for COM and CPn projections. More animals are needed to investigate whether $Egr3^{-/-}$ mice are deficient in serotonergic excitation of callosal/commissural (COM) projection neurons in PFC.

We also filled target neurons with biocytin when performed electrophysiology recording (Figure 20a). Brain slices were fixed with 4% PFA and biocytin-filled neurons were avidin conjugated with Alex Fluor 550 (Invitrogen). Then we reconstructed the labeled neurons using
Neurolucida neuron tracing software (MBF Bioscience, Williston, VT) (Figure 20b). Sholl analysis will be performed on reconstructed neurons to quantify and compare the complexity of dendritic arbors between Egr3 \(-/-\) and WT.

![Image of biocytin labeled neurons and neuron tracing](image)

**Fig. 20.** Representative images of biocytin labeled neurons and neuron tracing.

(a) Representative images of biocytin labeled layer 5 pyramidal neuron after electrophysiology recording in mouse prefrontal cortex. (b) Example of three-dimensional reconstruction of pyramidal neuron with the soma (red), basal dendrites (white), apical dendrites (green) using Neurolucida neuron tracing software (MBF Bioscience, Williston, VT).

**Troubleshooting and alternative strategies**

1. The SD project was performed over 4 years (2013 to 2016) and 5 cohorts of animals were included. Considering the feature of *Egr3* as an IEG that can be rapidly activated and degraded, I employed several strategies to decrease the variation between each study. First, schedule the SD experiment on the day when no construction is taking place in the building and fewer people are working in the animal colony room. Light and sound are stimuli that activate IEGs. So, control animals should not be disturbed more excessively than normal in their home cages. Second, try to dissect animals from matched pairs at the same time since *Egr3* mRNA degrades very quick. Otherwise, different dissecting time can cause gene expression differences within the same group. Third, if you have many cohorts, as I did, gene expression from animals which are sleep
deprived should be normalized to the control on the same day. It will be helpful for replication of the results.

2. For the ChIP-qPCR, I combined anterior and prefrontal cortical tissues because mouse prefrontal cortex is tiny (around 15mg). The exact amount of tissue needed for ChIP may vary depending on protein abundance, antibody affinity etc. and should be determined for each tissue type. Many publications and company manuals suggested more than 25mg of tissues for ChIP. Our qRT-PCR data of SD project in chapter 2 show Egr3 alters Htr2a expression only in prefrontal cortex but not in the anterior cortex. Unfortunately, there is no evidence about how the combination of anterior and prefrontal cortex would affect our ChIP results. In future, people in lab can combine prefrontal cortical tissues from serval animals of the same group.

3. Several questions should be considered to optimize experimental conditions for in vitro luciferase. 1) Which transfection reagent is appropriate for your cell type? I started with calcium phosphate transfection in neuro2a cells but have very low transfection efficiency. Calcium phosphate is probably effective in transfecting DNA into non-neuronal cell lines while Lipofectamine is more appropriate for neuronal-derived cell lines. 2) How long after transfection should we collect cell supernatant medium to check luciferase activities? It is necessary to check luciferase signals after transfection at a variety of different time points. This will depend on the gene promoter activities. 3) How much plasmid DNA should be transfected into cells? This is particularly important for weak gene promoter detection. In chapter 3, we can see that using the same amount of DNA, Arc promoter driven luciferase signal is higher than the Htr2a promoter driven luciferase signal. Different dilutions of plasmid DNA should be used for optimization of transfection for a specific promoter reporter clone.
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APPENDIX A

THE SEROTONIN (5-HT) RECEPTORS
The 5-HT₁ receptor family includes 5-HT₁A, 5-HT₁B, 5-HT₁D, 5-HT₁E and 5-HT₁F receptors. They are all Gᵢ/Gₒ-protein coupled proteins which couple negatively to adenylate cyclase and lead to membrane depolarization and inhibition of firing in cells (Hannon & Hoyer, 2008). What was previously termed the 5-HT₁c receptor has been reclassified as the 5-HT₂C receptor after it was found to be more closely related to the 5-HT2 family (D Hoyer et al., 1994).

Depending on the brain regions, the 5-HT₁A receptor can cause neuronal hyperpolarization by inhibition of adenylyl cyclase activity and/or the opening of potassium channels. In addition, the receptor autoradiography studies revealed that the density of 5-HT₁A Rs is higher in hippocampus, lateral septum, cortical areas and the mesencephalic raphe nuclei compared with the basal ganglia and cerebellum (Hannon & Hoyer, 2008; Pedigo, Yamamura, & Nelson, 1981). Moreover, in situ hybridization and immunocytochemical studies further demonstrate that 5-HT₁A Rs exist in cortical pyramidal neurons, pyramidal and granular neurons of the hippocampus. It has been found in the hippocampus, 5-HT₁A Rs are coupled to both effector systems while in the dorsal raphe nucleus, 5-HT₁A Rs are coupled only to the opening of potassium channels (Frazer & Hensler, 1999).

