Cellular Mechanisms Underlying the Effects of Repeated D_2-like Agonist Treatment on Prepulse Inhibition

by

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ABSTRACT

Patients with schizophrenia have deficits in sensorimotor gating, the ability to gate out irrelevant stimuli in order to attend to relevant stimuli. Prepulse inhibition (PPI) of the startle response is a reliable and valid model of sensorimotor gating across species. Repeated D_2-like agonist treatment alleviates prior PPI deficits in rats, termed a PPI recovery, and is observable 28 days after treatment. The aim of the current project is to illuminate the underlying mechanism for this persistent change of behavior and determine the clinical relevance of repeated D_2-like agonist treatment. Our results revealed a significant increase in Delta FosB, a transcription factor, in the nucleus accumbens (NAc) 10 days after repeated D_2-like agonist treatment. Additionally, we investigated if Delta FosB was necessary for long-lasting PPI recovery and discovered a bilateral infusion of dominant-negative Delta JunD prevented PPI recovery after repeated D_2-like agonist treatment. To further develop the underlying mechanism of PPI recovery, we observed that dominant negative mutant cyclic adenosine monophosphate (cAMP) response biding element protein (CREB) prevented repeated D_2-like agonist-induced Delta FosB expression in the NAc. We then compared our previous behavioral and intracellular findings to the results of repeated aripiprazole, a novel D_2-like partial agonist antipsychotic, to determine if repeated D_2-like receptor agonist action is a clinically relevant pharmacological approach. As compared to previous PPI recovery and Delta FosB expression after repeated D_2-like agonist treatment, we found similar PPI recovery and Delta FosB expression after repeated aripiprazole treatment in rats. We can conclude that repeated D_2-like agonist treatment produces persistent PPI recovery through CREB phosphorylation and Delta FosB, which is necessary for PPI recovery. Furthermore, this

pharmacological approach produces behavioral and intracellular changes similar to an effective novel antipsychotic. These findings suggest the underlying intracellular mechanism for sustained PPI recovery is clinically relevant and may be a potential target of therapeutic intervention to alleviate sensorimotor gating deficits, which are associated with cognitive symptoms of schizophrenia.

DEDICATION

I would like to dedicate my dissertation to my mother, Connie Maple. My mother has given me a lifetime of support and love. She always believes in my abilities to accomplish any goal, even when I didn't believe in myself. Her positivity and failure to quit even in the bleakest situations constantly inspires me to do my best no matter what the situation.

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Chapter 1

INTRODUCTION

Significance

Schizophrenia is a prevalent, chronic, and severe mental disorder that causes substantial financial, physical, and emotional burdens on society, families, and individuals (McEvoy, 2007). While antipsychotic drugs are the preferred and most effective treatment option, these drugs produce harmful side effects, resulting in non-compliance in patients (Tandon, Keshavan, & Nasrallah, 2008). Currently, there is no cure or sustainable treatment because the etiology and underlying mechanism of schizophrenia are mostly unknown. The outdated theory that a simple neurotransmitter dysregulation triggers symptoms does not address the complexity and multifaceted characteristics of the disorder; therefore, treatment derived from this theory is not adequate. Specifically, all typical and atypical antipsychotics non-specifically block dopamine (DA) D_2 receptors resulting in side effects, tolerance, and inability to treat negative and cognitive symptoms (Lieberman et al., 2005). This problem calls for a more sophisticated approach is needed in order to treat and better understand schizophrenia. It is believed that dopamine acting via neurons containing D_2–like receptors in the nucleus accumbens (NAc), affect positive and possibly negative symptoms of schizophrenia (Flagstad et al., 2004). These neurons are integral to the understanding of schizophrenia symptoms and future potential therapeutic options. Deficits in sensorimotor gating, the ability to selectively filter unnecessary stimuli and attend to relevant information, are common in patients with schizophrenia and are associated with cognitive symptoms (Braff, Geyer, & Swerdlow, 2001). We previously found that repeated D_2-like agonist

treatment alleviated prior prepulse inhibition (PPI), a measurement of sensorimotor gating (Berger, Green, Siegel, Nestler, & Hammer, 2011; Culm & Hammer, 2004; Culm, Lugo-Escobar, Hope, & Hammer, 2004). The goal of these experiments is to build on our current knowledge of the effect of repeated D_2-like agonist treatment on neurons in the NAc that regulate PPI. We plan on accomplishing this goal using pharmacological and molecular manipulations and observing behavioral and intracellular adaptations.

