Effect of *Toxoplasma gondii* on altering dopamine levels and neuroinflammation contributing to an increased risk of developing schizophrenia

By

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Toxoplasma gondii infection is common in humans and is a significant risk factor for developing the disease schizophrenia. Genetic risk factors are likely required for the disease of schizophrenia to develop. Nurr1 – heterozygous (+/-) mice and wild-type (+/+) mice were evaluated using immune activation of astrocytes within the prefrontal cortex, dopamine levels within the striatum, and measuring the acoustic startle response reaction time by using prepulse inhibition (PPI). T. gondii infected heterozygous (+/-) mice exhibited increased GFAP expression within the prefrontal cortex. Dopamine levels within the striatum were measured and T. gondii infected wild-type (+/+) mice exhibited increased dopamine levels. The acoustic startle response reaction time was measured using PPI and T. gondii infected mice exhibited slower reaction times when compared to controls. These data demonstrate that the Nurr1 (+/-) genotype predisposes mice to T. gondii-induced alterations in behaviors that involve dopamine neurotransmission and are associated with symptoms of schizophrenia.
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Toxoplasma gondii

*Toxoplasma gondii* is a parasite that infects all warm-blooded animals. In humans, it is one of the most common parasites. Approximately 1/3 of the global population has been exposed and probably infected with *T. gondii* (Campbell & Farrell, 2008). There are four ways that humans are infected with *T. gondii*. They include: (1) consuming raw or uncooked meat containing *T. gondii*, (2) ingesting food or water contaminated by oocysts that were shed in the feces of an infected cat, (3) receiving a contaminated blood transfusion or organ transplant, and (4) from mother to fetus through the placenta (Campbell & Farrell, 2008). The lifecycle of *T. gondii* includes both sexual and asexual reproduction. Sexual reproduction occurs only within the family Felidae, using either wild or domestic cat hosts. Asexual reproduction occurs in numerous warm-blooded animals, including humans. Thus, cats are the definitive host whereas humans and other warm-blooded animals can be intermediate hosts (Tenter et al., 2000). During acute *T. gondii* infection, people usually have mild flu-like symptoms such as swollen lymph nodes, muscle aches and pains. Because human hosts have such general symptoms, it is easy for a host to become infected with *T. gondii* without knowing it. Obtaining empirical data in clinical settings can be easy and convenient by testing the subject for the presence of *Toxoplasma* antibodies (Flegr, 2013). One important reason
for investigating *T. gondii* is that presence of antibodies to *T. gondii*, increases the risk of schizophrenia. Furthermore, infection with *T. gondii* has more recently been linked with suicide attempts, obsessive convulsive disorder, Alzheimer’s disease and Parkinson’s disease. Additionally, individuals with *T. gondii* antibodies have slower reaction time and are in more car accidents (Flegr, 2013).

**Schizophrenia**

Schizophrenia is a disease that affects approximately one percent of the world’s population with incidence rates that vary across countries and culture. Schizophrenia is typically diagnosed in early adulthood, particularly in males as females have, on average, a later onset of symptoms. Schizophrenia symptoms can be categorized as positive, negative, and cognitive symptoms. Positive symptoms generally imply occurrences beyond normal experience. These symptoms will include visual, tactile, and/or auditory hallucinations, delusions, and paranoia. Negative symptoms are often present and include social withdrawal, autistic like behavior, ambivalence, blunted affect and inability to experience pleasure from activities that are usually enjoyable. The cognitive symptoms in schizophrenia patients include disorganized thoughts, altered working memory and planning ability and loose associations (Meyer & Feldon, 2009). The positive symptoms of hallucinations and paranoid delusions are the symptoms more often associated with schizophrenia. Positive symptoms generally respond well to antipsychotic medication. The negative and cognitive symptoms, however, are less obvious in most cases, but contribute substantially to the pathology of the disease. Furthermore, the negative and cognitive symptoms are more resistant to treatments than the positive symptoms (Javitt & Coyle, 2004).
Developing schizophrenia is typically thought to be due to a combination of environmental - stressors and genetic predisposition. One important parameter that is altered in schizophrenia is dopamine neurotransmission (Meyer & Felden, 2009). This is why it is important for us to understand how specific genes will be affected by environmental stressors and interact due to genetic predisposition to dopamine neurotransmission. The current dopamine hypothesis for schizophrenia suggests elevated subcortical dopamine neurotransmission and attenuated cortical dopamine neurotransmission (Miyake et al., 2011). Negative and cognitive symptoms are thought to result from reduced dopamine levels or dopamine neurotransmission in the prefrontal cortex (Meyer et al., 2008).

Most of the central nervous system dopamine is located within the nigrostriatal, mesolimbic and mesocortical systems. The nigrostriatal pathway in the brain is particularly involved in the production and control of movement. Whereas the mesoaccumbens dopamine pathway plays an important role in reward-motivated behavior. Elevated dopamine neurotransmission in these regions have been linked with positive symptoms of schizophrenia. Evidence for a role for altered dopamine neurotransmission in schizophrenia includes the observation that antipsychotic drugs block dopamine D2 receptors (Seeman, 1987; Carlsson, 1998; Lieberman et al., 1990; Carlson et al., 1997; Arnt & Skarsfeldt, 1998). Additionally, dopamine agonists and drug that stimulate dopamine release, like amphetamine, can induce psychotic symptoms in normal people and can exacerbate psychotic symptoms in people with schizophrenia (Seeman et al., 2013). The desired clinical effect of reducing psychotic symptoms is by attenuating dopamine function in the mesolimbic pathway.
Although dopamine appears to be the final mediator of many of the symptoms of schizophrenia, a number of potential causes have been suggested. One potential contribution to schizophrenia is the reaction of the immune system. This immune hypothesis is focused on the changes involving both cellular and humoral immunity. The changes with T-lymphocytes in the adaptive immune system change such that the T-helper 1 cells that secrete interleukin-2 (IL-2) and interferon-\(\gamma\) are reduced but the T-helper 2 cells have increased secretion of interleukins-6 (IL-6) and IL-10 (Leonard, 2005). Increased levels of IL-6 have been reported in patients with schizophrenia (Leonard, 2005) while other data have demonstrated a link between the increase in IL-6 and an increase of dopamine and serotonin in the brain (Meyer et al., 2009).

