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## The Glutamate Hypothesis of Schizophrenia: Assessment of a Novel Antipsychotic in Zebrafish Larvae

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The Glutamate Hypothesis of Schizophrenia:

Assessment of the Motor Effects of  
a Novel Antipsychotic in Zebrafish Larvae

Senior Project submitted to  
The Division of Social Studies  
of Bard College

by  
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Abstract

The glutamate hypothesis is a new theory of schizophrenia which proposes that deficient glutamatergic transmission at the NMDA receptor underlies the positive, negative, and cognitive symptoms of the disorder. In addition to tracing the development of the glutamate hypothesis in depth, this senior project presents a study investigating the effects of a novel antipsychotic in zebrafish. Zebrafish are an emerging model of several CNS disorders, including schizophrenia, and it has been demonstrated that NMDA-R antagonism induces motor hyperactivity in zebrafish adults and larvae. Previous research supports an ability of typical and atypical antipsychotics to reverse these motor effects in zebrafish adults. The present study investigates the motor effects of MK-801 administration in TL zebrafish larvae ( $n = 208$ ) at two dose levels, as well as the ability of CHPG (an agonist at the metabotropic glutamate 5 receptor) to reverse these effects. The findings indicate that MK-801 decreased motor activity at a dose of 20  $\mu\text{M}$ . CHPG increased motor activity at a dose of 360  $\mu\text{M}$ , an effect that was blocked by co-administration of 2  $\mu\text{M}$  MK-801. The relevance of these findings to the development of antipsychotics based on the glutamate hypothesis is discussed.

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## Introduction

This senior project will focus on a new hypothesis of schizophrenia called the *glutamate hypothesis*. The glutamate hypothesis postulates that disruption of glutamatergic transmission at NMDA receptor sites underlies the positive, negative, and cognitive symptoms of schizophrenia. Since the 1950s, neurological hypotheses of schizophrenia have focused their attention on dopaminergic systems in the brain. The glutamatergic systems that are the focus of the glutamate hypothesis have a “downstream” effect on these dopaminergic systems, allowing the old ways of thinking to be incorporated into the new, and painting a compelling new picture of the neural mechanisms underlying schizophrenia symptomatology. Animal models play an important role in development of glutamate based antipsychotic agents, and in this project a larval zebrafish model is used to investigate the effects of CHPG, a positive modulator of the metabotropic glutamate receptor type 5. The findings of this research are discussed into the context of the literature surrounding novel pharmacotherapies for schizophrenia.

The first chapter will introduce the reader to schizophrenia. Following a brief examination of the psychological, social, and economic burdens of the disease, the three main categories of symptoms (positive, negative, cognitive) will be introduced and examples will be provided for each.

The second chapter will describe how the *dopamine hypothesis* has attempted to account for schizophrenic symptoms. Starting with the first use of a dopamine antagonist for antipsychotic treatment, the origins of the dopamine hypothesis will be explained alongside early evidence for the involvement of dopaminergic transmission in

schizophrenia. The chapter will explain the mechanisms of action of D2 antagonists and clozapine (an atypical antipsychotic), and will list the side effects of these drugs which have contributed in part to the continual development of novel antipsychotic agents. Perhaps most importantly, the chapter will summarize the dopamine hypothesis' theoretical gaps surrounding the negative and cognitive symptoms, as well as the unmet therapeutic needs left by the typical and atypical antipsychotics.

The third chapter will begin with a history of the glutamate hypothesis, which stems from the findings of neurological studies on dissociative anaesthetics such as PCP and ketamine, drugs that block the NMDA glutamate receptor. This will lead into a description of the proposed model of glutamate disruptions in pathways that act on the dopamine pathways described in the previous chapter. The chapter will end with an overview of the metabotropic glutamate receptors, with a specific focus on the metabotropic glutamate type 5 receptor and agents that modulate activity at this receptor.

The fourth chapter will review recent studies on zebrafish that support the glutamate hypothesis. The findings of these studies will in part provide a rationale for the study conducted in this senior project. The main component of this chapter is an empirical study testing the effects two different dose levels of MK-801, a non-selective NMDA receptor antagonist, and CHPG, an agonist at the metabotropic glutamate receptor type 5, on motor activity in larval zebrafish whether administered individually or together.

The final chapter will summarize the findings of the zebrafish study in the context of the glutamate hypothesis and will conclude with suggestions for future research.

## **Chapter I: Schizophrenia and its Symptoms**

Schizophrenia is the most prevalent and best known, as well as the most persistent and debilitating, of all the psychotic disorders. It is a chronic illness that ranks among the top ten causes of disability in developing countries (WHO 2002). The world wide prevalence of schizophrenia has been reported at 1-1.1%. In the United States, there are over 300,000 acute schizophrenic episodes annually. It is an illness that causes great psychological and emotional suffering to those who have it. Rates of depression and social isolation are high among those with schizophrenia. The mortality rate of people with schizophrenia is eight times as high as that of the general population, due in part to the fact that between 25-50% of people with schizophrenia will attempt suicide. Recent meta-analyses have concluded that 4-4.9% of schizophrenics commit suicide (Palmer et. al. 2005; Inskip et. al. 1998), compared to a 2-9% suicide rate among people with depression (Mayo Clinic 2000). The World Health Organization reports an average reduced life expectancy of 10 years (Samnaliev & Clark 2008). The illness also represents a great burden to society as a whole, with 15% of people with schizophrenia residing for long periods in state or county mental health facilities (Javitt & Coyle 2004). Patients with schizophrenia occupy about 25-30% of all beds in hospitals, as well as accounting for 40% of all long term care days in the U.S (Chavez-Noriega et. al. 2002). According to Javett and Coyle (2004) around 15% of people with schizophrenia end up incarcerated for petty crimes and vagrancy, 60% live in poverty, and 5% are homeless. The estimated annual costs for treatment are \$20-35 billion in the U.S. alone, an amount that jumps to over \$46 billion when the cost of lost productivity is factored in. These statistics hint at the importance of finding effective ways to manage symptoms and care

for people with this disease. Although a pharmacological approach is the most widely accepted and effective form of treatment for both acute and chronic schizophrenia, antipsychotics alleviate all symptoms in only about 20% of patients (Javitt & Coyle 2004), while 30% of patients are completely unresponsive to typical antipsychotic treatment (Chavez-Noriega et. al. 2002). Furthermore, even among those who do respond to treatment with antipsychotic drugs, a large number discontinue their medication due to the unpleasant side effects.

According to the DSM-IV-TR: “The essential features of Schizophrenia are a mixture of characteristic signs and symptoms (both positive and negative) that have been present for a significant portion of time during a 1-month period (or for a shorter time if successfully treated), with some signs of the disorder persisting for at least 6 months” (American Psychiatric Association, 2000, p. 298). The terms “positive” and “negative” respectively refer to whether a particular symptom is problematic because of the *presence* of a maladaptive behavior or cognitive pattern, or because of the *absence* of a usually adaptive behavior or pattern of cognition. *Positive symptoms* represent distortions and exaggerations of normal cognitive functions, and negative symptoms represent a diminution or loss of normal functions. According to the DSM-IV-TR, positive symptoms include distortions in thought (delusions), distortions in perception (hallucination), disorganized language and speech, and disorganized self-monitoring behavior; *negative symptoms* include affective flattening, alogia (poverty of speech), anhedonia (lack of pleasure), and avolition. In addition to the positive and negative symptoms, people with schizophrenia also suffer from *cognitive symptoms*, which can

include impaired attention, impaired informational processing, problems with serial learning, and problems with executive functioning (Stahl 2002).

Positive symptoms are further divided into two dimensions: psychotic and disorganized. Psychotic symptoms include delusions and hallucinations while disorganized symptoms includes abnormalities in the form and structure of thought, speech, and behavior.

Delusional beliefs have four key features: they are objectively false, idiosyncratic, illogical, and stubbornly maintained. Delusions of influence consist of the patient believing they are being controlled by an outside force (delusions of control), that alien thoughts have been “inserted” into their mind against their will (thought insertion), or that an outside force has taken away their thoughts (thought withdrawal). Delusions of self-significance consist of gross distortions of self-importance, the belief that environmental sources are continually referencing the patient, or the assumption of illogical and exaggerated guilt or responsibility.

The hallucinations present in schizophrenia are most often auditory (80%), although they can be visual, olfactory, gustatory, or tactile (Stahl 2002). These hallucinations are distortions or disruptions of normal perceptual functioning, and can be very distressing for the schizophrenic person. The most common hallucinations are auditory hallucinations in the form of spoken voices. A particularly problematic subtype of auditory hallucinations is called “command hallucinations”, in which voices tell an individual to hurt his/herself or others (Javitt & Coyle 2004).

While hallucinations and delusions represent the psychotic dimension of positive symptoms, the speech/thought disorder in schizophrenia, as well as agitation, represents

the disorganized dimension. This symptom dimension is expressed as exaggerations or distortions in speech and thought. In diagnostic interviews, people with schizophrenia may give answers that are irrelevant or incoherent, and have trouble maintaining a logical, cohesive flow to their speech; they may jump from one subject to another with no transitions, or speak in loose associations; they may use neologisms (newly created words or idioms that do not make any sense); or they may repeat certain words and phrases over and over (Stahl 2002). Agitation, another symptom of the disorganized dimension, may be expressed as stereotyped behavior or diminished impulse control, and can also be expressed in speech as “word salad” (Javitt & Coyle 2004). Additionally, motor symptoms appear in at least 50% of psychotic patients (Seibt et. al. 2010).

The negative symptoms of schizophrenia are characterized by a reduction in normal functioning. Affective flattening or “blunted affect”, a commonly observed negative symptom, consists of restrictions in the range and intensity of the person’s emotional expression, and is; alogia is a restriction in the fluency and productivity of thought and speech; avolition is a restriction in the initiation of goal-directed behavior, speech, or movement; anhedonia is a restriction in the ability to feel pleasure (Stahl 2002). Another negative symptom is “autism” (not to be confused with the disorder on the autism spectrum), which refers to a loss of interest in other people or a one’s own surroundings (Javitt & Coyle 2002).

Individuals with schizophrenia also display cognitive symptoms, deficits or disruptions in normal cognitive functioning. While individuals with schizophrenia are likely to exhibit impairment on a wide assortment of neuropsychological tasks, recent findings have specifically highlighted deficits in selective attention, executive function,

motor and tactile dexterity, special abilities, affect recognition, intellectual ability, language functions, and memory (Heinrichs & Zakzanis 1998). For instance, a characteristic cognitive symptom of schizophrenia is prepulse inhibition (PPI) impairment. PPI is the ability to adapt to an loud auditory stimulus that is preceded by a warning tone. People with schizophrenia have been found to have deficiencies in their PPI. It is believed to reflect a defect in attentional “filtering” of nonnovel stimuli (Goff & Coyle 2001). Additionally, individuals with schizophrenia tend to show impairment on tasks that test working memory, such as spatial delayed-response tasks, the Wisconsin Card Sorting Task, the Stroop test, and the Tower of London task (Goldman-Rakic 1994).

The severity of an individual’s cognitive symptoms has been shown to be the best predictor of long-term outcome (Chavez-Noriega 2002). This could be because the severity of cognitive symptoms may represent the severity of the individual’s illness as a whole, or because cognitive symptoms have functional consequences that affect long-term outcome. For instance, a literature review conducted by Green (1996) found that performance on secondary verbal memory (the ability to recall lists of words or stories after a time delay) and card sorting cognitive tasks predicted community functioning outcome in people with schizophrenia; secondary verbal memory and vigilance (the ability to discriminate targets from non-targets in cognitive response tasks) predicted social problem-solving ability; immediate verbal memory (the ability to repeat back a series of digits), secondary verbal memory, and vigilance predicted capacity for skill acquisition in psychosocial rehabilitation programs. A recent longitudinal study by Shrivastava et. al. (2011) showed that regardless of improvement on the Clinical Global

Rating scale, individuals with schizophrenia still displayed deterioration of visuo-motor integration, working memory, and executive functioning over a period of ten years.

There is no question that if cognitive deficits are present, an individual can be expected to experience difficulties in other areas of their life such as social and emotional functioning. However, despite the evidence that cognitive symptoms are a severely debilitating dimension of schizophrenia, current pharmacological treatments mostly offer relief from only the positive symptoms of the disease, with minimal or no effect on the negative and cognitive symptoms (Lindsley et. al. 2006). Therefore, relief from negative and cognitive symptoms represents a substantial unmet medical need.