Schizophrenia: Prevalence and Impact

Schizophrenia is a neuropsychiatric disorder that has affected numerous individuals throughout history. Currently, an estimated 1 percent of Americans and 24 million people worldwide live with the disorder (Regier et al., 1993). These statistics does not include individuals who are homeless or do not seek treatment, a major problem for people with schizophrenia. Prevalence is much higher than onset statistics because schizophrenia is a chronic condition (Mueser & McGurk, 2004). In most cases, a person is diagnosed with schizophrenia between ages 16 and 30 and will experience sporadic episodes of psychosis or 'psychotic breaks' throughout their lifetime (de Haan, Linszen, Lenior, de Win, & Gorsira, 2003; Wistedt, 1981). However, the progression of schizophrenia depends on the individual, environment, and treatment, which contribute to the large variability within the schizophrenic population. Schizophrenia is often described as one of the most debilitating mental illnesses because of the intensity of symptoms, high cost to society, and high rate of mortality (Mueser & McGurk, 2004). For instance, patients with schizophrenia have a 2 to 3 times higher rate of premature mortality, approximately between 10 to 25 years, than the general population (Crump, Winkleby, Sundquist, & Sundquist, 2013; Saha, Chant, & McGrath, 2007). In the United States,

high mortality rates are due to various factors, with suicide as the most frequent cause of death (Brown, 1997) and cancer as the second most frequent (Tran et al., 2009). Incidences of patient suicide are often attributed to depression related to the disorder or a side effect of treatment for the disorder (Hawton, Sutton, Haw, Sinclair, & Deeks, 2005). These alarming findings have influenced counties to begin experimental health care programs to increase suicide prevention and cancer screening in patients with schizophrenia (Kisely et al., 2013). In addition to high mortality rates, the intensity of symptoms associated with schizophrenia greatly contributes to the severity of this disorder.

Symptoms of Schizophrenia

Schizophrenia is a complex disease that is characterized by multiple different behaviors, which have been deconstructed into symptom categories to better diagnose, research, and treat the disorder. Schizophrenia includes varying degrees of positive, negative, and cognitive symptoms. Positive symptoms are psychotic behaviors that are not observed in the normal population and are usually the reason an individual or family member seeks treatment because the resulting behaviors are bizarre, distinct, and deviant from normal behaviors (Kimhy et al., 2013). The frequency and intensity of these symptoms vary in each individual throughout their life and include hallucinations, delusions, thought disorders, and movement disorders (Mueser & McGurk, 2004). Positive symptoms respond well to antipsychotic medication as compared to the other symptoms of schizophrenia (Risch, 1996). Negative symptoms of schizophrenia are more difficult to distinguish as part of the disorder because they are mistaken for depression or other conditions (Mueser & McGurk, 2004). These symptoms are associated with

disruptions to normal emotion or behavior and include flat affect, anhedonia, alogia, lack of motivation, and decreased social interactions. Finally, cognitive symptoms include poor executive functioning, trouble focusing or paying attention, and working memory deficits. The severity of cognitive symptoms is highly predictive of a patient's long-term prognosis, more than the positive symptoms (Green, 1996). As compared to positive symptoms, negative and cognitive symptoms are more resilient to antipsychotic medications. Specific brain regions and circuitry appear to mediate each group of symptoms (discussed in detail later) and this may explain the variance of antipsychotic medication among the symptoms.

The symptoms of schizophrenia can be overwhelming and can affect an individual's ability to work or financially contribute to society (McEvoy, 2007). In the United States in 2002, the estimated cost of schizophrenia was $62.7 billion. These costs are estimated from the direct and indirect financial effects of the disorder. Direct costs include treatments provided by inpatient and outpatient facilities, criminal justice, and medication costs. Indirect costs include productivity loss by patients with schizophrenia, family members, and caregivers (McEvoy, 2007). Family members and caregivers of patients with schizophrenia can be vulnerable to increased stress and often need support themselves (Scazufca & Kuipers, 1999).