Maternal infection during pregnancy is an environmental risk factor for the newborn to develop brain disorders including schizophrenia. Dopamine levels are altered in offspring as adults when the maternal immunological system is stimulated. This is evidenced by the level of the viral mimetic Poly I:C (Winter et al., 2008). Prenatal cytokines could have a role in linking maternal infection and the associated risk of mental illness disorders in offspring (Brown & Derkits, 2010). Therefore, it is believed that induction of pro-inflammatory cytokines during pregnancy may alter early brain development and increase the risk of schizophrenia (Meyer et al., 2008). Microglial activation, which is triggered by pro-inflammatory cytokines such as IL-1 and -6, has been detected in the fetal brain during development (Felger & Miller, 2012).

As mentioned above, infection with \textit{T. gondii} is a significant risk factor for schizophrenia. Two types of studies have been conducted linking \textit{T. gondii} with an
increased risk of developing schizophrenia. The first type involves comparing the titers of
*T. gondii* antibodies in schizophrenia patients and a matched control sample. These
studies have consistently found an increase in prevalence of *T. gondii* antibodies in
schizophrenia patients (Brown & Derkits, 2010). Based on data collected, infection with
*T. gondii* may not directly cause schizophrenia. However, it does put one at a greater risk
to develop schizophrenia for those with certain genetic factors. More recent studies have
also found that the presence of *T. gondii* antibodies in schizophrenia patients increases
the severity of the disease and exacerbates pathological changes (decreased cortical gray
matter) in the brain (Brown & Derkits, 2010). The second type of study compared *T.
gondii* antibody titers in mothers and the risk of children being diagnosed with
schizophrenia. Brown et al. (2005) conducted a study comparing mothers with increased
risk of developing schizophrenia in their offspring. The study focused on the
immunoglobulin levels within with in the mother. They compared IgG and IgM antibody
titers in this group which showed high levels of IgG and no IgM antibodies. IgM
antibodies will show recent exposure to *T. gondii* and have been detected in mothers
whose offspring developed congenital toxoplasmosis.

Other studies were conducted to determine the correlation between *T. gondii* IgG antibodies and schizophrenia. Mortensen et al. (2007) was able to determine that people
newly diagnosed with schizophrenia before the age 18 had increased *T. gondii* IgG
antibody yiters. Blomstrom et al. (2012) conducted a study to determine the role IgG
antibodies played in putting mothers with *T. gondii* at risk for developing schizophrenia
when compared to the general population.
These studies reported sound data showing an increased risk of schizophrenia in the pregnant population that had antibody levels greater than the 75th percentile following prenatal exposure to selected infections (odds ranging from 3.2 – 2.1 ratio). Therefore, data from these studies validated a solid correlation between developing schizophrenia in their offspring as evidenced by women prior to pregnancy being infected with *T. gondii* (Brown & Dekits, 2010).

**Toxoplasma gondii infection in rodents**

*Toxoplasma gondii* has been found to alter behavior in both mice and rats. Comparing the impact of the infection on rodent behavior may help us determine how *T. gondii* infection alters human behaviors and increases the risk of mental illness. *Toxoplasma gondii* infection affects the offspring of mice differently depending on stage of pregnancy when infection occurs. Congenital toxoplasmosis can cause fetal damage in humans and mice including causing significantly lower birth weight. The survival rate of offspring from mice infected at the early stage of pregnancy was significantly lower than those infected at the late stage of pregnancy (Wang et al., 2011). Additionally, gestational infection with *T. gondii* can also alter behavior in mice as adults (Wang et al., 2011). A more epidemiologically relevant mouse model for how *T. gondii* infection in a mother increases the risk of schizophrenia in her children is to investigate mice born to dams that were infected with *T. gondii* prior to pregnancy and thus exposed to IgG antibodies from chronic infection with *T. gondii*. To our knowledge, no studies have investigated this relationship.

Most studies investigating the effect of *T. gondii* infection on behavior have used mice with an adult acquired infection. The most common behavioral changes include a
loss of aversion to bobcat urine and an increase in open field activity (Eells et al., 2015). Wang et al., (2013), had one study that showed that mice with *T. gondii* infection had deficits in learning and memory based on the passive avoidance test that was similar to mice treated with MK-801 administration, an NMDA receptor antagonist. Behavioral changes in mice with *T. gondii* infection have been linked with alterations in dopamine neurotransmission. In infected rodents, a significant increase in dopamine when exposed to *T. gondii* has been reported (Wang et al., 2013). Therefore, it is believed that *T. gondii* can alter dopamine which can result in behavioral changes in mice.

*Toxoplasma gondii* contains two tyrosine hydroxylase enzymes that decrease the rate of dopamine synthesis *in-vivo*. These two enzymes metabolize phenylalanine as well as tyrosine. Then these enzymes catabolize phenylalanine to tyrosine and tyrosine to L-DOPA. The study identified an aromatic amino acid hydroxylase due to potential synthesis signaling molecule L-DOPA (3, 4 dihydroxy-L-phenylalanine). *Toxoplasma gondii* tissue cysts contained a parasite tyrosine hydroxylase as evidenced by data collected through the enzymatic assays and immunohistochemical labeling (Gaskell et al., 2009). There are several possible biological roles of *T. gondii* infection, one being tyrosine hydroxylase through protein synthesis. Then synthesized tyrosine will be converted to L-DOPA. *Toxoplasma gondii* infects the glial and neuronal cells that form cysts during latent infection. An increased dopamine level has been associated with observed behavioral changes. Therefore, behavioral changes are closely linked with alteration in dopamine neurotransmission (Webster et al., 2006; Gaskell et al., 2009; Xiao et al., 2012).
Nurr1, Dopamine, and Schizophrenia

Nurr1 is a nuclear receptor, similar to a steroid hormone receptor, which is necessary for development and function of dopamine neurons (Eells et al., 2002). Alteration in Nurr1 has been linked to schizophrenia-related behavioral abnormalities (Eells et al., 2015). Nurr1-null heterozygous (+/-) mice have reduced dopamine in the mesolimbic and mesocortical systems (Eells et al., 2002). *Toxoplasma gondii* synthesizes L-DOPA which is a precursor to dopamine. Increased levels of dopamine during infection has been associated with observed behavioral changes (Eells et al., 2002 & Gaskell et al., 2009). Nigrostriatal dopamine levels, however, are not affected (Eells et al., 2002). These mice consistently show elevated open field activity (Eells et al., 2014). This is a behavior linked with elevated dopamine neurotransmission that will increase spontaneous locomotor activity, whereas blocking dopamine neurotransmission with dopamine receptor antagonists will decrease locomotor activity. Additionally, the Nurr1 +/- mice have been shown to be more sensitive to environmental stressors linked with schizophrenia, including the developmental stressor of post-weaning isolation and adult infection with *T. gondii* (Eells et al., 2015).