Sine the 1950s, the gold standard for treatment of schizophrenia has been treatment with dopamine antagonists, which are generally efficacious for ameliorating positive symptoms while ineffective for treating negative and cognitive symptoms. First generation antipsychotics still represent a large percentage of the agents used, though recently atypical antipsychotic treatment and development has become more prominent. Atypical antipsychotics are compounds that have antagonist activity at serotonin (5HT) type 2A receptors in addition to antagonist activity at dopamine type 2 receptors. They have been shown to have less side effects than first generation antipsychotics – as well as encouraging (albeit inconsistent) efficacy for a broader variety of symptoms than first generation antipsychotics – but there is a lack of comprehensive understanding of why they work when they do.

While researchers have continued to attempt to illuminate the mechanisms of action of the atypicals antipsychotics, new psychotomimetic paradigms and the ongoing need to find therapeutic agents with efficacy for both negative and cognitive symptoms

has shifted the focus of much schizophrenia research onto the glutamate systems. Glutamate-based drug therapies may be a novel way to treat negative and cognitive symptoms alongside positive symptoms. In order to understand how this new class of drug will work to affect changes in the brain and differ from first and second generation antipsychotics, it is important to review the current theories of schizophrenia. The next chapter will briefly explain the theoretical underpinnings that have historically guided antipsychotic drug development, known as the *dopamine hypothesis*, as well as the mechanisms by which antipsychotic drugs work.

## Chapter II: The Dopamine Hypothesis and Antipsychotics

For the last 60 years, pharmacotherapies and neurological models of schizophrenia have focused heavily on one neurotransmitter: dopamine. Dopamine is a member of the catecholamine family and plays an important role in behavior and cognition, voluntary movement, punishment and reward, inhibition of prolactin production, sleep, mood, attention, working memory, and learning. The *dopamine hypothesis* states that dopamine dysregulation in the central nervous system results in the symptoms of schizophrenia. The origins of this theory lie in the history of the development of antipsychotic drugs. In 1950 a Naval surgeon named Henry Laborit noted that the secondary effects of promethazine, a drug intended for use as a surgical sedative, included drowsiness, indifference to pain, and general euphoria. Laborit began to consider other possible applications for the drug, while also encouraging Laboratoires Rhône-Poulenc to synthesize a similar compound with increased psychological effects. Paul Charpentier was the chemist who then designed chlorpromazine (Thorazine) in 1951, which was distributed to physicians and psychiatrists for use as an antipsychotic. By 1954 chlorpromazine was being used in the United States to treat schizophrenia, and as its therapeutic use grew more popular, research was targeted at discovering its neurological mechanism(s) of action.

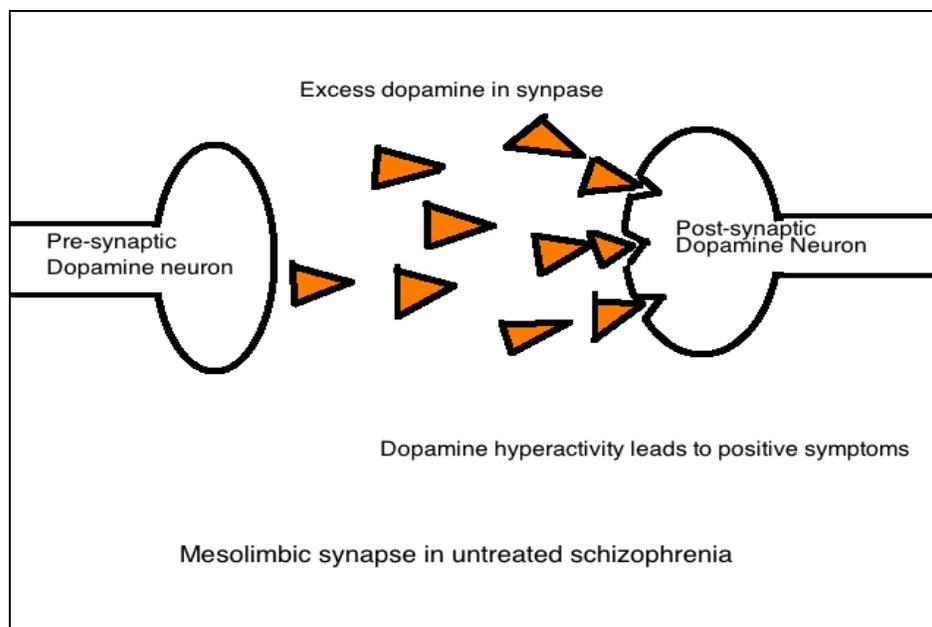
By the early 1960s, empirical findings had shown that the first generation of antipsychotics (commonly referred to as typical antipsychotics or neuroleptics) acted as antagonists on dopamine type D2 receptors, binding to these receptor sites and decreasing dopamine release. Furthermore, side effects resembling Parkinson's disease were

observed early on in clinical trials of antipsychotics, and these observations contributed to the understanding that typical antipsychotics acted on dopamine neurons. Research had already established Parkinson's disease as a result of deficient dopamine in the brain, especially in the nigrostriatal dopamine pathway (Mueser & Jeste 2008; Lauelle et. al. 2005; Stahl 2002). Around the same time, Arvid Carlsson demonstrated that amphetamines administered to healthy subjects induce hallucinations and delusions (analogous to the positive symptoms of schizophrenia) via indirect agonist activity at dopamine D2 receptors. Carlsson's research was inspired by earlier findings from studies of symptoms present in patients hospitalized for amphetamine psychosis: the patients frequently experienced auditory hallucinations much like those typically reported in schizophrenia – vague noises, voices that the patients occasionally conversed with – as well as visual hallucinations that resembled those reported by schizophrenic individuals during acute psychotic episodes (Snyder et. al. 1974). The dopamine hypothesis arose out of all of these early clinical observations. The current rationale for this hypothesis implicates dopamine as a causative neurotransmitter in schizophrenia based on the combined evidence that: 1) all clinically relevant antipsychotic agents display significant antagonistic activity at the dopamine D2 receptor; 2) indirect dopamine agonists, such as amphetamine and cocaine, have been shown to induce positive psychotic symptoms both human and animal subjects; and 3) many of the patients treated with first generation antipsychotics displayed Parkinsonian side effects, pointing towards dopamine antagonism as the mechanism of action by which these drugs acted on the brain.

Informed by pharmacological discoveries, the dopamine hypothesis implicates hyperactivity of dopamine in the mesolimbic dopamine pathway as the cause of the

positive symptoms of the disease. The dopamine neurons of this pathway have cell bodies located in the ventral tegmental area; dopamine is carried to the nucleus accumbens via the amygdala and hypothalamus. Hyperactivity of dopamine neurons in this pathway is thought to be responsible for the hallucinations and delusions present in schizophrenia (Stahl 2003).

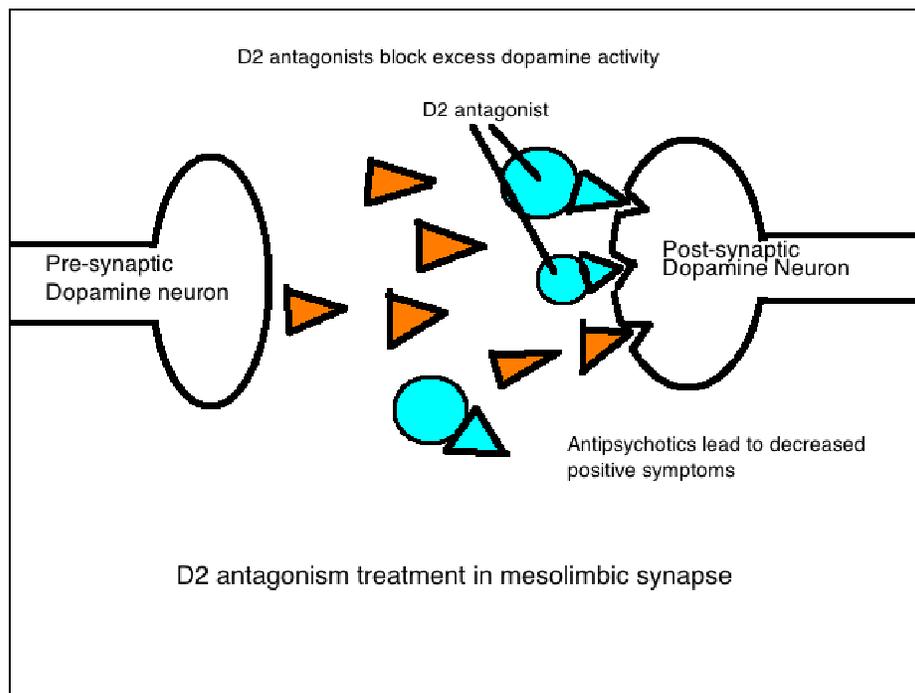
**Figure 1. Dopamine hyperactivity in mesolimbic pathway results in overactive post-synaptic dopamine neurons.**



Negative and cognitive symptoms are accounted for via a different dopamine pathway. The mesocortical dopamine pathway is comprised of dopamine neurons whose cell bodies are, like those of the mesolimbic pathway, located in the ventral tegmental area. However, unlike those neurons of the mesolimbic pathway, these neurons project dopamine to areas of the cerebral cortex, such as the dorsolateral prefrontal cortex, as well as many structures in the limbic system (Stahl 2002). According to the dopamine hypothesis, hypoactivity of these neurons is responsible for the negative and cognitive

symptoms seen in schizophrenia. While there has been considerable progress in the development of pharmacological agents to treat positive symptoms, biological treatments for the negative and cognitive symptoms remain unsatisfactory.

All first generation antipsychotics (also known as typical antipsychotics, or neuroleptics) inhibit dopamine 2 receptors. This ability to block D2 receptors is responsible for their clinical efficacy. By inhibiting D2 receptors in the mesolimbic dopamine pathway, neuroleptics can reduce hallucinations and delusions in people with schizophrenia (Stahl 2002). The efficacy of a neuroleptic in reducing positive symptoms is correlated with the compound's affinity for the D2 receptor (Snyder et. al. 1974; Stahl 2002).



**Figure 2. D2 Antagonists inhibit post-synaptic hyperactivity of dopamine neurons.**

While this method of drug treatment does provide reliable relief from positive symptoms in a majority of patients (roughly 70%), there are several drawbacks to neuroleptic treatment (Chavez-Noriega et. al. 2002). Antipsychotic drugs are delivered to the brain through oral ingestion, and therefore these compounds bind non-selectively to every D2 receptor in the brain, leading to decreased activity in both the mesolimbic pathway *and* mesocortical pathway. The result is that, while positive symptoms are alleviated due to antagonist activity in the mesolimbic pathway, negative and cognitive symptoms may be worsened due to antagonism in the mesocortical pathway – a condition referred to as the *neuroleptic-induced deficit syndrome* (Stahl 2002). Furthermore, many side effects are a result of the fact that in addition to the two pathways already mentioned, two other relevant dopamine pathways in the brain are blocked by neuroleptics – the nigrostriatal dopamine pathway, and the tuberoinfundibular dopamine pathway.

The nigrostriatal pathway is part of the extrapyramidal nervous system, responsible for controlling motor movements. In Parkinson's disease, deterioration of the nigrostriatal pathway is responsible for symptoms such as rigidity, akinesia (loss of control of voluntary movement), bradykinesia (impaired ability to adjust one's body position), and tremor. By blocking D2 receptors in this pathway, typical antipsychotics may cause drug-induced parkinsonism, as well as akathisia (a syndrome characterized by unpleasant "inner restlessness" that may manifest as an inability to sit still or remain motionless) and dystonia (characterized by twisting movements and abnormal postures due to sustained muscle contractions). Side effects on movement resulting from reduced dopamine in the nigrostriatal pathway are commonly referred to as extrapyramidal side effects, or EPS. Chronic blockade of D2 receptors in the nigrostriatal pathway may

produce a hyperkinetic movement disorder called tardive dyskinesia, characterized by constant chewing, tongue protrusions, facial grimacing, and jerky or choreiform limb movements (Stahl 2002). Annual incidence of neuroleptic-induced tardive dyskinesia is 5% (Stahl 2002), and in many cases it is an irreversible condition.

In addition to the side effects caused by blockade of the nigrostriatal pathway, patients may experience side effects caused by blockade of the tuberoinfundibular pathway. When D2 neurons in this pathway are blocked plasma prolactin levels increase, which may result in a condition called hyperprolactinemia. This condition is associated with breast secretions, irregular menstrual periods, and demineralization of bones in women. Further side effects may include sexual dysfunction and weight gain due to elevated prolactin levels (Stahl 2002).