Schizophrenia is a chronic and severe disorder that can be costly to an individual and community. The expression, complexity, and intensity of symptoms vary within the schizophrenic population and antipsychotics also vary in their effectiveness to treat symptoms. Treatment is difficult because the etiology and underlying mechanisms

responsible for schizophrenia are not completely understood, however there have been significant gains in research since Dr. Emile Kraepelin defined the disorder in 1887.

Etiology of Schizophrenia

Schizophrenia is caused by several factors including genetic and environmental influences. Research into the etiology of schizophrenia often focuses on genetic contributions because 10 percent of people with a first-degree relative with the disorder will develop schizophrenia as compared to 1 percent of the general population (Baron et al., 1985). Additional support is found in twin studies where identical twins have a high concordance rate of 40 to 65 percent (Cardno & Gottesman, 2000). Schizophrenia is not caused by a single gene mutation or deletion; rather researchers believe hundreds of different genes contribute to schizophrenia symptoms (Fanous et al., 2012). However, individuals with the disorder have higher rates of rare genetic mutations and single-nucleotide polymorphisms (SNPs) near genes involved in neurodevelopment, neuroprotection, or neurotransmission (Fanous et al., 2012; Walsh et al., 2008). Although schizophrenia has a strong genetic component, genetics alone cannot predict the disorder. Since the concordance rate among identical twins is not 100 percent, many believe environmental factors are also involved in the occurrence of this disorder.

A variety of environmental factors have been associated with the onset of schizophrenia. Early environmental insults, such as prenatal infections or malnutrition, maternal substance misuse, and obstetric complications are more common in the schizophrenic population as compared to the general population (Lieberman et al., 2001). There is growing evidence that social and psychological factors, such as stressful life events, ethnicity, urbanization, childhood trauma, and illicit drug use are also associated with

schizophrenia (Howes et al., 2004). This is supported by studies that found an increased risk of schizophrenia after an urban birth and upbringing as compared to rural residents (Allardyce et al., 2000). Cannabis is the most common illicit drug used by patients with schizophrenia and associated with an earlier age of onset (Di Maggio, 2001). Researchers are interested in this association because it may give insight into the underlying neurotransmission of schizophrenia and its potential precursors. The environmental factors listed previously are highly associated with schizophrenia, however because many of these studies are correlational it is difficult to determine the causal relationship and there may be additional contributing factors.

The identification of genetic and environmental factors that may produce schizophrenia has led to the "Two-Hit" hypothesis of schizophrenia. This hypothesis suggests that a prenatal genetic or environmental "first hit" disrupts brain development and increases vulnerability in the individual. The "second hit" is usually an environmental insult that compromises the functional integrity of the brain and exposes the vulnerability caused by the "first hit" (Maynard, Sikich, Lieberman, & LaMantia, 2001). This theory addresses the discrepancy in full concordance of the disorder among twins and the high association with environmental factors. Some potential mechanisms include early disruptions to central nervous system (CNS) development; specifically in cell-signaling pathways involved in induction, morphogenesis, and differentiation. These disruptions are then "primed" for later insults on the same pathways that are redeployed for neural maintenance during adolescence or early adulthood (Maynard et al., 2001). Research is still ongoing examining the validity of the "Two-Hit" hypothesis, however

previous research examining cell-signaling pathways has provided some evidence of these abnormalities that underlie symptoms of schizophrenia.

Neurotransmitter Systems Underlying Schizophrenia

Schizophrenia is a multifaceted disorder; therefore multiple brain regions, neurotransmitter systems, and cell-signaling cascades are involved in the underlying mechanisms. Specific brain regions or neurotransmitters systems are more closely associated with certain symptoms as compared to others. However, there is no simple direct region or system relationship with one symptom or behavior because many of these neurotransmitter systems are complex and interrelated. With that being said, the DA system, specifically the DA mesolimbic pathway, has been a focal point schizophrenia research and has been associated with positive and negative symptoms of schizophrenia.

Several neurotransmitter systems are involved in schizophrenia. DA and glutamate are the most examined, thus resulting in the DA and glutamate hypothesis of schizophrenia. DA has been a focal point of schizophrenia research and treatment since it was discovered that chlorpromazine attenuated psychotic symptoms by antagonizing the D_2 receptor (Seeman & Lee, 1975). DA is a modulating neurotransmitter that has been implicated in a multitude of behaviors and psychological disorders.