Prepulse inhibition (PPI) of the acoustic startle response is used to measure sensorimotor gating. Alterations in dopamine levels have been shown to disrupt sensorimotor gating. Furthermore, disrupted sensorimotor gating has been found in patients with schizophrenia. This altered sensorimotor gating correlates with positive symptoms of schizophrenia. Post-weaning isolation of Nurr1 +/- mice results in disruption in PPI (Eells et al., 2014). Furthermore, Nurr1 +/- mice infected with *T. gondii* show a significantly greater increase in open field activity (Eells et al., 2014). These
mice, therefore, represent a model for schizophrenia that combines an environmental stressor (*T. gondii*) with a genetic predisposition to disrupted behaviors that are linked with dopamine neurotransmission (Nurr1 (+/-)) (Eells et al., 2014).
CHAPTER II

GLIA-FIBRILLARY ACIDIC PROTEIN AND DOPAMINE LEVELS IN WILD-TYPE AND NURR1-NULL HETEROZYGOUS MICE AFTER TOXOPLASMA GONDII INFECTION

Introduction

Developing schizophrenia is typically thought to rely on a combination of environment-stressors and genetic predisposition. One important parameter that is altered in schizophrenia is dopamine neurotransmission (Meyer & Felden, 2009). The current dopamine hypothesis for schizophrenia correlates development of the disease with elevated subcortical dopamine neurotransmission and attenuated cortical dopamine neurotransmission (Eells et al., 2014). Negative and cognitive symptoms are thought to result from reduced dopamine levels or altered dopamine neurotransmission in the prefrontal cortex (Meyer et al., 2008). One proposed theory is that \textit{T. gondii} affects behavior through elevating dopamine neurotransmission. This theory is supported by 14\% elevated dopamine levels in the brain tissue of mice infected with \textit{T. gondii} (Gaskell et al., 2009).

\textit{Toxoplasma gondii} is a parasite that infects nearly all warm-blooded animals. In humans, it is one of the most common parasites. Approximately one-third of the global population has been exposed to and probably infected with \textit{T. gondii} (Campbell & Farrell, 2008). There are four ways that humans are infected with \textit{T. gondii} including
consuming raw or uncooked meat containing *T. gondii*, ingesting contaminated food or water through oocysts that have been shed in the feces of an infected cat, undergoing a contaminated blood transfusion or organ transplant, and transfer from mother to fetus through the placenta (Campbell & Farrell, 2008). The lifecycle of *T. gondii* includes sexual and asexual reproduction. Sexual reproduction occurs only within the family Felidae, including wild or domestic cats. Asexual reproduction can occur in cats and other warm-blooded animals, which includes humans. Because sexual reproduction of the parasite occurs only in cats, these are the definitive host whereas humans and other warm-blooded animals are intermediate hosts (Tenter et al., 2000). During acute *T. gondii* infection, people usually have mild flu-like symptoms such as swollen lymph nodes, or muscle aches and pains. Because humans have such non-specific symptoms, it is easy for a host to become infected with *T. gondii* without knowing it. One very important reason for investigating *T. gondii* is that presence of antibody to *T. gondii*, based on data from multiple studies, increases the risk of schizophrenia (Brown et al., 2005; Flegr et al., 2013 & Goodwin et al., 2012).

We know that infection with *T. gondii* does not directly cause schizophrenia, but *T. gondii infection* may interact with potential susceptibility genes such as Nurr1. Nurr1 is a nuclear receptor that is necessary for appropriate development and function of dopamine neurons (Kadkodaei et al., 2009). Alteration in Nurr1 has been linked to schizophrenia-related behavioral abnormalities (Eells et al., 2014). Based on these observations, Nurr1 (+/-) may alter how *T. gondii* infection affects the dopamine transmission in mice. The purpose of this study is to determine how *T. gondii* infection
influences the development of schizophrenia. We will be looking at alterations in dopamine levels and neuroinflammation in the brain tissue of mice.

**Materials and Methods**

All procedures were performed in accordance with the National Institute Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at Mississippi State University approved all study protocols. The individual room temperature was maintained between 18-22 degrees Celsius.

Animals were located in an AAALAC accredited facility at Mississippi State University, with their food and water available *ad libitum* being kept on a 12 hour light/dark cycle. Nurr1-null heterozygous mice used in this study were from a colony bred at Mississippi State University, originally produced in the laboratory of Dr. Vera Nikodem at the National Institute for Diabetes and Digestive and Kidney Diseases (Castillo et al., 1998). The Nurr1 +/- mice were bred and at about 19-21 days of age, weaned and placed in cages with other mice all in the same room.

**Measurement of Tissue Dopamine Levels**

After initial behavior testing, mice were injected subcutaneously with 200 ul Hank’s Buffered Salt Solution (HBSS) containing *T. gondii* ME49 (1,000 tachyzoites) or HBSS without tachyzoites. We used a type II strain (*T. gondii* ME 49) because it is the most common found in human *T. gondii* infections.

Behavior testing was repeated six weeks post-injection. After behavioral testing was completed, mice were euthanized by CO2 asphyxiation. After euthanasia, all mice were decapitated, the brain removed and trunk blood samples collected. Samples were
placed into vacutainer tubes containing heparin. The blood samples allowed us to
determine antibody titers to *T. gondii*. Evidence of seroconversion helped us determine
the probability of detecting cysts when we were still considering the tyrosine hydroxylase
hypothesis. The brain was divided into two distinct regions of the midbrain and forebrain
by making a coronal cut in the brain at approximately 1mm from bottom to top which
divided into the midbrain and forebrain. The fore brain tissue was cut sagittally into right
and left and then frozen by using dry ice and kept at a temperature of -80 degrees Celsius
(Eells et al., 2014). Blood samples were centrifuged at 2,000 x g for five minutes and
then the plasma was collected from these tubes to be used in serological testing.