Clearly there are many risks inherent in conventional antipsychotic treatment despite the benefits of alleviated positive symptoms. Side effects are associated with discontinuation of medical treatment by patients at a rate of 10% per month, resulting in a 50% relapse rate by 6 months after medication has been discontinued (Stahl 2002). Furthermore, 30% of individuals with schizophrenia do not respond to typical antipsychotics (Chavez-Noriega et. al. 2002; Sajatovic et. al. 2008). The development of atypical antipsychotics was intended to remedy this situation. The goal in the development of the atypicals has been to decrease the prevalence of unpleasant side effects as well as increase clinical efficacy for more symptoms in a larger portion of the patient population. The improved efficacy and reduced extrapyramidal side effects of treatment with clozapine, the prototype drug of the atypical class, has led to the development of atypicals such as risperidone, olanzapine, quetiapine, ziprasidone,

aripiprazole, and paliperidone. According to a European review (Seshamini 2002) treatment with clozapine improves patient symptoms and quality of life, as well as reduces the number of hospitalizations in individuals with schizophrenia. Clozapine has antagonist activity at dopamine type 2 receptors much like haloperidol. Additionally, clozapine blocks serotonin type 2A (5HT-2A) receptors. Serotonin and dopamine interact in the nigrostriatal dopamine pathway of the brain, the pathway responsible for the Parkinsonian side effects that can be caused by treatment with typical antipsychotics such as haloperidol. In this pathway, serotonin acts as a brake by inhibiting dopamine neurons. By blocking 5HT-2A receptors here, 5HT-2A antagonism can reverse the dopamine type 2 antagonism resulting from blockade of dopamine receptors, leading to fewer or no extrapyramidal symptoms than those that result from the dopamine antagonism of typical antipsychotics (Stahl 2002).

However, while atypical antipsychotics are not associated with the extrapyramidal side effects that characterize neuroleptic treatment, the atypicals have their own side effect profile which can contribute to the problem of treatment discontinuation in the same way that typical antipsychotic side effects have. When compared to haloperidol, clozapine is associated more with hypersalivation, temperature increase, and drowsiness, but fewer motor side effects and less dry mouth (Sajatovic et. al. 2008). Clozapine side effects can include agranulocytosis (dangerous lowering of white blood cell count leading to increased risk of infections), granulocytopenia (another lowering of white blood cell count), sedation, seizures, fevers (100-103°F), neuroleptic malignant syndrome (a life-threatening neurological disorder characterized by muscle rigidity, fever, autonomic instability, and delirium), development of obsessive-compulsive symptoms, tachycardia

(potentially dangerous increase of resting heart rate), orthostatic hypotension (low blood pressure), prolongation of QTc interval (which may lead to palpitations, fainting, or sudden death), deep vein thrombosis (blood clotting in various limbs), myocarditis (inflammation of the heart muscles with a 20% mortality rate), cardiomyopathy (deterioration of heart muscles), sialorrhea (excessive drooling), urinary retention, constipation, gastrointestinal obstruction, and enuresis (involuntary urination) (Sajatovic et. al. 2008). In addition, McGurk et. al. (2005) suggested that the anticholinergic effects of clozapine may be responsible for the worsening of spatial working memory in individuals with schizophrenia. Furthermore, research has failed to find consistent significant data supporting atypical drugs efficacy for cognitive and negative symptoms. On a final note, clozapine use is limited to about 5% of patients in clinical settings due to its adverse side effect profile, need for regular laboratory monitoring, and cost (Satajovic et. al. 2008).

The dopamine hypothesis has laid the foundations for an understanding of the neural mechanisms underlying many symptoms of schizophrenia, as well as leading to the development of antipsychotic agents. However, there are still unanswered questions about the neural mechanisms of negative and cognitive symptoms, and current antipsychotic treatments have been inconsistent in ameliorating these symptoms. Additionally, the adverse side effect profiles of antipsychotics demonstrate that the current state of pharmacotherapies for schizophrenia is one that can be improved upon. In recent years a new hypothesis of schizophrenia, the glutamate hypothesis, has led to the possibility of the development of antipsychotic treatments which are efficacious for a broader range of symptoms, and which may also avoid the side effects of current

antipsychotic administration. The next chapter will review the glutamate hypothesis, as well as antipsychotic agents that have been developed based on this new direction in schizophrenia research.

### **Chapter III: The NMDA-R Hypothesis of Schizophrenia**

In the last twenty years, researchers investigating the etiology of schizophrenia have shifted their focus from dopamine systems to “upstream” glutamate systems that have modulating effects on the relevant dopamine pathways implicated in schizophrenia. Glutamate is the primary excitatory neurotransmitter in the brain, which means it activates many neurons. It is fairly ubiquitous (60% of neurons contain glutamate, and almost all neurons have some type of glutamate receptor), and plays a role in prenatal and childhood development, learning, and memory (Harvard Mental Health Letter 2009). Glutamate also has a modulating effect on the dopamine pathways implicated in the dopamine hypothesis. By looking at glutamate’s role in schizophrenia, researchers have begun to formulate a model of the disease which incorporates the whole of the dopamine hypothesis while also accounting for aspects of schizophrenia which the dopamine hypothesis has been unable to explain.

The glutamate hypothesis comes out of research with the dissociative anesthetics PCP and ketamine. According to Javitt & Coyle (2004) studies first drew parallels between the effects of PCP and the symptoms of schizophrenia as early as the 1960s. PCP acts as a nonselective antagonist at the NMDA glutamate receptor, binding to a site within the ion channel and blocking the influx of cations that leads to an action potential (Goff & Coyle 2001). The NMDA receptor is a ligand- and voltage-gated calcium/sodium channel that is believed to play a role in both learning and memory (Sison & Gerlai 2011; Javitt 1987). Ketamine has the same mechanism of action at the NMDA receptor, with a lower affinity than PCP. As psychotomimetics, there is

compelling evidence that these NMDA receptor antagonists can produce a psychotic state more closely resembling the symptom profile of schizophrenia than dopamine agonists such as amphetamine or methylphenidate. While dopamine agonists only induce effects similar to the positive symptoms of the disease, PCP and ketamine can give rise to a state that resembles the full range of positive and negative symptoms in schizophrenia including hallucinations, thought disorder, specific cognitive impairment, emotional withdrawal, and apathy (Javitt 1987; Stone 2009). PCP can produce a syndrome in normal individuals that closely resembles schizophrenia and exacerbates symptoms in patients with chronic schizophrenia (Luby et. al. 1959). Ketamine can produce positive symptoms in the form of suspiciousness, disorganization, and auditory and visual hallucinations; negative symptoms in the form of blunted affect, withdrawal, and psychomotor retardation; and cognitive symptoms including impaired performance on the Wisconsin Card Sorting Test (which is used to test a number of cognitive abilities including attention, memory, visual processing, and other executive functions) and on verbal declarative memory, delayed word recall, and impairment on verbal fluency tests (Goff & Coyle 2001). In fact, both ketamine and PCP mimic schizophrenia so well that patients treated with either of these drugs cannot be readily distinguished from individuals with schizophrenia in clinical settings (Javitt & Zukin 1991).

Observations on the psychomimetic effects of these NMDA receptor antagonists have led to the development of a NMDA-R hypothesis of schizophrenia, referred to as *the glutamate hypothesis*. The glutamate hypothesis attributes the symptoms of schizophrenia to hypofunctional NMDA receptors that have a downstream effect on the two major dopamine pathways implicated in the dopamine hypothesis. There is an

important descending glutamatergic pathway which projects from cortical pyramidal neurons to dopamine neurons in the ventral tegmental area (VTA), which normally acts as a brake on the mesolimbic dopamine pathway by exciting  $\gamma$ -aminobutyric acid (GABA) interneurons in the VTA to inhibit dopamine release from the mesolimbic pathway. Hypoactivity at NMDA receptors in schizophrenia would lead to a decrease in activation of GABA interneurons, thereby leading to hyperactivity of the mesolimbic pathway – responsible for the positive symptoms of the disease (hallucinations, delusions, thought disorder, agitation).

**Figure 3. NMDAR hypofunction leads to positive symptoms (Stahl 2002).**

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decompressor  
are needed to see this picture.

Another important glutamate pathway extends from the cortical regions of the brain caudally to the brainstem, and the neurons of this pathway normally have an excitatory effect on the dopamine neurons of the mesocortical dopamine pathway. Hypoactivity at

NMDA receptors in schizophrenia would lead to decreased dopamine activity in the mesocortical pathway – thereby accounting for the negative symptoms (blunted affect, withdrawal, avolition) and cognitive symptoms (working memory impairment, executive functioning deficits) of the disorder (Stahl 2007).

**Figure 4. NMDAR hypofunction leads to negative and cognitive symptoms (Stahl 2002).**

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Several neurological findings are consistent with the glutamate hypothesis: studies have shown significantly lower levels of glutamate in the cerebrospinal fluid and postmortem brain tissue of individuals with schizophrenia than in controls; cerebrospinal fluid glutamate levels have been shown to be inversely correlated to the severity of positive symptoms in unmedicated individuals with schizophrenia; levels of kynurenic acid (an endogenous ionotropic glutamate antagonist with activity at the glycine site of NMDA receptors) have been found to be elevated in the cerebrospinal fluid and cortical

tissue of schizophrenics compared to controls; and a number of studies have reported alterations in gene and receptor expression in cortical, hippocampal, and thalamic regions for several glutamate receptors including the NMDA receptors (Chavez-Noriega et. al. 2002).

Genetic findings from animal models also support the glutamate hypothesis. Several studies have shown that decreased expression of the NR1 subunit, required in mice for normal NMDA receptor function, leads to a range of phenotypic symptoms analogous to schizophrenia such as increased locomotor activity, stereotypy and deficits in social and sexual interactions, which can be ameliorated by treatment with haloperidol and clozapine (Chavez-Noriega et. al. 2002); furthermore, symptoms such as disruption of pre-pulse inhibition, spatial working memory, and GABA interneuron expression are more likely to occur if NR1 deletion occurs during early developmental windows – consistent with the neuro-developmental aspect of schizophrenia (Gordon 2010). NR2 knockout display schizophrenic-like behaviors that can be ameliorated with antipsychotic drug treatment, and NR1 glycine site knockout mice display impairment in LTP and learning (Linsley et. al. 2006). A study using metabotropic glutamate type 5 receptor knockout mice demonstrated a significant reduction in the NMDA-component of synaptic transmission, as well as long-term potentiation, in hippocampal field CA1 and the dentate gyrus (Lu et. al. 1997). Kinney et. al. (2003) were able to demonstrate that mGluR5 knockout mice had significant deficits in PPI when compared to wild-type controls.

In light of the growing body of evidence in support of the glutamate hypothesis, it has been suggested that modulation of glutamatergic transmission be used as a novel pharmacotherapy for schizophrenia, and thus several compounds that have action at

glutamate receptors have been developed for research. Metabotropic glutamate receptors (mGluRs) have emerged as promising targets for development of new ligands. The relative uneven distribution in the brain of mGluRs, compared to ionotropic glutamate receptors (iGluRs), gives rise to the possibility that selective modulators of mGluR function might be used to target specific aspects of glutamatergic activity in specific neural circuits. While none of these agents are currently approved for clinical use in humans because they are still so early in the development and research process, several studies have shown that modulation of glutamatergic transmission at metabotropic receptor sites may be able to ameliorate symptoms of schizophrenia.