Dopamine Circuitry Involved in Schizophrenia

DA is produced in the midbrain within cell bodies in the substantia nigra and ventral tegmental area (VTA). The nigrostriatal pathway projects from the substantia nigra to the basal ganglia or striatum and controls motor function and movement. Compensation in the nigrostriatal pathway after antipsychotic medication contributes to motoric side effects (discussed later). The mesolimbic pathway projects from the VTA to

the NAc and is involved in many behaviors related to motivation, drug abuse, and schizophrenia. The mesolimbic pathway synapses onto gamma Aminobutyric acid (GABA) medium spiny neurons, a class of GABAergic cells, and is hyperactive in schizophrenia patients (Lee & Seeman, 1980). Hyperactivity within this pathway is believed to induce the positive symptoms such as hallucinations and delusions (Creese, Burt, & Snyder, 1976). This was first concluded after observations that drugs that increased DA release enhanced or produced positive symptoms and drugs that decrease dopamine decreased or stop positive symptoms (Meltzer & Stahl, 1976). These observations contributed to the DA hypothesis of schizophrenia or more specifically the mesolimbic DA hypothesis of positive symptoms of schizophrenia (Stahl, 2007). This overactivity can be expressed as excess of DA release from the nerve terminals, more DA receptors, supersensitivity of DA receptors, or a hyperactivity of the DA receptor-signaling cascade. Conflicting findings of DA influx or increased D_2 receptors detected by position emission tomography (PET) led to doubts about the DA hypothesis (Nordstrom, Farde, Eriksson, & Halldin, 1995). However, significant increases in D_2 receptor density were found after single photon emission computer tomography (Kelz et al.) in early-diagnosed schizophrenia patients before exposure to antipsychotic drugs (Corripio et al., 2011), which supports the hypothesis. In addition to the mesolimbic pathway, the mesocortical pathway also projects from the VTA but sends axons to areas of the prefrontal cortex (PFC), specifically the dorsolateral prefrontal cortex (DLPFC), synapsing on pyramidal neurons that may have a role in mediating cognitive and negative symptoms (Morrissette, 2011). The specific role of the mesocortical pathway in regulating cognitive and negative symptoms is still not clearly understood, but many

believe the symptoms are due to a deficit in DA activity in the pathway to the DLPFC (Morrissette, 2011). While DA pathways were the first identified contributors to schizophrenia symptoms, increasing evidence suggests they are the result of pathophysiology within the glutamate system; therefore it is imperative to understand the interconnections between the DA hypothesis and the expanding glutamate hypothesis of schizophrenia.

Glutamate Regulation of Dopamine Transmission

Recently, glutamate has become the focus of research and resulting theories to explain the pathophysiology of schizophrenia. Glutamate is the major excitatory neurotransmitter in the brain and glutamate projections interact with the dopamine projections in the PFC, NAc, and VTA as well as other key brain areas associated with schizophrenia. Glutamate involvement was first hypothesized after phencyclidine (PCP), an *N*-methyl-D-aspartate receptor (NMDAR) antagonist, produced positive, negative, and cognitive deficits in users and exacerbated symptoms in schizophrenic patients (Itil, Keskiner, Kiremitci, & Holden, 1967). Also, reduced glutamate was found in schizophrenic patients' cerebral spinal fluid (CSF) as compared to other patients (Kim, Kornhuber, Schmid-Burgk, & Holzmuller, 1980). These observations supported the glutamate hypothesis, that NMDAR hypofunction disrupts glutamate pathways and results in positive, negative, and cognitive symptoms of schizophrenia (Stahl, 2007). The glutamate hypothesis does not negate the dopamine hypothesis of schizophrenia; rather it is believed the two hypothesizes are dependent on each other through a circuitry based explanation.