Antibody titer data was obtained by using an indirect immunofluorescent antibody
test (IFAT) with *T. gondii* (RH) antigen, murine plasma diluted 2-fold from 1:25, and a
fluorescein-isothiocyanate-labled secondary antibody. The antibody titers were then
calculated at the reciprocal dilution producing the last visible fluorescence on the IFAT.
A cut-off titer of 50 was used in order to determine the status of seroconversion to *T.
gondii* (Eells et al., 2014).

Brain dissection and catecholamine measurements were performed. In order to
collect tissue to determine catecholamine measurements, the right frozen forebrain tissue
was placed in a custom slicer with O.C.T. compound (Sakura Finetek, Torrance, CA).
The device cut frozen sections of about 600-800 µm which were mounted on glass slides.
Dorsal striatum micropunches were obtained using a blunt 20 gauge needle. For the
estimation of neurotransmitter levels, the dissected brain region was weighed and internal
standard DHBA was adjusted for each sample. For catecholamine isolation, 0.1M
perchloric acid was added to the collected tissue and homogenized. Two successive centrifuges at 10,000 g was used to separate the supernatant.

A Pierce BCA Protein Assay was performed following the manufactures specifications on each supernatant sample. This method combines the reduction of Cu $^{+2}$ to Cu $^{+1}$ by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu $^{+1}$) using a unique reagent containing bicinchoninic acid. The purple-colored reaction product of the assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000 ug/mL).

**GFAP Immunohistochemistry**

Immunohistochemistry testing was used to measure the GFAP level. The left portion of the forebrain was sectioned into 10 μm sections using a cryostat. The serial sections were labeled with GFAP. In order to do this, the sections were fixed for 30 minutes in a 4% paraformaldehyde solution that is dissolved in PBS. The sections were washed three times in PBS-BSA then incubated in blocking serum containing (PBS with 1% Triton –X 100, 4% normal goat serum, 1% BSA) for a total of 30 minutes. The sections were then incubated in either a rabbit polyclonal GFAP antibody (Calbiochem, San Diego, CA) diluted 1:1,000 or anti-Iba1 and kept at a temperature of 4 degrees Celsius overnight. The following day the sections were rinsed 10 times with PBS containing 1% BSA and 0.2% Triton X-100, incubated in biotinylated anti-rabbit IgG for the next two hours. Once time has elapsed, tissue was rinsed 3 times with PBS then incubated in avidin-biotin complex with horse radish peroxidase for 2 h. Following this
incubation antibody labeling was visualized with diaminobenzidine. Once sections were coverslipped, 60X images within the prefrontal cortex were collected using a Stereo Investigator Stereology Software from MicroBrightField Inc and an Olympus BX51 microscope with a CCD camera and a motorized Z-stage which was connected to a computer. The number of immunoreactive cells (GFAP) were counted and the distribution of immunolabeling was quantified as described by ImageJ software. The Stereo Investigator system allows a researcher to obtain accurate and unbiased images in our tissue specimens. The Stereo Investigator procedures were followed for every section on prepared slides within each of our stains for GFAP. This aided us in determining which sections of our forebrain tissue deserved a closer unbiased observation within the prefrontal cortex area. It was very important to keep an accurate reference point. If not, all subsequent steps would be greatly affected and ultimately would affect our results. The accurate way to select a reference point is to choose something that is present on each of the prepared slides. The number of sections used depended on how many were on our prepared slides. It typically ranged from 12-16 sections per slide. A block advance of 80 µm and a set tissue thickness of 10 µm was used. Once this first section was completed, we continued to choose the next section until all were completed. Once the section images were taken, we put them all on a single powerpoint slide. At the top of the slide was the mouse number and stain type and below were multiple rows containing approximately 6 sections per row. Once this was prepared, it provided us with a better insight into which sections of the prefrontal cortex needed to be contoured. Once we had determined which prefrontal cortex sections needed to be contoured, the entire area is encircled. Then the software knows to obtain unbiased image fields at 60x.
magnification within this encircled area. We made sure to adjust appropriate camera settings for the type of stain and it is extremely important to use the same settings for all the images. The appropriate camera setting for our GFAP stain are: Exposure = 6.46 ms, Target Intensity = 95%, Gain = 1.000, Live Setting: Red = 1.0000, Green = 1.0000, and Blue = 1.0000. These procedures were repeated multiple times, thus leaving us with many unbiased prefrontal cortex images at 60x magnification. The next step was to isolate the tissue of interest from the rest of the section. In our GFAP stain, we isolated astrocytes. In order to isolate our tissue of interest, we used Adobe Photoshop software. Our first objective was to define our template color. We identified the color of astrocytes within the images to quantify our respective immune activation within our stains. This allowed us to move toward quantifying immune system activation.

**Data Analysis**

Comparisons of differences between means of the treatment groups were analyzed using analysis of variance (ANOVA) with genotype, sex and treatment used as co-variants followed by Fisher’s least significant difference post-hoc comparisons where appropriate (Eells et al., 2014). All data was collected and analyzed for normality using comparisons appropriately. ANOVA was used for striatum dopamine data collection by using treatment, genotype, treatment genotype and residual. All statistical analysis was performed using *STATVIEW* (JMP) computer software obtained from www.jmp.com/en_us/software/jmp.html. A p-value of less than 0.05 was considered statistically significant.
Results

GFAP Stain of Astrocytes

*Toxoplasma gondii* tachyzoites have a high affinity for brain tissue, it is believed that their presence in the central nervous system will induce a local acquired immune response. Astrocytes are also a type of glial cell performing many functions, including biochemical support of endothelial cells that form the blood–brain barrier, provision of nutrients to the nervous tissue, maintenance of extracellular ion balance, and a role in the repair and scarring process of the brain and spinal cord following traumatic injuries.

When considering immune activation within the forebrain tissue, we used the prefrontal cortex area of the brain because of CNS dopamine pathways. We used the variables (genotype, treatment, area of immune activation) to assess this area. When observing prepared brain tissue slides, we found no significant effect of either genotype (F[1,6]=3.059, p=0.1309) or *T. gondii* infection (F[1,6]=0.035, p=0.858) on GFAP expression when grouped together (Figure 1A,B). When comparing the immune system activation of Nurr1-homozygous to Nurr1-heterozygous mice, we observed a drastic difference between the Nurr1-homozygous and Nurr1-heterozygous mice (Figure 1C). Because of the differences between the Nurr1-homozygous and Nurr1-heterozygous mice no statistical significance was found (F[1,4]=4.579), however, *T. gondii* infection increased GFAP in Nurr1-heterozygous mice but decrease GFAP in Nurr1-homozygous mice.