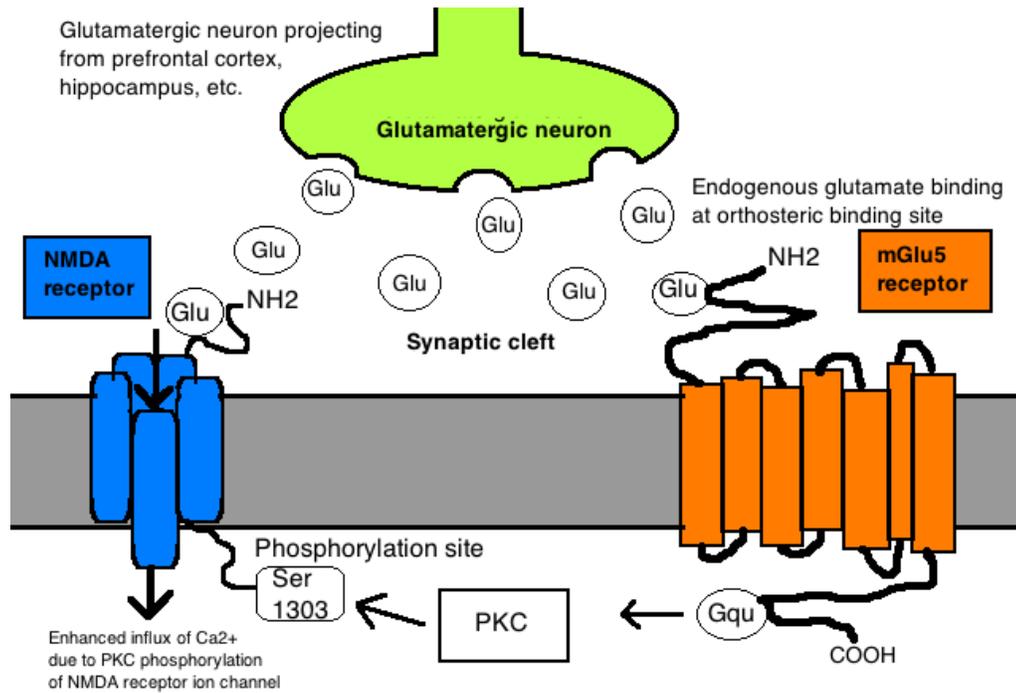
The metabotropic glutamate receptors belong to the family C of seven transmembrane receptors that couple with G proteins and control to the activity of membrane enzymes and ion channels in the neuron (Kanuma et. al. 2010). There are eight known types of mGluRs classified into groups I, II, and III according to primary structure, second messenger coupling, and pharmacological profile. Group I (containing mGluR1 and mGluR5) and group II (containing mGluR2 and mGluR3) have both been identified as potential targets for novel antipsychotic agents.

**Figure 5. Classification of metabotropic glutamate receptors.**

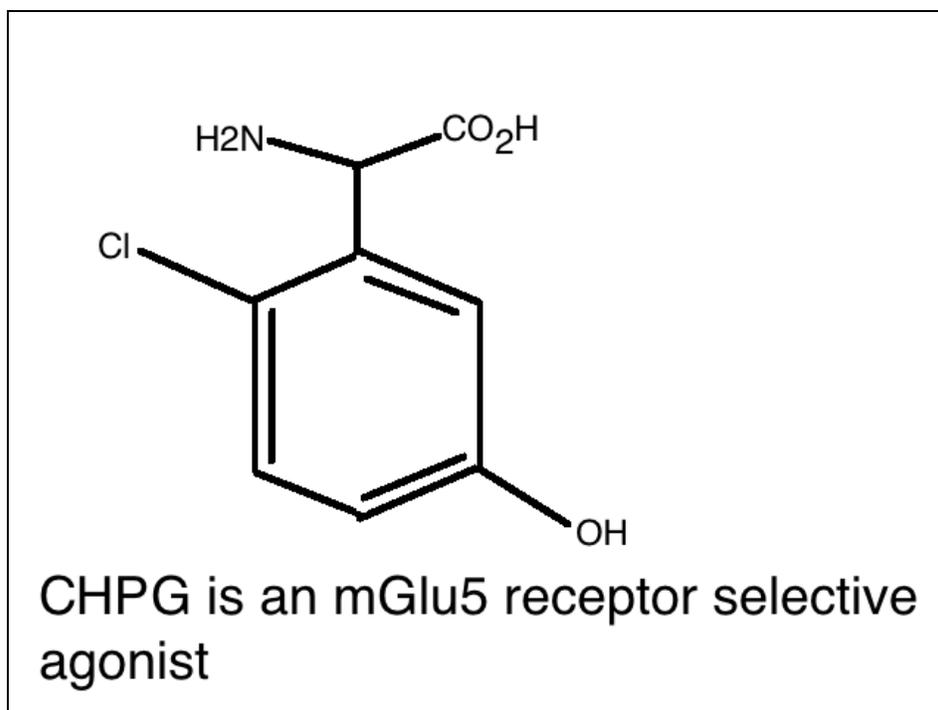
Group	Receptor	Signal transduction mechanism	Primary localization
I	mGluR1	↑PLC	Postsynaptic; forebrain/midbrain
I	mGluR5	↑PLC	Glia, postsynaptic; forebrain/midbrain
II	mGluR2	↓AC	Pre/postsynaptic; forebrain
II	mGluR3	↓AC	Glia, postsynaptic; forebrain
III	mGluR4	↓AC	Pre/postsynaptic; cerebellum
III	mGluR6	↓AC	Postsynaptic; retina
III	mGluR7	↓AC	Pre/postsynaptic
III	mGluR8	↓AC	Pre/postsynaptic

AC = adenylate cyclase; PLC = phospholipase C; ↑ indicates increase; ↓ indicates decrease.

Multiple studies have shown that activation of group I mGluRs induces potentiation of NMDA receptor currents in a variety of regions in the brain (Sou et. al. 2006; Chan et. al. 2008). In particular, activation of mGluR5 has been shown to alleviate locomotor, sensorimotor, and cognitive deficits induced by NMDA receptor antagonists. Chavez-Noriega et. al. (2002) have hypothesized that activation of mGluR5 may normalize hypofunctional NMDAR transmission in schizophrenia, thereby representing a useful approach for the development of novel antipsychotic drug treatments. There is supporting evidence from studies on mGluR5 antagonists, which have demonstrated that the mGluR5 antagonists MPEP and MTEP can worsen motor and cognitive symptoms induced by NMDA receptor antagonists (Krystal et. al. 2010). According to Kanuma et. al. (2010) the mechanism by which activation of mGluR5 with agonist agents enhances NMDAR function is that it is likely that activation of mGluR5 leads to PKC phosphorylation of the ion channel associated with NMDA receptors, thereby resulting in increased NMDA receptor sensitivity and activity, and leading to an influx of calcium ions. Figure 6, adapted from Kanuma et. al. (2010), is an illustration that shows this chain of events: glutamate binds to the mGluR5 site (drawn as a 7-transmembrane protein), triggering the activation of a second messenger (G $\alpha$ ), leading to PKC phosphorylation, resulting in enhanced ionic influx.

**Figure 6. mGluR5 activation enhances NMDAR function (Kanuma et. al. 2010)**

One particular compound that has been shown to have antipsychotic effects in rodent models of psychosis is the mGluR5 agonist (RS)-2-chloro-5-hydroxyphenylglycine (CHPG). Sou et. al. (2006) administered CHPG via intracerebralventricular (i.c.v.) injections to male NMRI mice that had been treated with either ketamine or propofol (an anaesthetic which binds to GABA subtype A neurons). Results indicated that CHPG decreased the duration of ketamine-induced loss of right reflex (LORR) at 3 dose levels (0.5, 2.5, and 5.0 nmol in a 5 $\mu$ L injection) in a dose dependant manner (5.0 nmol dose resulted in the shortest duration of LORR), but did not have an affect on propofol-induced LORR. This supports the hypothesis that CHPG acts via modulation of NMDA receptors.

**Figure 7. Structure of CHPG.**

In another study, Chan et. al. (2008) examined whether CHPG could reverse ketamine induced locomotor hyperactivity, motor incoordination, sensorimotor gating deficit, and learning impairment in male NMRI mice (a Swiss-bred type) and ICR (Inherited Cataract Rat, bred for research in neurobiology and ophthalmology) rats. CHPG reversed ketamine-induced hyperactive locomotor activity at a dose of 5 nmol delivered via a 5 $\mu$ L i.c.v. injection, but did not affect motor activity when delivered alone. CHPG also improved ketamine-induced deficits in the novel object recognition test at doses of 1 nmol and 5 nmol, respectively. CHPG did not alter sensorimotor gating, motor coordination on the rotarod test, or cognitive abilities in the novel object recognition test when delivered alone. This study supports the hypothesis that CHPG may be binding directly to the orthosteric (direct) glutamate binding site and thus increasing the intrinsic

effect of endogenous glutamate, leading to the enhancement of NMDA receptor function; at the same time, the fact that the drug had no effect on these measures when administered alone opposes the possibility that the reversal of ketamine-induced hyperactivity was due to a sedative effect, or that the reversal of cognitive deficits in the novel object recognition test was due to anxiolytic effects.

Current investigations into the ability of mGluR5 agonism to alleviate the effects of NMDA-R antagonism have been limited to rodent models of psychosis. Zebrafish (*Danio rerio*) is an emerging animal model of the behavioral and cognitive disruptions caused by NMDA-R antagonism. A small number of studies have demonstrated the ability of first and second generation antipsychotic treatment to alleviate the behavioral and brain changes induced by MK-801 (a non-selective NMDA-R antagonist). Taken together, these findings suggest a rationale for investigating whether or not mGluR5 agonism can reverse the behavioral disruptions caused by administration of MK-801. The next chapter will include the rationale, methods, results, and discussion of a pilot study conducted to investigate the effects of CHPG on MK-801-induced motor symptoms in zebrafish larvae.

## **Chapter IV: Assessing CHPG Reversal of Locomotor Effects of MK-801 in Zebrafish Larvae**

### **Background**

Schizophrenia is a central nervous system disorder characterized by positive, negative, and cognitive symptoms. All currently approved antipsychotic drugs share the trait of reducing dopaminergic function via antagonist or partial antagonist activity at dopamine type 2 receptors. One consequence of this is that most antipsychotics are only effective for the treatment of the positive symptoms of the disorder (hypothesized to be a result of dopaminergic hyperactivity in the mesolimbic dopamine pathway), while negative and cognitive symptoms (hypothesized to be a result of dopaminergic hypoactivity in the mesocortical dopamine pathway) are alleviated inconsistently, if at all. Thus, there is an incentive to develop pharmacotherapies that will be efficacious for the whole range of symptoms present in schizophrenia. In the last 10 years, research has begun to explore modulation of glutamatergic systems as a novel approach to antipsychotic treatment.

The dopamine pathways implicated in schizophrenia are “downstream” from key glutamate systems in the brain. Hypoactivity of the glutamate systems that act on the mesolimbic and mesocortical dopamine pathways has recently been implicated in the etiology of schizophrenia. In particular, it has been suggested that dysfunction at the *N*-methyl-D-aspartate (NMDA) glutamate receptor may play an important causal role in mediating positive, negative, and cognitive symptoms. The NMDA receptor is a ligand- and voltage-gated calcium/sodium cation channel that is believed to play a role in both learning and memory (Sison & Gerlai 2011; Javitt 1987). Non-selective NMDA-R

antagonists, such as PCP and ketamine, have been found to elicit symptoms in healthy controls that resemble the variety of symptoms found in individuals with schizophrenia. Importantly, the state induced by administration of NMDA-R antagonists more accurately models the full range of symptoms in schizophrenia than the psychosis induced by treatment with amphetamines, which increase dopaminergic activity and have heretofore been the most popular method of eliciting schizophrenic-like symptoms (Javitt 1987; Stone 2009).

Animal models of psychosis provide opportunities for investigation into different aspects of schizophrenia. The zebrafish (*Danio rerio*) is emerging as a model organism for examining the interactions of toxicology, neurodevelopment, and behavior (Padilla et. al. 2011). The small (4 cm long) freshwater teleost that inhabits slow moving streams and small lakes of the Indian sub-continent has been argued to represent an excellent compromise between neurological complexity and practical simplicity (Sison & Gerlai 2011). The many advantages to zebrafish include low cost, ease of handling and maintenance, and 70-80% genetic homology to humans (Seibt et. al. 2011). Recent findings suggest that zebrafish may be useful in modeling schizophrenic symptoms via administration of NMDA-R antagonists.

Dizocilpine (MK-801) is a non-selective NMDA-R antagonist that has been used in animal models of psychosis to elicit motor and cognitive effects. Treatment with MK-801 has been demonstrated to lead to a dose-dependent increase in motor activity in both rodent and adult and larval zebrafish models analogous to the positive symptom dimension in schizophrenia. The motor effects of MK-801 are hypothesized to be mediated by blockade of glutamatergic transmission leading to enhanced dopaminergic

activity. This mechanism of action is consistent with the mechanism of positive symptoms proposed by the NMDA-R hypofunction hypothesis of schizophrenia.