Glutamate can have actions at nearly all neurons in the brain; however, there are five main glutamate pathways relevant to schizophrenia. The cortical brainstem glutamate projection projects from the cortical pyramidal neurons to brainstem neurotransmitter centers and regulates neurotransmitter release. The corticostriatal glutamate pathway projects from the prefrontal cortex to the striatum. Thalamocortical glutamate pathways ascend from the thalamus and innervate the cortex. Corticothalamic glutamate pathways descend from the prefrontal cortex to the thalamus. Finally, intracortical pyramidal neurons communicate with each other via corticocortical glutamatergic pathways. The pathway believed to be most critical to schizophrenia and specifically DA modulation is the corticobrainstem pathway, which projects from the cortical pyramidal neurons, primarily in lamina 5, to brainstem neurotransmitter cell bodies, including the raphe nucleus for serotonin (5-HT), VTA and substantia nigra for DA, and locus coeruleus for norepinephrine (NE). In the DA system, the corticobrainstem glutamate pathway is usually the "break" for the mesolimbic pathway via its excitatory inputs onto GABA interneurons synapsing onto DA neurons and the "accelerator" for the mesocortical pathway via its direct synapse onto DA neurons. Therefore, NMDAR hypofunction in the PFC dysregulates the glutamatergic corticobrainstem pathway to the VTA resulting in lack of stimulation onto GABA interneurons and hyperactivity of the mesolimbic dopamine pathway while at the same time lack of direct stimulation onto the DA mesocortical pathway produces hypoactivity (Stahl, 2007). Through their interconnections, glutamate and DA both contribute to the positive, negative, and cognitive symptoms of schizophrenia.

Dopamine and the Nucleus Accumbens

There is an existing theory that hyperactivity of the mesolimbic dopamine pathway is responsible for the positive symptoms and the hypoactivity of the mesocortical dopamine pathway is responsible for the negative symptoms. This explanation is oversimplified as evidenced by the complex interactions and modulations of glutamate and new evidence suggesting the striatum may also be involved in the cognitive or negative symptoms. For example, high distractibility or sensory overload is often included within cognitive or negative symptoms and the striatum is believed to modulate the "filter" by which sensory information is processed (Simpson, Kellendonk, & Kandel, 2010). The cortico-striatal-thalamic-cortical (CSTC) loop underlies the regulation of sensory overload and the thalamus is usually identified as the "thalamic sensory filter", however the striatum is also involved in this process. Within the striatum, the dorsal striatum is more closely associated with the CSTC loop; however, the ventral striatum may also be involved in regulation of behaviors related to cognitive and negative symptoms.

Within the ventral striatum, the NAc has been implicated in several behavioral and cognitive functions, such as translation of stimuli to behavioral response (Mogenson, 1980 #2902), coding of reward and reward expectation (Koob, Sanna, & Bloom, 1998), impulse control (Cardinal, Pennicott, Sugathapala, Robbins, & Everitt, 2001), and instrumental learning (Balleine & Killcross, 1994). The NAc is often considered the point of connection between sensory and associative brain areas processing and evaluating stimuli and the motor systems mediating responses to these stimuli (Mogenson, 1980 #2902). Specific to schizophrenia, the NAc is critical for sensorimotor gating, which may

lead to sensory flooding and cognitive fragmentation (Swerdlow, Braff, Taaid, & Geyer, 1994). The NAc has also been implicated in the negative symptoms of schizophrenia, where schizophrenic patients have decreased blood flow in the NAc when reporting feelings of anhedonia (Crespo-Facorro et al., 2001).

In summary, hyperactivity of the mesolimbic dopamine pathway via dysregulation of the corticobrainstem glutamate pathway produces positive symptoms of schizophrenia. Within the mesolimbic pathway, the NAc may also be involved in negative and cognitive symptoms of schizophrenia (Howes & Kapur, 2009). Due to glutamate's influence on dopamine, researchers are beginning to examine drugs that target glutamate receptors as possible therapeutic targets for schizophrenia treatment. However, since all current antipsychotic drugs target D_2 receptors it is important to understand these receptors and their role in schizophrenia and treatment.

Dopamine Receptors

Endogenous DA can bind to five different G-protein coupled receptors in the mammalian brain, D_1, D_2, D_3, D_4, and D_5. These receptors are categorized into two groups, based on the resulting activation or inhibition of adenylate cyclase following stimulation of a particular receptor subtype. Adenylate cyclase is an important part of the G-protein signaling cascade that can initiate intracellular changes via activation of the cyclic adenosine monophosphate (cAMP) pathway. The D_1-like receptors include the D_1 and D_5 and are G*s* protein coupled receptors that and activate adenylate cyclase. The D_2-like receptors include the D_2, D_3 and D_4 and are G*i* protein coupled receptors that and inhibit adenylate cyclase. Furthermore, D_2 receptors are a heterogeneous population comprised of short, D_2S, and long D_2L isoforms (De Mei, Ramos, Iitaka, & Borrelli,

2009). D_2S receptors are often described as autoreceptors and generally located on presynaptic DA neurons, specifically cell soma/dendrites and regulates transmitter synthesis and release (Roth, 1979). D_2S receptors are more sensitive as compared to D_2L receptors as evidenced by low doses of DA agonists preferentially stimulate D_2S (White & Wang, 1986). D_2L are located on the postsynaptically where D_2L mRNA are contained within 90 percent of total D_2 receptor containing neurons in the striatum (Neve, Neve, Fidel, Janowsky, & Higgins, 1991).