Striatal Tissue Dopamine Levels

When considering dopamine levels obtained from our striatum micropunches, we saw that *T. gondii* infected mice had higher levels of dopamine within the striatum when
compared to our controls (Figure 2A), although this was not statistically significant (F[1,62]=2.846). When separated by genotype (F[1,70]=1.639, p=0.2047), there was a slight reduction in tissue dopamine levels (Figure 2B). When analyzed by genotype and treatment, the *T. gondii* infected wild-type mice showed higher dopamine levels as compared to the uninfected controls (F[3,64]=4.059, p=0.0105). In the heterozygous mice, no change in dopamine levels were observed in the infected heterozygous mice (Figure 2C).
Treatment (1.A). Found no significant effect of toxoplasma gondii infection ($F[1,6] = 0.035$, $p = 0.858$).

Genotype (1.B). Found no significant effect of either genotype ($F[1,6] = 3.059$, $p = 0.1309$). Treatment and Genotype (1.C). When comparing immune activation of Nurr1 (+/+) and Nurr1 (+/-), drastic differences were observed. Because of the difference between the two, no statistical significance was found ($F[1,4] = 4.579$). Toxoplasma gondii infection increased GFAP expression in Nurr1 (+/-) and decreased expression in Nurr1 (+/+) mice. Significant genotype (*) and treatment (#) effects based on ANOVA with Fisher’s post hoc comparisons. N= 1 female (+/+) control, 1 female (+/+) T. gondii infection, 1 female (+/-) T. gondii infection, 1 male (+/+) control, 2 male (+/-) controls, 1 male (+/+) T. gondii infection, & 1 male (+/-) T. gondii infection.
Figure 2. Treatment and Genotype of dopamine levels within the striatum.

Treatment (2.A). T. gondii infected mice had an increase in dopamine within the striatum compared to controls, although not statistically significant ($F[1,62] = 2.846$). Genotype (2.B). There was a slight reduction in tissue dopamine levels within our Nurr1 (+/-) mice ($F[1,70] = 1.639, p = 0.2047$). Treatment & Genotype (2.C). Toxoplasma gondii infected Nurr1 (+/+) mice exhibited higher dopamine levels compared to Nurr1 (+/+) mice controls ($F[3,64] = 4.059, p = 0.0105$). No observable change in Nurr1 (+/-) mice T. gondii infected dopamine levels compared to Nurr1 (+/-) mice controls. Significant genotype (*) & treatment (#) effects based on ANOVA with Fisher’s post hoc comparisons. N = 11 female (+/+) controls, 6 female (+/-) controls, 4 female (+/+) T. gondii infection, 9 female (+/-) T. gondii infection, 15 male (+/+) controls, 8 male (+/-) controls, 8 male (+/+ ) T. gondii infection, 3 male (+/-) T. gondii infected. Including: 4 male (+/+ ) unknown treatment, 3 male (+/-) unknown treatment, & 1 female (+/-) unknown treatment.
**Discussion**

Due to the association between *T. gondii* infection and schizophrenia, we need to better understand the relationship between antibody titers to *T. gondii* and neuroinflammation and alteration in dopamine levels as both alterations in dopamine neurotransmission and inflammation have been demonstrated in schizophrenia patients. Our study investigated the interaction between heterozygous deletion of Nurr1 gene and *T. gondii* infection on prefrontal GFAP levels and striatal dopamine levels. Both the Nurr1 heterozygous genotype and T. gondii infection can affect GFAP levels and dopamine levels.

Two possible mechanisms through which *T. gondii* infection can alter behaviors in rodents and contribute to elevated risk of schizophrenia is through elevated inflammation and dopamine levels. Stibbs (1985) found 14% elevation of dopamine in the whole brain of mice chronically infected with *T. gondii*. The serotonin and 5HIAA neurotransmission levels were unchanged.

In another study, Prandovszky et al., (2011) found high levels of dopamine located in cysts in the brain. Additionally, infected PC12 cells release more dopamine following stimulation (Prandovszky et al., 2011). This study (Prandovszky et al., 2011) showed a direct correlation between the numbers of infected *T. gondii* cells when immunostaining brain sections of infected mice which resulted in an increase of dopamine being released through intense staining of encysted parasites. The *T. gondii* amplified levels of K+ induced release of dopamine several fold. Dopamine was also evidenced by immunostaining brain sections of the *T. gondii* infected mice with dopamine antibody exhibiting a high degree of encysted parasites. Additional evidence
for a role of dopamine in T. gondii infection was supported by an experiment in which
the dopamine receptor antagonist haloperidol and the dopamine transporter inhibitor
GBR 12909 altered behavior changes in T. gondii-infected rodents (Skallova et al., 2006;
Webster et al., 2006). Recently, it was reported that T. gondii has two replications of
tyrosine hydroxylase gene with encoded proteins (97.5%) in bradyzoite-stage parasites
and high-degree of homology (53%) with mammalian tyrosine hydroxylases
(Prandovszky et al., 2011). To locate these parasite-encoded tyrosine hydroxylase within
brain tissue an antibody for T. gondii tyrosine hydroxylase (TgTH) was developed that
was designed uniquely from mammalian tyrosine hydroxylases. Test confirmed TgTH
was specific to T. gondii and tyrosine hydroxylases (Prandovszky et al., 2011). This
suggests that T. gondii infection can directly increase dopamine production through
parasite synthesis by converting amino acid tyrosine to L-DOPA which is a precursor to
producing dopamine. The level of dopamine is regulated by tyrosine hydroxylase
activity through selective dopaminergic neuronal death. Tyrosine hydroxylase is
inactivated by catecholquinones and converted to redoxycyclingquinoprotein which can
treat Parkinson’s disease which caused an increase of dopamine production (Prandovszky
et al., 2011 & Ogawa et al., 2005).