The 5-HT₁B and 5-HT₁D receptor also inhibit adenylyl cyclase activity. The 5-HT₁B receptor was originally thought to be rodent-specific, whereas 5-HT₁D is limited to non-rodents. However, studies found high similarities in transduction features, function, and brain distribution between these two receptors (Daniel Hoyer & Middlemiss, 1989). Therefore, the rodent ‘5-HT₁B’ and non-rodent ‘5-HT₁D’ receptors are now thought to be species homologues. But, this opinion is questioned again because two genes encoding the human 5-HT₁D receptor, 5-HT₁Dα and 5-HT₁Dβ have been discovered (D Hoyer et al., 1994). Further studies are needed to determine whether the 5-HT₁B and 5-HT₁D receptors are species homologs or distinct 5-HT receptor subtypes.

in situ hybridization has localized the expression of 5-HT₁E receptors. It has been found the 5-HT₁E receptors mostly exist in the caudate putamen, parietal cortex and olfactory tubercle. The exact function of the 5-HT₁E receptor in vivo is not clear due to the lack of selective agonists or antagonists. In addition, the homology with the 5-HT₁D receptor of 5-HT₁E receptor is 64%, which is higher than any other 5-HT1 receptor (D Hoyer et al., 1994). However, both the 5-HT₁E
and 5-HT\textsubscript{1F} receptor inhibit adenylyl cyclase activity \textit{in vitro} (Lucas & Hen, 1995). In addition, the 5-HT\textsubscript{1F} receptor is 61% homologous with 5-HT\textsubscript{1E} receptors. The 5-HT\textsubscript{1F} receptor is widely distributed throughout the nervous system including in the cortex, hippocampus, and spinal cord (D Hoyer et al., 1994).

The 5-HT\textsubscript{2} receptor family includes the 5-HT\textsubscript{2A}, 5-HT\textsubscript{2B} and 5-HT\textsubscript{2C} receptors. They share 46-50% sequence identity and couple preferentially to Gq/11 to increase inositol phosphates and cytosolic Ca\textsuperscript{2+} to induce muscle contraction and neuron activation in the brain (Julius, Huang, Livelli, Axel, & Jessell, 1990). The 5-HT\textsubscript{2A}R encoding gene \textit{HTR2A} is highly expressed in the cerebral cortex (especially in laminae I and IV-Va), the olfactory bulb, and in the brain stem, but at only very low levels in the cerebellum, thalamic nuclei and hippocampus. It has been further identified that 5-HT\textsubscript{2A} receptors are expressed in pyramidal cells and interneurons in the neocortex (Hannon & Hoyer, 2008). In addition, \textit{HTR2A} gene promoter polymorphisms are associated with the response to clozapine in schizophrenia patients (Arranz, Munro, & Owen, 1998). The T102C polymorphism in \textit{HTR2A} was found to be associated with schizophrenia in several populations (Lin et al., 2015; Sujitha et al., 2014). Moreover, mice lacking 5-HT\textsubscript{2A} receptors shows a paradoxical constitutive behavioral sensitization to amphetamine (Salomon et al., 2007), as well as changes in sleep pattern opposite to WT mice (Popa, Prenat, Gingrich, Escourrou, & Hamon, 2005). Those findings suggest that 5-HT\textsubscript{2A} receptors also play an important role in sleep-wakefulness regulation. Furthermore, it is found that MDL100907, a selective 5-HT\textsubscript{2A} receptor antagonist, does not impact short term memory (STM) but facilitates long term memory (LTM). However, the selective 5-HT\textsubscript{1B} receptor antagonist SB-224289 facilitated both STM and LTM (Meneses, 2007). These findings indicate that 5-HT mediates STM and LTM via binding to 5-HT\textsubscript{1B} receptors, whereas other receptors are also involved in memory.

In rat, the 5-HT\textsubscript{2B} mRNA is extensively detected in peripheral organs, but is not detected in brain using quantitative polymerase chain reaction (qPCR) (D Hoyer et al., 1994). In contrast, 5-HT\textsubscript{2B} receptor immunoreactivity has been detected in rat brain but only restricted to a few brain regions such as cerebellum, dorsal hypothalamus and medial amygdala. Moreover, the cells expressing 5-HT\textsubscript{2B} receptor immunoreactivity are more like neurons but not astrocytic cells.
(Bonhaus et al., 1995). Thus, further studies are needed to decipher the controversial of 5-HT$_{2B}$ receptor’s presence in the brain, especially that of the rat. In humans, 5-HT$_{2B}$ receptor mRNA has been found both in peripheral organs and in brain regions such as cerebellum, cerebral cortex (D Hoyer et al., 1994). It has been reported that 5-HT2B receptors on endothelial cells mediate vaso-relaxation via nitrogen oxides (NO) release (Linder, Gaskell, Szasz, Thompson, & Watts, 2010). In addition, in human small intestine, 5-HT$_{2B}$ receptors mediate the longitudinal muscle contraction (Fitzgerald et al., 2000). Moreover, the deletion of 5-HT$_{2B}$ receptor in mice causes severe embryonic defects and is lethal. However, the overexpression this receptor in mice leads to severe cardiovascular disorder (Nebigil et al., 2003).