Swain et. al. (2004) used a zebrafish model to examine the effects of MK-801 on circling behavior, swimming activity, latency to enter (as well as preference for) an enriched chamber. Consistent with findings that treatment with PCP or other NMDA receptor antagonists increase circling behavior in rodents, it was demonstrated that MK-801 significantly increased circling behavior in adult zebrafish at a dose of 2 and 20  $\mu\text{M}$  in a dose-dependent manner. Treated fish circled “almost continuously”, whereas control fish displayed motor activity but rarely completed a full 360 degree circle. Preference for the enriched chamber was also disrupted in fish treated with MK-801 as compared to controls, suggesting a possible cognitive disruption due to MK-801 treatment.

Seibt et. al. (2010) demonstrated that a dose of 20  $\mu\text{M}$  MK-801 increased locomotor activity in adult zebrafish as measured by parameter line crossings, distance traveled, and mean speed when compared to control animals. Co-administration of either 9  $\mu\text{M}$  haloperidol, 100  $\mu\text{M}$  olanzipine, or 250  $\mu\text{M}$  sulpiride was shown to reverse the changes in locomotor behavior induced by MK-801. In another study, Seibt et. al. (2011) demonstrated that treatment with 20 $\mu$  MK-801 significantly decreased Na<sup>+</sup>, K<sup>+</sup>, ATPase activity (which is altered in various neuropsychiatric illnesses including schizophrenia) in adult zebrafish. This effect was reversed by administration of antipsychotics (haloperidol 9  $\mu\text{M}$ , olanzipine 100  $\mu\text{M}$ , or sulpiride 250  $\mu\text{M}$ ). Taken together, the findings of these two studies provide a rationale for investigating whether a novel antipsychotic agent could have the ability to reverse the effects of MK-801 in zebrafish.

The previously cited findings are from studies on adult zebrafish, and to date there are very few studies on larval models that exist in the current literature. However, in light of the proposed neurodevelopmental aspect of schizophrenia there may be knowledge to gain from investigations into zebrafish larvae as potential models of schizophrenic symptoms and antipsychotic effects. A recent study provides support for the conservation of the motor effects of MK-801 in zebrafish larvae. Chen et. al. (2010) demonstrated a dose-dependent effect of MK-801 on locomotor activity in zebrafish larvae 5-7 d.p.f.. MK-801 was found to significantly increase average swim speed over a 3 hour period when administered at doses of 5, 10, 20, 50, and 100  $\mu\text{M}$ . The dose that elicited the maximal increase in average swim speed was 20  $\mu\text{M}$ . This study provides evidence that the hyperactive motor effects of MK-801 previously demonstrated in adult zebrafish are conserved in zebrafish larvae, contributing to a rationale for investigating the effects of potential antipsychotics in larval models. The dose effects from the study also contribute to the rationale for using a 20  $\mu\text{M}$  dose of MK-801 in the present study.

(RS)-2-chloro-5-hydroxyphenylglycine (CHPG) is a novel glutamate-based compound which selectively binds to the metabotropic glutamate type 5 receptor (mGluR5). It has previously been shown to have antipsychotic activity in rodents. CHPG is hypothesized to modulate glutamatergic transmission via enhancement of NMDA-R function. According to Alioto & Ngai (2006) zebrafish have a genetic ortholog that codes for an mGlu5 receptor. While previous studies have examined the effects of NMDA-R antagonism in zebrafish, to date the ability of glutamate based compounds to enhance NMDA-R function have never been investigated in zebrafish. Furthermore, despite the fact that a number of studies have demonstrated that mGluR-

binding ligands have behavioral effects in rodents, there are very few studies that have assessed the degree to which these findings can be replicated in a zebrafish model. However, the ability of the mGluR5 antagonist MPEP (an mGluR5 antagonist which has been shown to increase the effects of NMDA-R antagonists) to affect the behavior of zebrafish in an addiction model (Tucker et. al. 2006) is sufficient reason to investigate whether an mGluR5 agonist can also affect larval behavior.

### **Experiment 1**

The purpose of this study was to assess whether treatment with the mGluR5 agonist CHPG has any independent effects on motor activity in zebrafish larvae, as well as whether treatment with CHPG can reverse the locomotor hyperactivity MK-801 has previously been demonstrated to induce in zebrafish. In order to achieve this, zebrafish larvae were observed at 5 days post fertilization following treatment with either vehicle (egg water), MK-801, CHPG, or a mixture of MK-801 and CHPG. The previously cited studies by Swain et. al. (2004), Chen et. al. (2010), and Seibt et. al. (2011) provide compelling evidence to hypothesize that the zebrafish treated with MK-801 would exhibit increased motor activity from baseline as compared to controls. Additionally, it was hypothesized that the motor activity of zebrafish treated with CHPG alone would not differ significantly from controls. The rationale for this prediction was based on findings of Kinney et. al. (2003) and Chan et. al. (2008) which demonstrated that while CHPG was able to reverse NMDA-R antagonist-induced motor and cognitive symptoms in rodents, there was no effect on these measures when administered alone. This is consistent with the hypothesis that CHPG may be binding directly to the orthosteric

glutamate binding site and increasing the intrinsic effect of endogenous glutamate, leading to the enhancement of NMDA receptor function. It was also hypothesized that fish treated with both MK-801 and CHPG would exhibit significantly lower motor activity than the group treated with MK-801 alone, and would not differ significantly from the control group or the group treated with CHPG alone. This prediction was based on observations from rodent studies that indicated an ability of CHPG to reverse NMDA-R antagonist-induced motor effects, while not having an independent effect on spontaneous motor activity.

### **1.1 Subjects**

Fertilized zebrafish eggs were obtained from Z-FIN Lab in Oregon, and housed on Bard College campus on a 14-hour lights on/off cycle. Zebrafish larvae were tested at 5 d.p.f. (days post-fertilization).

### **1.2 Materials**

MK-801 was obtained from Research Biochemicals International (Natick, MA). CHPG was obtained from Tocris Bioscience, Inc. CHPG sodium salt was used based on optimal water solubility. 6.7 mg MK-801 was dissolved in 10 mL egg water to yield a stock solution of 2000  $\mu\text{M}$  MK-801; the stock was then diluted to yield 30 mL solution of 20  $\mu\text{M}$  MK-801. 5 mg CHPG was dissolved in 60 mL egg water to yield a stock solution of 360  $\mu\text{M}$  CHPG, which was then diluted with egg water to yield a solution of a solution of 180  $\mu\text{M}$  CHPG. Stock solution of MK-801 and CHPG were diluted to yield a single solution of 20  $\mu\text{M}$  MK-801 and 180  $\mu\text{M}$  CHPG.

Zebrafish behavior was observed using a mounted Ikegami camera over 8 well plates on a 24 well plate dish, and recorded with Noldus EthoVisionXT at an applied

sampling rate of 29.97 samples/second. Detection settings were set as follows: Dynamic Subtraction; frame weight: 20; dark contrast: 19-134; subject size minimum: 8 pixels, subject size maximum: 1519 pixels. Data output was exported into Microsoft Excel and then transferred into SPSS. All data analyses were conducted using SPSS.

### 1.3 Procedure

Zebrafish were observed in round well plates each 2 cm in diameter. 8 fish were observed at a time, with a total of 104 fish. First, 1 mL egg water was added into each individual well. Zebrafish were transferred into each well by a pipette. Baseline activity was recorded for 5 minutes. The egg water was removed from the wells, leaving just enough for the larvae to swim in the perimeter. During the test period subjects received either: a) 1 mL of egg water (control group;  $n = 26$ ); b) 1 mL containing 20  $\mu\text{M}$  MK-801 ( $n = 26$ ); c) 1 mL containing 180  $\mu\text{M}$  CHPG ( $n = 26$ ); or d) 1 mL containing 20  $\mu\text{M}$  MK-801 and 180  $\mu\text{M}$  CHPG ( $n = 26$ ). We did not counter-balance the location of treatment administration in the well plates, because based on past experience the location of treatment administration would not be expected to affect the results. Motor activity was recorded for 15 minutes during the test period. After testing, zebrafish were left in the well plates for the rest of the day and observed for lethal effects of CHPG and MK-801.

### 1.4 Measures

Fourteen movement parameters were recorded and analyzed. These were as follows: Total distance traveled (millimeters); duration of movement in zone B (seconds); frequency of movement in zone B; latency to first move in zone B (seconds); duration of movement in zone C (seconds); frequency of movement in zone C; latency to first move in zone C (seconds); duration of movement overall (seconds); frequency of movement;

latency to first move (seconds); duration of immobility overall (seconds); frequency of not moving; latency to first stop moving (seconds); mean velocity (millimeters/seconds).

### 1.5 Data Analysis

The analysis output was exported from EthoVision as a Microsoft Excel file. Data was analyzed in a one-way ANOVA with condition (1 = control, 2 = MK-801, 3 = CHPG, 4 = MK-801 + CHPG) as the independent variable. A separate one-way ANOVA was conducted to determine if baseline differences in activity existed. One-way ANOVA tests were performed separately for each of the dependent measures described in 1.4. Tukey post-hoc tests were performed in order to make specific group expansions. All significance levels were set at  $p < 0.05$ .

### 1.6 Results

ANOVA tests conducted for all dependent variables at baseline showed that no significant differences existed between groups prior to drug administration. Although there was a trend for groups 3 and 4 (CHPG and MK-801/CHPG, respectively) to be less active at baseline, there were no significant differences detected. Tables 1 and 2 display the mean distance traveled for each group during baseline and test recordings.

**Table 1. Pre-Treatment Mean Distance Traveled (5 minutes)**

Condition	<i>N</i>	Mean Total Distance Traveled (mm)	ANOVA Statistics
Control	23	390.9 ± 37.7	$F(3, 96) = 1.422$
MK801	26	344.2 ± 43.5	$p = .241$
CHPG	26	287.1 ± 38.5	
MK801+CHPG	25	290.9 ± 41.4	
Total	100	326.8 ± 20.4	

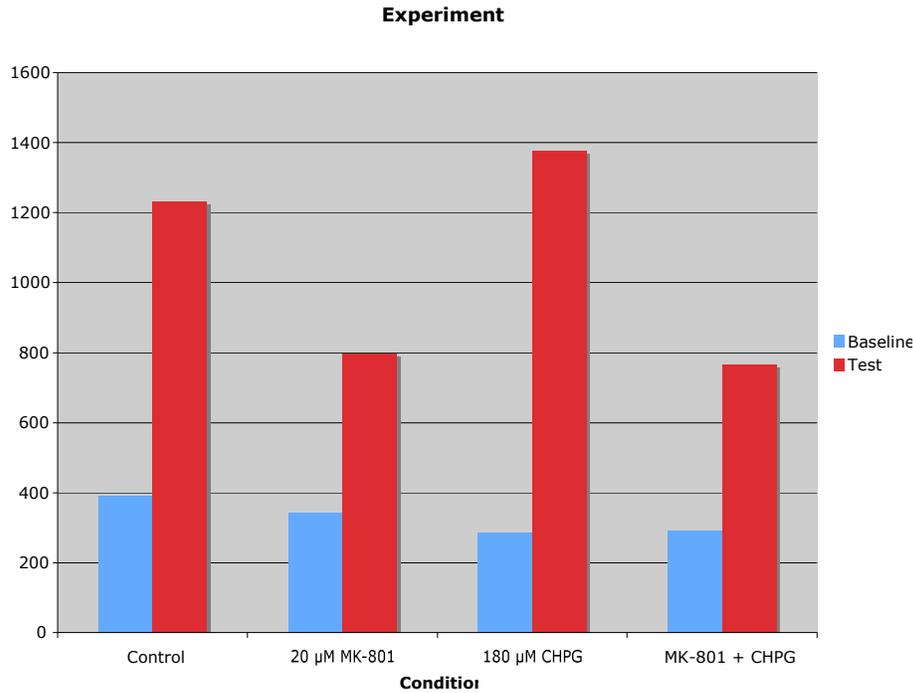
**Table 2. Post-Treatment Mean Distance Traveled (15 minutes)**

Condition	<i>N</i>	Mean Total Distance Traveled (mm)	ANOVA Statistics
Control	26	1232.4 ± 122.2	$F(3, 98) = 9.735$
MK801	26	797.8 ± 90.0	$p < .001$
CHPG	25	1377.5 ± 87.5	
MK801+CHPG	25	766.1 ± 87.6	
Total	102	1042.9 ± 55.2	

Table 1 shows that there were no pre-treatment differences in the total distance traveled by the larvae. Table 2 shows that there was a significant difference between groups after treatment. ANOVA tests conducted for all dependent variables at baseline showed that no significant differences existed between groups prior to drug administration. The results of a one-way ANOVA indicated the groups differed significantly on total distance moved,  $F(3, 98) = 9.735$ ,  $p < .001$ . Post-hoc analysis indicated that Group 1 (control) and Group 3 (CHPG alone) were more active than Group 2 (MK-801) and Group 4 (MK-801 + CHPG). A Tukey HSD test indicated that controls traveled a greater mean total distance than larvae treated with MK-801 (mean difference = 434.6,  $p = .011$ ) and larvae treated with MK-801/CHPG ( $MD = 466.3$ ,  $p = .006$ ); larvae treated with CHPG alone traveled a greater mean distance than larvae treated with MK-801 ( $MD = 579.6$ ,  $p < .001$ ) and larvae treated with MK-801/CHPG ( $MD = 611.3$ ,  $p < .001$ ).