The experiments that have investigated dopamine have had conflicting results.
Stibbs (1985) found a significant increase in dopamine levels after T. gondii infection.
However, Goodwin et al., (2012) used a virulent Type I T. gondii strain and found no
effects on tissue dopamine levels. One interesting result from these studies was that the
heterozygous mice showed no effect on dopamine levels, although they did show
behavioral changes (Eells et al., 2014, Stibbs, 1985 & Goodwin et al., 2012). One
potential explanation is that changes in dopamine are not necessary for alterations in behavior. Another possibility is that *T. gondii* cysts indirectly alter dopamine synthesis by elevating endogenous activity of tyrosine hydroxylase. Since, Nurr1 heterozygous mice have reduced tyrosine hydroxylase activity, *T. gondii* infection may not be able to elevate tissue dopamine levels (Eells et al., 2006). Dopamine-related behavior changes may be related to the interaction of genetic predisposition as well as environmental and neurodevelopment (Eells et al., 2006).

Our current hypothesis for schizophrenia suggests elevated subcortical dopamine neurotransmission and attenuated cortical dopamine neurotransmission. It is not known how *T. gondii* affects dopamine neurotransmission across these defective pathways. It still is a complex task to determine the exact defective dopamine pathway that leads to schizophrenia.

Other studies have found elevated dopamine neurotransmission in central nervous systems including the nigrostriatal pathway in the brain which have been linked with positive symptoms of schizophrenia. Evidence for a role for altered dopamine neurotransmission in schizophrenia includes the observation that antipsychotic drugs block dopamine D2 receptors (Seeman, 1987; Carlsson, 1998; Lieberman et al., 1990; Carlson et al., 1997; Arnt & Skarsfeldt, 1998). Additionally, dopamine agonists and drug that stimulate dopamine release, like amphetamine, can induce psychotic symptoms in normal people and can exacerbate psychotic symptoms in people with schizophrenia (Eells et al., 2006).

Data from the current study using GFAP stains, found that treatment and genotype of astrocyte activation within the prefrontal cortex measured controls had a
slight increase of astrocyte activation. When considering the genotype of the wild-type mice, their astrocyte activation appeared to be much higher astrocyte activation. There was no noticeable differences among *T. gondii* infected mice within the prefrontal cortex. But there was a drastic increase in astrocyte activation within our controls in our wild-type mice.

McConkey et al. (2013) believed that the location of the parasites in the host, specific brain regions associated with fear processing, could manipulate behavior of infected rodents. But according to Prandovszky et al. (2011), the exact location of infection to certain brain regions does not correlate to specific changes in behaviors, therefore other proximate mechanisms are needed to contribute to these effects.

Additionally our dopamine levels within the striatum micropunches revealed that the *T. gondii* infected mice and our wild-type mice both had increased levels, whereas our controls had no observable differences. When compared, controls had a slight increase of astrocyte activation within the prefrontal cortex of our *T. gondii* subjects. It was important to note that our control subjects had a much higher standard error range that could contribute to any discrepancies.

Another mechanism that could alter dopamine neurotransmission is the immune system reaction to infection. Therefore making one potential contribution to schizophrenia symptoms is the reaction of the immune system. The changes with the T-lymphocytes in the adaptive immune system are that the T-helper 1 cells that secrete interleukin-2 and interferon-γ are reduced whereas the T-helper 2 cells have increased secretion of interleukins-6 and -10 (Leonard, 2005). Increased levels of IL-6
have been reported in patients with schizophrenia (Leonard, 2005) while other data has demonstrated a link between the increase in IL-6 and an increase of dopamine and serotonin in the brain (Meyer et al., 2008). Additional studies have reported that the presence of *T. gondii* antibodies in schizophrenia patients increases the severity of the disease and exacerbates pathological changes (decreased cortical gray matter) in the brain (Brown & Derkits, 2010).

A more recent study (McConkey et al., 2013), states that immune responses to *T. gondii* infection may affect the degree of neurotransmission. This is evidenced by increased interferon-gama (IFN-y), interleukin-12 (IL-12) and CD8+ T-cells. IFN-y is important in controlling the degree in which *T. gondii* infection is transmitted. Many believe that IFN-y prevents the reactivation of tissue cysts by decreasing tachyzoite growth.

The underlying mechanism responsible for altered different behaviors that links *T. gondii* infection with schizophrenia is still unknown.
CHAPTER III
EFFECT OF TOXOPLASMA GONDII INFECTION ON THE ACOUSTIC STARTLE REFLEX

Introduction

*Toxoplasma gondii* has been found to alter behavior in both mice and rats. Comparing the impact of the infection on rodent’s behavior has helped us determine how *T. gondii* infection alters human behaviors and increases the risk of mental illness.

*Toxoplasma gondii* infection affects the offspring of mice differently depending on the stage of pregnancy of the dam when infected. Congenital toxoplasmosis can cause fetal damage in humans and mice which includes significantly lower birth weight. The survival rate of offspring from mice infected at the earlier stage of pregnancy was significantly lower than those infected at the late stage of pregnancy (Wang et al., 2011). Additionally, gestational infection with *T. gondii* can also alter behavior in mice as adults (Wang et al., 2011). A more epidemiologically relevant mouse model for how *T. gondii* infection in a mother increases the risk of schizophrenia in their children may be to investigate mice born to dams that were infected with *T. gondii* prior to pregnancy and exposed to antibodies to *T. gondii*. Most studies investigating the effect of *T. gondii* infection on behavior have used an adult acquired infection. The most common behavioral changes include a loss of aversion to bobcat urine and an increase in open field activity (Eells et al., 2014). One study reported mice with *T. gondii* infection having
deficits in learning and memory based on the passive avoidance test were similar to mice treated with MK-801, an NMDA receptor antagonist. The N-methyl-D-aspartate receptor (NMDA) is a glutamate receptor and ion channel protein found in nerve cells. It is activated when glutamate and glycine bind to it, allowing positively charged ions to flow through the cell membrane. It is also very important for controlling synaptic plasticity and memory function. Behavioral changes in mice with *T. gondii* infection have been linked to alterations in dopamine neurotransmission.