Both the 5-HT$_{2C}$ receptor and mRNA expression are detected in the choroid plexus and limbic structures include hippocampus, amygdala and anterior olfactory of rodent (Daniel Hoyer, Waeber, Schoeffter, Palacios, & Dravid, 1989). In addition, the 5-HT$_{2C}$ receptor cys23ser polymorphism is found associated with vulnerability to affective disorders (Lerer et al., 2001). Furthermore, both typical and atypical antipsychotics have inverse agonist activity at the human 5-HT$_{2C}$ receptors (Rauser, JE, HY, & BL, 2001). Another -759C/T polymorphism also affects the 5-HT$_{2C}$ receptor-encoding gene HTR2C expression; the -759T containing promoter has a higher transcriptional activity than the more common -759C allele, which underlies the vulnerability to antipsychotic-induced weight gain in schizophrenia patients (Buckland et al., 2005). Moreover, the HTR2C/- mouse is severely overweight because of abnormal food intake regulation and may also experience fatal seizures (Tecott et al., 1995).

**The 5-HT3 receptor family** is LGICs that can induce the rapid depolarization of neurons by a transient current of cations (Na$^+$, Ca$^{2+}$ influx, K$^+$ efflux) (D Hoyer et al., 1994). Electron microscopy has identified that the native 5-HT3 receptor is a pentamer composed of 5 subunits surrounding a central channel (Boess, Beroukhim, & Martin, 1995). Using receptor radioligand binding assay, 5-HT$_3$ receptor has been found highly distributed in at the dorsal vagal complex mediating the vomiting reflex (Hannon & Hoyer, 2008). In addition, it is found that 5-HT$_3$ receptor-immunoreactive GABAergic interneurons in the rat telencephalon also contain cholecystokinin (CCK), which indicates serotonin may regulate GABA and CCK activity via binding to the 5-HT$_3$
receptor (Morales & Bloom, 1997). Recently, other 5-HT₃ receptor subtypes such as 5-HT₃C, 5-HT₃D, 5-HT₃E, have also been reported (Niesler, Frank, Kapellner, & Rappold, 2003). However, more information pertaining to their specific features has yet to be clarified.

**5-HT₄, 5-HT₅, 5-HT₆, and 5-HT₇ receptors** include a group of serotonin receptors preferentially coupled to the Gs-proteins and mediate intracellular levels of adenyl cyclase. The 5-HT₄ receptor is highly expressed in the nigrostriatal and mesolimbic systems, and is involved in learning and memory (Dumuis, Bouhelal, Sebben, Cory, & Bockaert, 1989). In addition, 5-HT₄ receptor KO mice exhibit abnormal feeding and locomotor behavior in response to stress and novelty, and hypersensitivity to seizures (Compan et al., 2004). The transduction pathways and the function of 5-HT₅ receptors have not been well established. 5-HT₅A receptor KO mice show increased exploratory activity in a novel environment, which suggests a putative role of 5-HT₅A receptors in adaptation under stress (Wesołowska, 2002). Rat and human 5-HT₆ receptors are found in central nervous system regions such as striatum, hippocampus, and cortex, but not in peripheral tissues (Frazer & Hensler, 1999). It has been found that various antipsychotic and antidepressant drugs have a high affinity for the 5-HT₆ receptor, and the use of antagonists increase cholinergic neurotransmission and have positive effects on learning and memory (Hannon & Hoyer, 2008). 5-HT₇ receptors have been identified in peripheral tissues, such as vascular smooth muscle cells, and in central nervous system tissues, such as suprachiasmatic nucleus, thalamic nuclei and cortical regions (Leung, Walsh, Pulido-Rios, & Eglen, 1996), which suggests this receptor may regulate sleep, circadian rhythmic activity and mood. It has been reported that 5-HT₇ receptor KO mice exhibit reduced immobility in the forced swim test, suggesting an ‘antidepressant-like’ phenotype. Treatment of wildtype mice with a selective 5-HT₇ receptor antagonist did not decrease immobility unless tested in the dark (or active) cycle, which indicates a circadian influence on receptor function (Guscott et al., 2005).

In summary, the 5-HT receptor subtypes are complex and involved in many biological functions. Given the diversity of symptoms in schizophrenia, it is difficult to identify a single gene which accounts for all types of abnormalities. A comprehensive examination of serotonergic
system in various developmental stages under wide range of environmental stimuli in schizophrenia patients will be helpful to find potential therapeutic targets.
APPENDIX B

THE LETTER FOR RESEARCH USING ANIMALS
Verification of Review and Approval
by the Institutional Animal Care and Use Committee
PHS Assurance Number A3248-01 - USDA Registration Number 86-R-0003

This protocol has been reviewed by the Institutional Animal Care and Review Committee (IACUC) and the work may commence at this time. This approval only authorizes the activities reviewed by the IACUC as described on the final version of the Protocol Form.

Principal Investigator: Amelia Gallitano
Department: Basic Medical Sciences

Protocol Number: 13-481
Title: Mechanisms of 5HT2AR regulation by EGR3

Approval Date: 10/16/2013
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