	Control	20 µM MK-801	180 µM CHPG	MK-801 + CHPG
Total distance traveled (mm) (Mean ± S.E.M.)	1232.4 ± 122.2 + ^	797.8 ± 90.0 * #	1377.5 ± 87.5 + ^	766.1 ± 87.6 * #

\*different than controls; + different than MK-801; # different than CHPG; ^ different than MK-801 and CHPG



**Figure 1.** Larvae receiving MK-801 or co-administration of MK-801/CHPG traveled significantly less than controls or larvae treated with CHPG alone ( $F(3, 98) = 9.735, p < .001$ ). Pre- and post-treatment data are shown.

This particular result was unexpected because it was hypothesized that larvae treated with MK-801 would travel greater distance than controls, and that larvae treated with MK-801/CHPG would not differ from controls. However, as is shown below, the larvae in those both of those groups were less active than controls on several activity parameters. Controls and larvae treated with CHPG alone entered the perimeter (zone B) of the well more frequently than larvae treated with MK-801 or MK-801/CHPG ( $F(3,100) = 7.631, p < .001$ ), while not differing significantly from each other. A post-hoc Tukey HSD test indicated that controls differed from larvae treated with MK-801 ( $MD = 60.2, p < .008$ ) and from larvae treated with MK-801/CHPG ( $MD = 65.3, p = .003$ ). Larvae treated with

CHPG alone entered the perimeter significantly more than larvae treated with MK-801 ( $MD = 59.3, p = .009$ ) and larvae treated with MK-801/CHPG ( $MD = 64.3, p = .004$ ).

	Control	MK-801	CHPG	MK-801 + CHPG
Zone B frequency	110.1 ± 17.6 + ^	49.8 ± 8.6 * #	109.1 ± 15.3 + ^	44.8 ± 7.5 * #

\*different than controls; + different than MK-801; # different than CHPG; ^ different than MK-801 and CHPG

In addition to the result that larvae treated with MK-801 or MK-801/CHPG entered the perimeter less frequently than controls, a one-way ANOVA indicated a significant between groups difference on frequency of entering the center of the well that followed a similar trend ( $F(3, 100) = 7.448, p < .001$ ). Tukey post-hoc analysis indicated that controls entered more frequently than larvae treated with MK-801 ( $MD = 59.96, p = .007$ ) and larvae treated with MK-801/CHPG ( $MD = 63.57, p = .004$ ); larvae treated with CHPG alone entered the center more frequently than larvae treated with MK-801 ( $MD = 57.73, p = .010$ ) and larvae treated with MK-801/CHPG ( $MD = 61.34, p = .006$ ). Taken together, these two results suggest that larvae treated with MK-801 moved less overall than controls.

	Control	MK-801	CHPG	MK-801 + CHPG
Zone C frequency	108.0 ± 17.6 + ^	48.08 ± 8.5 * #	105.8 ± 14.7 + ^	44.4 ± 7.5 * #

\*different than controls; + different than MK-801; # different than CHPG; ^ different than MK-801 and CHPG

This was confirmed by results indicating a significant between groups difference for movement duration ( $F(3, 98) = 4.007, p = .010$ ). A post-hoc Tukey test indicated that larvae treated with MK-801 moved for a shorter amount of time than controls ( $MD = -$

134.1,  $p = .027$ ) and larvae treated with CHPG alone ( $MD = -131.39$ ,  $p = .034$ ); the other three groups did not differ significantly from each other.

	Control	MK-801	CHPG	MK-801 + CHPG
Movement duration (s)	694.8 ± 28.2 +	560.7 ± 37.7 * #	692.1 ± 22.7 +	601.3 ± 42.1

\*different than controls; + different than MK-801; # different than CHPG; ^ different than MK-801 and CHPG

In addition to the difference in movement duration, there was significant difference between groups for movement frequency ( $F(3, 100) = 6.905$ ,  $p < .001$ ), and a post-hoc Tukey test indicated that larvae treated with MK-801 moved less frequently than larvae treated with CHPG ( $MD = -71.65$ ,  $p = .002$ ), and larvae treated with CHPG alone moved more frequently than larvae treated with MK-801/CHPG ( $MD = 70.6$ ,  $p = .002$ ).

	Control	MK-801	CHPG	MK-801 + CHPG
Movement frequency	139.7 ± 15.3	89.6 ± 12.6 #	161.27 ± 13.7 + ^	90.5 ± 12.7 #

\*different than controls; + different than MK-801; # different than CHPG; ^ different than MK-801 and CHPG

Given that the groups differed on duration of mobility, it would be expected that they would also differ on duration of immobility. This was confirmed by results indicating that there was a significant between groups difference for duration of time spent immobile ( $F(3, 96) = 6.384$ ,  $p = .001$ ), and a post-hoc Tukey test indicated that larvae treated with MK-801 spent less time moving than controls ( $MD = 133.3$ ,  $p = .015$ ) and larvae treated with CHPG alone ( $MD = 157.95$ ,  $p = .003$ ); larvae treated with MK-801/CHPG spent less time moving than larvae treated with CHPG alone ( $MD = 134$ ,  $p = .017$ ) but did not spend significantly less time moving than controls ( $p = .073$ ).

	Control	MK-801	CHPG	MK-801 + CHPG
Not moving duration (s)	202.3 ± 26.6 +	335.7 ± 35.5 * #	177.7 ± 14.9 + ^	311.7 ± 41.4 #

\*different than controls; + different than MK-801; # different than CHPG; ^ different than MK-801 and CHPG

A one-way ANOVA indicated a significant difference between groups for not moving frequency ( $F(3, 100) = 6.891, p < .001$ ), as would be expected from the results indicating a difference between frequency of movement in each group. A post-hoc Tukey test indicated that larvae treated with CHPG alone were more frequently immobile than larvae treated with MK-801 ( $MD = 71.6, p = .002$ ) and larvae treated with MK-801/CHPG ( $MD = 70.5, p = .002$ ). These results are consistent with the earlier results indicating significant differences in movement frequency.

	Control	MK-801	CHPG	MK-801 + CHPG
Not moving frequency	139.4 ± 15.3	89.1 ± 12.7 #	160.8 ± 13.7 + ^	90.27 ± 12.7 #

\*different than controls; + different than MK-801; # different than CHPG; ^ different than MK-801 and CHPG

*F* scores for Experiment 1 are reported below in Table 3.

**Table 3. F Statistics for Dependent Variables in Experiment 1**

Dependent Variable	<i>F</i> score	<i>p</i> value
Total distance moved	9.735	<.001*
Zone B duration	.992	.400
Zone B frequency	7.631	<.001*
Zone B latency to enter	1.862	.141
Zone C duration	2.425	.071
Zone C frequency	7.448	<.001*
Zone C latency to enter	.950	.420

Movement duration	4.007	.010*
Movement frequency	6.905	<.001*
Latency to first move	1.479	.225
Not moving duration	6.384	.001*
Not moving frequency	6.891	<.001*
Latency to first stop moving	.925	.432
Mean velocity	2.132	.101

In summary, fish treated with either 20  $\mu\text{M}$  MK-801 or 20 $\mu$  MK-801/180  $\mu\text{M}$  CHPG tended to be more active than controls on total distance moved, frequency of entering the perimeter of the well, frequency of entering the center of the well, movement duration, and movement frequency, while fish treated with CHPG alone tended not to differ from controls on any movement parameters.

### Experiment 2

The results of the first experiment prompted us to modify the dosage of both MK-801 and CHPG in Experiment 2. It was hypothesized that lowering the dose of MK-801 to 2  $\mu\text{M}$  could effect motor behavior in the larvae that would be more consistent with previous literature demonstrating that MK-801 can induce hyperactivity in zebrafish. It was thought that the dose of 20  $\mu\text{M}$  may have been extremely high for the larvae and thus reduced motor activity, so a lower dose was investigated. Furthermore, because CHPG did not appear to have an effect at the dose of 180  $\mu\text{M}$  it was hypothesized that a higher dose would be necessary in order to significantly reduce the motor effects of MK-801, and therefore in the second experiment larvae received a dose of 360  $\mu\text{M}$  CHPG. Aside from dose levels the procedure was not modified because the data suggested that significant differences were due to drug effects, rather than an effect of the procedure itself.

## 2.1 Procedure

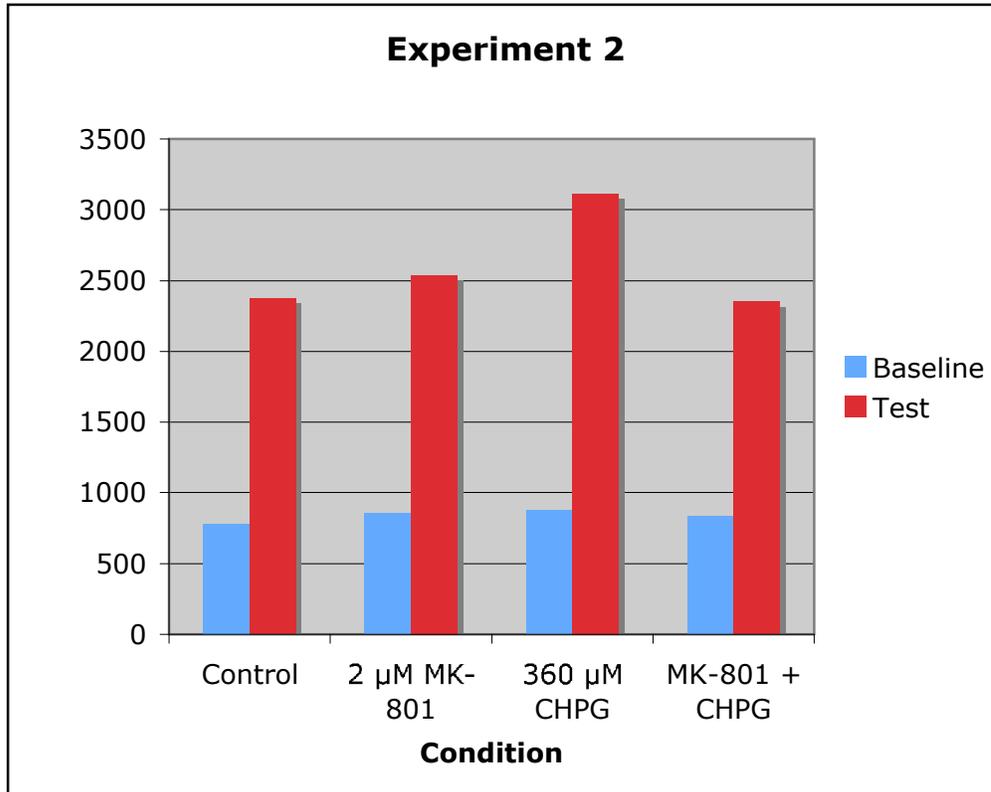
In the second experiment a lower dose (2  $\mu\text{M}$ ) of MK-801 was used and a higher dose (360  $\mu\text{M}$ ) of CHPG was used. During the test period subjects received either: a) 1 mL of egg water (control group;  $n = 26$ ); b) 1 mL containing 2  $\mu\text{M}$  MK-801 ( $n = 26$ ); c) 1 mL containing 360  $\mu\text{M}$  CHPG ( $n = 26$ ); or d) 1 mL containing 2  $\mu\text{M}$  MK-801 and 360  $\mu\text{M}$  CHPG ( $n = 26$ ). The same procedure that was used in Experiment 1 was used in Experiment 2. The measures that were recorded were the same in both experiments. Baseline activity was also compared across both experiments to determine if there were differences between the subjects on the two separate days. One minor change in the second experiment was a reduction in ambient light, which necessitated systematically turning the lights on and off during the procedure.