Pearce et al., (2013) conducted a study showing how *T. gondii* infection affects the time it takes to elicit a response through a three-synapse neural process. These authors concluded that there was a decreased processing speed with chronic *T. gondii* infection and the slowest response was in *T. gondii* seropositive schizophrenia group. *T. gondii* infected rodents exhibit impaired psychomotor performance and learning ability (Yolken et al., 2009). It has been also reported that *T. gondii* seropositive humans without psychiatric illness exhibit not only psychomotor slowing (Havlicek et al., 2001) and impaired learning (Yolken et al., 2009) but also an increased rates of motor vehicle accidents (Flegr et al., 2002; Flegr et al., 2009; Kocazeybec et al., 2009 and Yereli et al., 2006). Prolonged acoustic startle latency was found associated with *T. gondii* infected seropositive in schizophrenia and control subjects. Prolonged latency in schizophrenia was studied (Pearce et al., 2013) with 183 adult schizophrenia patients and 137 healthy control subjects. The slowest acoustic startle response was found in the schizophrenia subjects who tested positive for *T. gondii* infection.

The acoustic startle reflex has been defined as a simplex reflex brought out by a sudden intense stimulation. Latency of the startle response is the waiting time to receive...
the reflexive response. This allows one to determine the speed of neural processing through the three-synapse subcortical circuit (Koch, 1999; Felger & Miller, 2012). Alteration in dopamine levels have been shown to disrupt sensorimotor gating. Furthermore, disrupted sensorimotor gating has been found in patients with schizophrenia. This altered sensorimotor gating correlates with positive symptoms of schizophrenia. Post-weaning isolation of Nurr1 +/- mice results in disruption in PPI (Eells et al., 2014). Furthermore, Nurr1 +/- mice infected with T. gondii show significantly greater increase in open field activity. These mice, therefore, represent a model combining an environmental stressor with a genetic predisposition for disrupted behaviors that are linked to dopamine neurotransmission. This reproduces the concept of combining an environmental risk factor with a genetic predisposition that contributes to schizophrenia (Eells et al., 2014).

To test the hypothesis that T. gondii infection slows reaction time and interacts with the Nurr1 +/- genotype, +/+ and +/- mice were infected with T. gondii for 6 weeks and tested for startle response reaction time. The reaction time for the acoustic startle response was determined in adults as the time to the maximal startle. Based on our observation, T. gondii infection reduces the reaction time in the acoustic startle response which is an indicator of reaction time. Deficits in prepulse inhibition manifest in the inability to filter out the unnecessary information.

Based on the observations that Nurr1 (+/-) genotype alters dopamine neurotransmission and acoustic startle response, the goals of the study were to determine how T. gondii infection interacts the (+/-) genotype to affect dopamine levels with changes in behavior. Our results demonstrated susceptibility of (+/-) mice to behavioral
changes of *T. gondii* infection with altered dopamine levels which decreased acoustic startle response reaction time. Additionally we demonstrated that *T. gondii* infected mice have an increased risk of developing schizophrenia.

**Materials and Methods**

All procedures were performed in accordance with the National Institute Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at Mississippi State University approved all study protocols.

Animals were located in an AAALAC accredited facility at Mississippi State University, with their food and water available *ad libitum* being kept on a 12 hour light/dark cycle. The individual room temperature was maintained between 18-22 degrees Celsius. Nurr1-null heterozygous mice used in this study were obtained from a colony bred at Mississippi State University, originally produced in the laboratory of Dr. Vera Nikoderm at the National Institute for Diabetes and Digestive and Kidney Diseases (Castillo et al., 1998). The Nurr1 +/- mice were bred and following parturition were weaned at about 19-21 days of age and placed in cages with other mice of this study all in the same room.

**Behavioral Tests**

In order to perform and evaluate behavioral data, a type II strain of *T. gondii* was used because this type is more common in humans with *T. gondii* infections. The initial behavioral tests were performed and then mice were given an injection subcutaneously with 200 ul Hank’s Buffered Salt Solution (HBSS) containing 1,000 tachyzoites (*T. gondii* ME49) or HBSS without tachyzoites. The 120 day old mice were placed in a
startle chamber on a transducer, calibrated to 1Newton to measure the startle responses. Each procedure consisted of a 5 minute acclimation period followed by 65 stimulation trials presented in a pseudorandom order using a 70 dB background white noise. The first five and last five stimulations were the 120 dB startle, and 10 trials each of a 72 dB prepulse, 74 dB prepulse, and 78 dB prepulse were performed 100 ms prior to the 120 dB startle. The first five and last five stimulations were chosen to be analyzed because this gave us an unbiased population. There were also 4 trials of both each dB alone and 4 trials of no stimulation (Eells et al., 2006). We observed the Max Time (ms) within the Startle Pretest, Startle Post-test, and Startle Response. The five response times of each category were averaged. We entered these averages into STATVIEW observing such factors as genotype, sex, treatment and time. By using this method, we were able to determine the time it takes an individual to reach its maximum startle response and how an infected individual would have these responses affected. Comparisons of differences between means of the treatment groups were analyzed using two analysis of variance (ANOVA) with genotype, sex and treatment used as co-variants followed by Fisher’s least significant difference post-hoc comparisons (Eells et al., 2001). All statistical analysis was performed using StatView Software. Once our data was compiled and an average was made per subject, we were able to get a better understanding of the results by inputting describing factors such as genotype, sex, treatment, and area. We chose ANova, Means, Interaction Bar Graph, and Fisher’s Test. We also set our dependent variable (y) as a function of area, our independent variable (x) as a function of treatment (control/TG), and genotype (heterozygous/homozygous). Results were considered significant.
Results

Acoustic startle response in *T. gondii* infected mice acoustic startle response data were analyzed across genotype, sex, and prepulse intensity. We used repeated measures ANOVA. By choosing the 120 dB range, we were able to evaluate both the first five startle reaction times and the last five startle reaction times. We first compared homozygous (wild type) mice to heterozygous (Nurr1) mice (figure 3, C). When evaluating the results, it showed an elevated or slower reaction time within our heterozygous mice when compared to the homozygous mice. Within our first five startle reaction times, we also observed slower reaction times in *T. gondii* infected mice as compared to controls (F[1,92]=5.067, p=0.0268). This assessment showed an elevated or slower reaction times within our *T. gondii* infected mice when compared to controls (figure 3, A).

The last five startle reaction times compared homozygous (wild type) mice to heterozygous (Nurr1) mice (figure 4, C). When evaluating the results, it showed an elevated or slower reaction time within our heterozygous mice when compared to the homozygous mice, but was not statistically significant (Treatment: F[1,90]=3.842, p=0.5531; Genotype: F[1,90]=3.212, p=0.0765). Within our last five startle reaction times (figure 4, A), we also observed slightly elevated or slower reaction times within our controls when compared to *T. gondii* infected mice, although this was not statistically significant (F[1,87]=2.365, p=0.1277). This was unexpected and it was the opposite result expected to have when comparing controls and *T. gondii* infected mice. Overall, we showed that *T. gondii* infected mice had a slower reaction time in the acoustic startle response when compared to controls. Our expectations were mostly correct, with the
exception of our evaluation of the last five startle response times, where the controls
showed slower reaction times. This is unexpected and additional data may be required to
explain the results. We were also able to show this slower reaction time to be more
dramatic in *T. gondii* infected Nurr1 heterozygous mice than infected homozygous mice.
This was shown in both our first five and last five startle response reaction times with
Nurr1 infected mice having much slower reaction times
Treatment (3.A). *T. gondii* infected mice exhibited slower acoustic startle response reaction time ($F[1,92] = 5.067$, $p = 0.0268$).


Significant genotype (*) & treatment (#) effects based on ANOVA with Fisher's post hoc comparisons. $N =$ 13 female (+/+) controls, 6 female (+/-) controls, 7 female (+/-) *T. gondii* infection, 13 female (+/+) *T. gondii* infection, 19 male (+/+) controls, 11 male (+/-) controls, 12 male (+/+) *T. gondii* infection, & 8 male (+/-) *T. gondii* infection.

Figure 3  Treatment, Genotype & Gender, Treatment & Genotype of the first five acoustic startle response reaction time was measured in 120 day old female and male *Nurr1* (+/+) wild-type and *Nurr1* (+/-) heterozygous mice.
Discussion

Our study investigated the role of the heterozygous deletion of the Nurr1 gene and *T. gondii* infection in sensory processing, based on reaction time in the acoustic startle response, as it may relate to aspects of schizophrenia. The current investigation includes data on how Nurr1-null heterozygous (+/-) mice and wild-type mice (+/+) behaviors were affected by using the acoustic startle response prior to and at 6 weeks after infection of *T. gondii*.

The major finding of these experiments is that *T. gondii* infection significantly increase the reaction time in the acoustic startle response. Based on our current study, we used 120 dB for the startle stimulus and we evaluated both the first five and last five startle reaction times within a 66 trial prepulse inhibition session. The data showed an elevated or slower reaction time within our heterozygous mice when compared to the wild-type mice, as well as in *T. gondii* infected mice as compared to uninfected controls. In the last five startle reaction tests, comparing wild-type mice to heterozygous (Nurr1) mice, there was an elevated or slower reaction time within our heterozygous mice when compared to the homozygous mice. The average startle reaction time in the last five startle trials was lower, or faster in the *T. gondii* infected mice, although this was not statistically significant. This was unexpected and was the opposite result expected, therefore additional scientific data may be needed to help explain the results. We were able to show a more dramatic slower reaction time in *T. gondii* infected Nurr1 heterozygous than the infected wild-type mice. Therefore, our data demonstrated that in both our first and last five startle response reaction tests, Nurr1 *T. gondii* - infected mice had slower reaction times.
One possible reason for delayed startle response is that *T. gondii* cysts contained 2 genes encoded for enzyme tyrosine hydroxylase which determine the limits of the dopamine synthesis (Gaskell et al., 2009). *T. gondii* has active tyrosine hydroxylase which can lead to increased dopamine synthesis (Webster & McConkey, 2010). This could lead to slowing down the startle latency response due to increased dopamine level (Prandovszky et al, 2013). This enzyme regulates the chemical process which could explain the reason for behavioral changes in humans related to the level of dopamine transmitted as a result of *T. gondii* infections (McConkey et al., 2013). Another possible reason for delayed startle response comes from the host immune response to *T. gondii* infection. *T. gondii* required tryptophan for replication and if this pathway is affected by other receptors, it could slow the neural process (Pearce et al., 2013; Flegr, 2013; Leonard, 2005). Therefore, there could be an immunological basis for schizophrenia.

Prepulse inhibition (PPI) is a neurological condition in which a weaker prestimulus (prepulse) will inhibit the reaction time of someone to subsequent stronger startling stimulus (pulse). The startle response is normally seen as a defensive response to a sudden or threatening stimuli. The brain uses sensorimotor gating as a way to filter unnecessary or redundant stimuli and mount an appropriate response. The dopamine neurotransmission pathway regulates PPI. A deficit in sensorimotor gating is observed in schizophrenia patients. Our study used 120 dB as our decibel range to evaluate the acoustic startle response in our animal model. Slower reaction times are present in schizophrenia patients with *T. gondii* infection which correlates with sensorimotor gating deficits.
Therefore, mice infected with *T. gondii* had longer reaction time similar to what has been found in schizophrenia patients infected with *T. gondii*. Finally, the exact cause of schizophrenia is unknown, but it is known that there are many predisposing factors that could lead to schizophrenia. Therefore, understanding the predisposing factors and their mechanics of action will help us to diagnose and treat the illness of schizophrenia.

**Future Focus of Research**

There needs to be additional research to help us better understand the underlying mechanisms of *T. gondii*. There should be additional immunohistochemistry on the tissues of the striatum, nucleus accumbens, and prefrontal cortex performed within our adult mice. The Iba1 stain of microglia in adults would be beneficial to help us understand immune activation within the brain. Microglia are much like macrophages which have a seeking and quiet mode. It would be interesting to see how much more active they would become given a *T. gondii* infection. We should also include micropunches of the nucleus accumbens to determine dopamine levels within that area, as well as if this dopamine pathway is affected given our variables. Our current dopamine hypothesis suggests elevated subcortical dopamine levels. This study included the target of the nigrostriatal pathway dopamine levels within the striatum. The other subcortical pathways target of the mesolimbic pathway dopamine levels within the nucleus accumbens. More research is needed to determine the effects of *T. gondii* infection on newborns born to infected mothers. This would include performing the same staining GFAP/Iba1 on astrocytes and microglia of the newborn pups.
Results from this current study raise additional questions regarding the shared relationship between *T. gondii* infection and the development of schizophrenia. These questions warrant more focused research to understand schizophrenia.


Seeman, P. (1987). Dopamine receptors and the dopamine hypothesis of schizophrenia. Synapse, 1, 133-152.