## 2.2 Results

Baseline activity was analyzed by a one-way ANOVA and the results and indicated no significant differences between groups prior to drug administration. One-way ANOVA tests indicated significant differences between groups on several measures of activity after drug administration. The results were surprising in that they indicated an independent effect of CHPG on locomotor activity that was unexpected. In general, larvae treated with CHPG alone were more active than larvae receiving other treatments or controls.

	Controls	2 $\mu$ M MK-801	360 $\mu$ M CHPG	MK-801 + CHPG
Total distance traveled (mm) (Mean $\pm$ S.E.M.)	2371.2 $\pm$ 156.3 #	2540.4 $\pm$ 206.0	3112.6 $\pm$ 161.2 * ^	2349.1 $\pm$ 136.3 #

\*different than controls; + different than MK-801; # different than CHPG; ^ different than MK-801 and CHPG



**Figure 2.** Larvae treated with CHPG traveled significantly greater distance than controls.

Groups differed on total distance traveled ( $F(3, 100) = 4.562, p = .005$ ); a Tukey post-hoc test indicated that larvae treated with 360  $\mu$ M CHPG traveled greater total distance than controls ( $MD = 741.4, p = .012$ ) and larvae treated with MK-801/CHPG ( $MD = 763.5, p = .009$ ), but not larvae treated with 2  $\mu$ M MK-801 ( $MD = 572.1, p = .079$ ).

QuickTime™ and a  
decompressor  
are needed to see this picture.

**Figure 3. Tracks from Trial 18.** From left to right, each column of two represents the different groups: controls, MK-801, CHPG, MK-801 + CHPG. Above is the plotted tracks of movement over the 15 minute test period, representing the total distance moved.

A one-way ANOVA indicated that groups differed significantly on duration of time spent in zone B ( $F(3, 99) = 3.116, p = .030$ ); a Tukey post-hoc test indicated that larvae treated with 2  $\mu\text{M}$  MK-801 spent more time in zone B than larvae treated with MK-801 and CHPG ( $MD = 117.03, p = .046$ ). It is not clear exactly what this result indicates.

	Controls	MK-801	CHPG	MK-801 + CHPG
Zone B duration (s)	672.1 $\pm$ 28.4	687.7 $\pm$ 33.3 ^	681.3 $\pm$ 24.9	570.7 $\pm$ 37.1

\*different than controls; + different than MK-801; # different than CHPG; ^ different than MK-801 and CHPG

Similarly, differences in latency to enter the center of the well seem ambiguous as to their meaning. Analysis with a one-way ANOVA test indicated that there was a significant between groups difference in latency to enter zone C ( $F(3, 99) = 3.293, p = .024$ ); a Tukey post-hoc test indicated that the larvae receiving MK-801/CHPG took significant longer to enter zone C than controls ( $MD = 58.7, p = .044$ ).

	Controls	MK-801	CHPG	MK-801 + CHPG
Zone C latency to enter (s)	4.1 $\pm$ 1.4 ^	5.2 $\pm$ 1.4	9.7 $\pm$ 6.0	62.8 $\pm$ 30.4 *

\*different than controls; + different than MK-801; # different than CHPG; ^ different than MK-801 and CHPG

A one-way ANOVA test indicated significant between groups differences on movement duration ( $F(3, 100) = 3.320, p = .023$ ); a Tukey post-hoc test indicated that larvae receiving 360  $\mu\text{M}$  CHPG moved for longer than the control group ( $MD = 129.1, p = .033$ ).

	Controls	MK-801	CHPG	MK-801 + CHPG
Movement duration (s)	586.3 $\pm$ 38.5 #	595.2 $\pm$ 40.8	715.5 $\pm$ 25.1 *	608.5 $\pm$ 23.7

\*different than controls; + different than MK-801; # different than CHPG; ^ different than MK-801 and CHPG

These results are consistent with the findings of a one-way ANOVA that indicated a significant between groups difference on duration of not moving ( $F(3, 100) = 3.335, p = .022$ ); a Tukey post-hoc test indicated that the larvae treated with CHPG alone spent significantly less time immobile than controls ( $MD = -129.1, p = .033$ ), and there was a marginally significant difference between the larvae treated with CHPG and larvae treated with 2  $\mu\text{M}$  MK-801 ( $MD = -120.3, p = .054$ ).

	Controls	MK-801	CHPG	MK-801 + CHPG
Not moving duration (s)	314.6 $\pm$ 38.4 #	305.7 $\pm$ 40.8	185.4 $\pm$ 25.1 *	292.4 $\pm$ 23.6

\*different than controls; + different than MK-801; # different than CHPG; ^ different than MK-801 and CHPG

Analysis by one-way ANOVA indicated a significant between groups difference on latency to first stop moving ( $F(3, 100) = 4.579, p = .005$ ); a Tukey post-hoc test indicated that larvae treated with 2  $\mu\text{M}$  MK-801 were quicker to first stop moving than

larvae treated with MK-801/CHPG ( $MD = -16.4, p = .019$ ), and larvae treated with MK-801/CHPG took significantly longer to first stop moving than controls ( $MD = 16.7, p = .016$ ) and larvae treated with CHPG alone ( $MD = 17.04, p = .014$ ). It is interesting to note here that the standard error of the mean is significantly higher for the MK-801/CHPG group and this could explaining the discrepancy.

	Controls	MK-801	CHPG	MK-801 + CHPG
Not moving latency (s)	2.4 ± 0.7 ^	2.7 ± 0.5 ^	2.1 ± 0.4 ^	19.2 ± 7.7 * + #

\*different than controls; + different than MK-801; # different than CHPG; ^ different than MK-801 and CHPG

Lastly, a one-way ANOVA indicated a significant between groups difference on mean velocity ( $F(3, 100) = 4.059, p = .009$ ); a post-hoc Tukey test indicated that larvae treated with 360  $\mu$ M CHPG had higher mean velocity than controls ( $MD = .793$  mm/s,  $p = .015$ ) and larvae treated with MK-801/CHPG ( $MD = .756, p = .023$ ). Taken along with the results indicating larvae receiving CHPG moved for longer than other larvae, the higher average velocity of these larvae is consistent with the results indicating that this group traveled a greater total distance than controls.

	Controls	MK-801	CHPG	MK-801 + CHPG
Mean velocity (mm/s)	2.6 ± 0.1 #	2.8 ± 0.2	3.4 ± 0.1 * ^	2.7 ± 0.1 #

\*different than controls; + different than MK-801; # different than CHPG; ^ different than MK-801 and CHPG

All *F* scores for Experiment 2 are reported below in Table 4.

**Table 4. F Statistics for Dependent Variables in Experiment 2.**

<b>Dependent Variable</b>	<b><i>F</i> Score</b>	<b><i>p</i> Value</b>
Total distance traveled	4.562	.005*
Zone B duration	3.116	.030*
Zone B frequency	1.343	.265
Zone B latency to enter	.364	.779
Zone C duration	2.391	.073
Zone C frequency	1.366	.257
Zone C latency to enter	3.293	.024*
Movement duration	3.320	.023*
Movement frequency	.504	.680
Latency to first move	.301	.825
Not moving duration	3.335	.022*
Not moving frequency	.511	.676
Latency to first stop moving	4.579	.005*
Mean velocity	4.059	.009*

In summary, larvae treated with CHPG alone tended to be more active than controls (traveling greater total distance, moving for longer duration, and moving with a greater velocity), while larvae receiving MK-801 or MK-801/CHPG generally did not exhibit different motor behavior than controls.

### **Discussion**

Previous research has demonstrated that adult zebrafish treated with MK-801 exhibit increased motor activity (Swain et. al. 2004; Seibt et. al. 2010; Seibt et. al. 2011), consistent with the effects of NMDA-R antagonism in other rodent models. The findings of Chen et. al. (2010) demonstrated that these effects are conserved in larval zebrafish. Based on these findings, administration of MK-801 was expected to increase larval

zebrafish motor activity in our first experiment. However, this was not observed in Experiment 1. Rather, the zebrafish larvae treated with MK-801 or the combination of MK-801 and CHPG showed significantly decreased motor activity compared to controls and larvae treated with CHPG alone. Larvae treated with MK-801 traveled less total distance than controls and larvae treated with. Furthermore, analyses of several of the dependent variables from both experiments indicate a trend for larvae treated with MK-801 and MK-801/CHPG to be less active than controls or larvae treated with CHPG alone. The results of Experiment 1 were part of the rationale for changing the dose levels in Experiment 2. It was considered a possibility that at the dose level of 20  $\mu\text{M}$ , MK-801 was acting as an anesthetic and thereby impairing the larvae's ability to move normally. Indeed, very high doses of MK-801 have also been shown to impair the control of motor movement in rats, and it is a possibility that the decreased motor activity seen in Experiment 1 is representative of a disruption of the larvae's ability to control their swimming motions (Kovacic & Somanathan 2010). Therefore, we hypothesized that administering a smaller dose of MK-801 would be more likely to elicit an increase in motor activity that would be more consistent with the current literature.

Contrary to this hypothesis, MK-801 did not affect larval motor activity at the dose level of 2  $\mu\text{M}$  in Experiment 2. In fact, there were no significant differences between controls and larvae treated with 2  $\mu\text{M}$  MK-801, suggesting that at this dose level MK-801 does not have an effect on motor activity in zebrafish larvae. However, it is interesting to note that results seemed to indicate an interaction between MK-801 and CHPG at this dose level. Larvae treated with 360  $\mu\text{M}$  CHPG were more active than controls on several parameters, but larvae who received MK-801 and CHPG co-

administration frequently did not differ from controls on the same parameters. This suggests that although MK-801 was not behaviorally active by itself at the 2  $\mu\text{M}$  dose level, some drug interaction between MK-801 and CHPG did prevent an overall motor activity increase such as was seen in the larvae that received CHPG alone.

CHPG was not expected to have any effects on motor activity when administered alone, as Kinney et al. (2003) and Chan et. al. (2008) demonstrated that CHPG does not alter motor activity in rodents when administered alone, despite reversing motor effects of NMDA-R antagonism in these models. While the data from Experiment 1 supports the hypothesis that CHPG by itself will not affect motor activity, the data from Experiment 2 indicated increased activity on several motor parameters at a dose of 360  $\mu\text{M}$ . At this dose level, CHPG administration led to an increase in (1) total distance traveled, (2) duration of movement, and (3) mean velocity, as well as a decrease in the duration of time spent immobile, in comparison to controls. This is contrary to the expectation that activation of mGluR5 would not affect motor behavior on its own due to the proposed mechanisms by which mGluR5 enhances NMDA-R function. According to Kanuma et. al. (2010) the mechanism by which activation of mGluR5 with agonist agents enhances NMDAR function is that it is likely that activation of mGluR5 leads to PKC phosphorylation of the ion channel associated with NMDA receptors, thereby resulting in increased NMDA receptor sensitivity and activity, and leading to an influx of calcium ions. However, this hypothesis comes out of findings from rodent studies, and it may not be possible to extrapolate this proposed mechanism to a zebrafish model. In the zebrafish brain the mGluR5 may have the ability to more directly affect glutamatergic transmission in neuronal circuits governing motor activity, and thus CHPG-induced activity at the

receptor leads to increased motor activity. Future research could investigate whether administration of a high dose of another mGluR5 agonist causes the same increase in motor activity, as well as whether inhibition of the receptor via mGluR5 agonism can lead to a decrease in motor activity at any dose level. The findings of the present study may suggest that in the central nervous system of the larval zebrafish mGluR5 may be more directly linked to neuronal systems governing motor activity than in the rodent or human nervous system. The data indicated that at 5 dpf the larval nervous system is sufficiently developed for mGluR5 agonism to have an effect on behavior. Further investigation into the nature of this connection could lead to a more comprehensive and refined understanding of the glutamatergic systems in zebrafish and their relationship to motor behavior. It should be noted that there is also always the possibility that CHPG increased motor activity by some means other than mGluR5 agonism. The ontogeny (development) of mGluR5 does not appear to be available in the literature regarding mGluR5 orthologs yet. The data evidences that CHPG is behaviorally active in zebrafish larvae at 5 d.p.f.. If the mGluR5 is not expressed by 5 dpf, it would appear that CHPG is acting on different systems than previously thought.

With regard to potential toxicity of MK-801 and CHPG, during both experiments well plates were kept in another room of the lab after treatment and observation, in order for us to assess any lethal toxicity of both drugs. None of the fish died at either dose administered. This indicates that at this dose range, lengthy exposure to either MK-801 or CHPG is not lethal to zebrafish larvae at these dose levels.

The data from experiments do not support an ability of CHPG to reverse the effects of MK-801 at a dose of either 180  $\mu\text{M}$  or 360  $\mu\text{M}$ . A lack of significant

differences between the controls and the larvae treated with MK-801/CHPG (on dependent variables where larvae treated with MK-801 alone differed from control) would suggest that CHPG was reversing the motor effects of MK-801. In Experiment 1, the group receiving MK-801 and CHPG did not move for a significantly different amount of time than the controls or the group receiving MK-801 alone. However, both groups that received MK-801 traveled for significantly less distance than the groups that did not receive MK-801. There seems to be the possibility that in the group that received a co-administration of both drugs, MK-801 was able to rescue the subjects from increased immobility due to MK-801 administration. This tendency to spend more time immobile than controls may indicate a disruption by NMDA-R antagonism that is reversible by MK-801. However, it would be premature to make this conclusion in the absence of other results supporting MK-801's ability to reverse MK-801 effects. It is likely that this effect of MK-801 on movement duration is caused via the same mechanism by which a higher dose of MK-801 increased motor activity in the second experiment. Though what exactly this mechanism is remains for the moment unknown, the results of this study do suggest a dose-dependent effect of MK-801 on motor activity.

It should also be considered that MK-801 may not be potent enough to have reversed the effects of the high dose of MK-801 in Experiment 1 on the majority of movement parameters. Recent literature reviews have cited lower relative potency of some mGluR5 agonists as a rationale for the development of positive allosteric modulators (PAMs) of mGluR5, such as the compounds CDPPB and ADX47273 (Kanuma et. al. 2010; Niswender and Conn 2010). Given that MK-801 did not demonstrate a rescue effect at a dose of 180  $\mu$ M, while at a dose of 360  $\mu$ M increased

motor activity when administered alone, the data of the present study may indicate that such a high dose of CHPG is needed to have an antipsychotic effect that there is a risk of increased motor side effects. In the context of the development of new antipsychotics, the implication is that positive allosteric modulation is most likely a more desirable pharmacotherapy to investigate for the treatment of schizophrenic symptoms. Future research could assess whether administration of an mGluR5 PAM would lead to similar behavioral results as the dose of 360  $\mu\text{M}$  CHPG.

It was hypothesized that in Experiment 1 the differences between the group receiving MK-801/CHPG and the controls would not be statistically significant. However, most of the differences between controls and larvae co-treated with 2  $\mu\text{M}$  MK-801/360  $\mu\text{M}$  CHPG were indeed significant. The only non-significant differences were (1) a Tukey post-hoc test indicated no significant difference from controls for movement duration and movement frequency, and (2) a Tukey post-hoc test on duration and frequency of immobility indicated no significant differences from controls. The findings the first experiment suggested that a higher dose of CHPG was needed to reverse motor effects of MK-801, providing the rationale for investigating the dose level of 360  $\mu\text{M}$  in Experiment 2. In the second experiment, larvae co-treated with 2  $\mu\text{M}$  MK-801 and 360  $\mu\text{M}$  CHPG did not differ significantly from the control group except on: (1) duration of time in zone B, (2) latency to enter zone C, (3) latency to first stop moving. Given that overall the larvae treated with 360  $\mu\text{M}$  exhibited locomotor hyperactivity on several measures, it would seem that the 2  $\mu\text{M}$  dose of MK-801 is preventing the increases in motor activity induced by CHPG administration, while by and large having no effect on motor activity when administered alone.

These results are surprising, but one plausible explanation is that the 2  $\mu$ M dose of MK-801 could have decreased activity level by such a small amount that it was not statistically significant by itself, but was enough to prevent the motor effects that were seen when CHPG was administered alone. One factor that could have contributed to this effect is the fact that the drugs were administered simultaneously. It could be the case that the simultaneous blockade of the NMDA-R by MK-801 prevented CHPG from affecting motor behavior. CHPG has been proposed to modulate transmission at the NMDA receptor, but if MK-801 was already preventing glutamatergic transmission it would make sense that CHPG was unable to modulate motor activity by this proposed mechanism. Further research could explore the mechanism by which this effect is caused, but it would seem that currently not enough is known about metabotropic modulation of the glutamatergic systems in the zebrafish CNS to come to a clear and compelling conclusion for why the larvae that received co-treatment did not differ from controls on the measures that larvae treated with CHPG did.

While the data does not largely suggest the presence of confounding variables, the possibility should never be ignored. Importantly, it should be noted that the subjects in the two experiments differed on several parameters of baseline activity. The subjects in Experiment 2 tended to be more active overall than the subjects in Experiment 1. For instance, though the movement duration of the subjects in both experiments did not differ in a statistically significant manner, subjects in Experiment 2 moved roughly 2.5 times greater total distance than subjects in Experiment 1. This suggests that the subjects in Experiment 2 moved at a higher mean velocity, which is confirmed by the data ( $t = -3.298$ ,  $df = 202$ ,  $p < .001$ ). This increased activity could be an effect of differences in

light level between the two experiments : during the first experiment, we did not cover the window when we began running subjects. After a few hours of testing ambient light levels increased due to sunlight coming in the window. A majority of light was blocked for the remaining trials. In contrast, when we ran the second experiment the window was covered the entire time. However, because the low light level made handling the larvae and drug administration difficult, the following procedure was followed: 1) When placing fish in wells, lights were turned on; 2) before recording 5 minutes of baseline activity, lights were turned off; 3) before administering the drug treatments, lights were turned on; 4) before recording 15 minutes of post-treatment activity, lights were turned off. There has been a noted effect of light transitions on motor activity in zebrafish. Padilla et. al. (2011) demonstrated that both level of light and the order of light presentation can influence motor activity in larval zebrafish 6 d.p.f.. It is unfortunate that these baseline differences exist because they suggest possible extraneous variables affecting the data. However, in neither experiment were there significant differences between groups during baseline activity, suggesting that the observed drug effects can be considered valid.

Another important factor that should be mentioned is that the zebrafish larvae used in both studies were wild-type (tupel long fin) TL zebrafish, a strain that has not been used before in assessing the behavioral effects of MK-801 administration. Chen et. al. (2010) reported conservation of MK-801 motor effects in zebrafish larvae, but those were a wild-type AB strain. It may be that the TL strain is more sensitive to MK-801 treatment, and therefore the dose level of 20  $\mu$ M may have had an effect that is only seen at much higher dose levels in other strains of zebrafish. It may also be the case that there are developmental differences in the glutamate systems between the two strains: Chen et.

al. found that MK-801 was not behaviorally active in the AB larvae until 5 d.p.f.. It may be that there exist subtle developmental differences at 5 d.p.f. between AB and TL larvae which could account in part for the discrepancy between the results of Chen et. al.'s study and the results of the present study. Additionally, it could be the case that the exposure time (15 min) was not long enough to elicit the expected increase in motor activity in either experiment. Because our 20  $\mu$ M dose elicited a decrease in motor activity, but our 2  $\mu$ M dose did not elicit any significant response, it would be interesting to conduct further research to assess how larval TL zebrafish respond to doses in between 2-20  $\mu$ M. Lastly, there is always the possibility that having a different commercial source than that of the research previously cited impacted MK-801 responsiveness. Further research should investigate at what dose, if any, MK-801 can elicit increased motor activity in TL larvae.

In summary, although the current study failed to demonstrate an increase in motor activity following treatment with MK-801, we did demonstrate behavioral effects of a novel mGluR5 agonist, CHPG, at a dose of 360  $\mu$ M. These effects were generally inhibited by co-administration of 2  $\mu$ M MK-801. Furthermore, the results of the current study are consistent with the findings of Padilla et. al. (2011) which indicate that light-to-dark transitions can influence motor activity in zebrafish larvae as early as 5 dpf. The present data support further investigation into how the glutamate systems in the larval zebrafish brain effect motor behavior.

## Chapter 5: Conclusion

The emergence of the glutamate hypothesis of schizophrenia represents an important shift in focus away from dopaminergic systems and onto glutamatergic transmission at the NMDA receptor. Dissociative anesthetics that antagonize the NMDA receptor, such as MK-801, have begun to be utilized as psychotomimetics due to their ability to induce deficits that more accurately resemble the symptoms of the disorder. Several agents that enhance NMDA-R function via activation of metabotropic glutamate receptors have been developed and are currently being explored as alternative approaches to antipsychotic drug therapy.

The use of animal models is an essential part of the process of understanding and developing the pharmacological profile of novel antipsychotics. MK-801's ability to schizophrenic-like behavior in rodent and zebrafish models makes a full assessment of its behavioral effects important and relevant to current progress towards improved treatment models. The assessment of the behavioral effects of MK-801 in animal models is relevant to the development of novel antipsychotic drugs based on the glutamate hypothesis. The purpose of the study conducted in this senior project was to determine whether zebrafish larvae could serve as a model to investigate the antipsychotic effects of CHPG, a novel metabotropic glutamate receptor 5 selective agonist. The principle questions of interest were: a) can treatment with MK-801 disrupt motor activity in zebrafish larvae in a manner consistent with the current scientific literature?; b) what, if any, are the motor effects induced by treatment with CHPG by itself?; and c) can co-administration of CHPG with MK-801 rescue larvae from MK-801-induced motor

disturbances? While in the second experiment the co-administration of CHPG and MK-801 did not result in any significant differences in motor activity from controls, it is unclear by what mechanism these results were demonstrated. The motor effects of the highest dose of CHPG could be indicative that in the larval zebrafish mGluR5 agonism affects behavior in a different and perhaps more direct way than in previously explored rodent models. Furthermore, the present findings support the findings of Chen et. al. (2010) that at a dose of 20  $\mu$ M MK-801 can disrupt behavior in zebrafish larvae, but whereas Chen et. al. found an increase in motor activity, we consistently found a decrease in motor activity at this dose level. These findings suggest that further research ought to be conducted regarding the character of larval zebrafish's behavioral response to MK-801 administration. Further investigation of the mechanisms underlying the reasons that the larvae responded the way they did to MK-801 administration could help provide a more comprehensive model for NMDA-R mediated behavior in zebrafish. Additionally, further research into dose-dependent responses to mGluR5 agonism could help to illuminate how this particular receptor affects motor behavior in zebrafish.

Because this particular area of research is a growing one, easy explanations for the results of the present study are not yet readily available in the literature. Therefore, what this senior project seeks to contribute is several questions for possible empirical studies in the future: Are the motor effects of 20  $\mu$ M MK-801 confined to TL larvae, or can these findings be replicated in other strains such as AB larvae? At what dose levels, if any, will MK-801 increase spontaneous motor activity in TL larvae (5 dpf) in a way that is consistent with the existing literature? For instance, what would be the results of treatment with a dose of MK-801 in between the two dose levels (2  $\mu$ M and 20  $\mu$ M)

administered in the present study? What would be the motor effects of longer exposure time – for instance 30, 60, or 90 minutes? Are the motor effects of a 20  $\mu\text{M}$  dose of MK-801 different in TL larvae at 6 dpf and 7 dpf than at 5 dpf? Additionally, what are the independent motor effects of CHPG in zebrafish larvae at doses that fall in between the range of 180-360  $\mu\text{M}$ ? Can CHPG reverse MK-801-induced motor disturbances if larvae are pre-treated with it? Does a 360  $\mu\text{M}$  dose of CHPG increase motor activity in larvae at 6 dpf or 7 dpf? Research that seeks to address these question will serve to clarify the use of zebrafish larvae as a model of NMDA-R mediated psychosis by providing a more sophisticated picture of how glutamate systems affect behavior in this model.

The rising popularity of the zebrafish as a model organism for CNS disorders has come at the same time as the developers of antipsychotic agents have shifted their focus onto ligands that enhance glutamatergic transmission at the NMDA-R receptor. It has been hypothesized that glutamatergic modulation presents a more comprehensive strategy for ameliorating schizophrenic symptoms while causing less side effects than conventionally prescribed antipsychotics. A clearer understanding of the way that glutamate transmission effects behavior in the larval zebrafish will inform researchers as to whether or not the larval zebrafish can be a useful model to investigate the antipsychotic efficacy of many of these new agents. If larval zebrafish can indeed serve as a model, it represents a cost-effective and efficient way to evaluate the efficacy of new antipsychotics which may have far-reaching effects in combating the personal and social costs of schizophrenia.

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