D_2-like receptors are located throughout the brain, mainly in regions receiving DA projections. In the striatum, D_2-like receptors are located ubiquitously throughout the dorsal and ventral regions; however this is only true for D_2 receptors as D_3 receptors are specifically located in the ventral striatum (Curran & Watson, 1995). D_2-like receptors in the NAc are mainly located on GABAergic medium spiny neurons, which receive dopaminergic input from the VTA and glutamatergic input from PFC, thalamus, hippocampus, and basolateral amygdala. NAc medium spiny neurons project out to the ventral pallidm (VP), lateral hypothalamus, and mesencephalic dopaminergic areas (Groenewegen, Berendse, & Haber, 1993).

D_2-like receptors were first associated with schizophrenia after the discovery of the D_2 antagonist action of antipsychotics (Seeman & Lee, 1975). Furthermore, the clinical antipsychotic potencies of these drugs are directly related to their binding affinity for the D_2 receptor (Kapur et al., 2002). Also, post-mortem examination of the brains of schizophrenic patients, who had not taken antipsychotic medication for several years prior to death, displayed significant increases in D_2 receptors; specifically, this increase was observed in the NAc which may be a compensatory mechanism due to an increased

amount of endogenous DA in the area (Kestler, Walker, & Vega, 2001). This finding would support the DA hypothesis of schizophrenia, specifically the hyperactivity of the mesolimbic pathway, however increases of D_2-like receptors in the NAc have been difficult to replicate in patients with schizophrenia. Researchers have also hypothesized that D_2-like receptors might contribute to positive symptoms through a change in receptor function as compared to amount of receptors in the NAc. Patients with schizophrenia are supersensitive to their own endogenous DA and have exacerbated symptoms when challenged with dopamine-stimulating drugs (Lieberman, Kane, & Alvir, 1987). This suggests there is an increased amount of D_2 receptors in a high affinity state (D_2High) in schizophrenic patients. Although this was found in animal models of schizophrenia, this has not been replicated in humans (Seeman et al., 2005).

The exact mechanism of how D_2 receptor function in the NAc is related or causative of schizophrenic symptoms is unknown. We do know that D_2 -like receptors are somehow involved in the alleviation of positive symptoms of schizophrenia from the direct relationship of D_2 receptor affinity and clinical effectiveness (Kapur et al., 2002). However, there are negative side effects associated with current antipsychotics, therefore newer antipsychotic with different pharmacological targets or mechanisms are needed.

Antipsychotic Drugs

Antipsychotic drugs are characterized as either typical or atypical based on their pharmacological properties. Typical antipsychotics antagonize the D_2 receptor and block muscarinic cholinergic, histamine-1, or alpha-1 adrenergic receptors. These drugs are effective in treatment of positive symptoms, but have little effect on negative or cognitive symptoms. The first antipsychotic was developed as an anesthetic (Lacomme, Laborit, Le

Lorier, & Pommier, 1952), which can induce neurolepsis, an extreme form of slowness or absence of motor movements. Since typical antipsychotics non-specifically antagonize the D_2 receptor, they are effective at targeting the dysregulation in the mesolimbic pathway. In the nigrostriatal pathway, continued use of typical antipsychotics can cause an up-regulation of D_2 receptors in the dorsal striatum. This up-regulation disrupts basal ganglia functioning resulting in extra pyramidal symptoms (EPS), such as tardive dyskinesia, a common side effect of antipsychotics. Typical antipsychotics are still used today, but usually not for long-term treatment. These findings lead to the development of a second class of drugs with the goal to decrease EPS.

Atypical antipsychotic drugs vary within their pharmacological properties, however they do share some similarities. This second generation of antipsychotic drugs mostly decreases EPS and is slightly more effective at treating negative symptoms. The pharmacological property of atypical antipsychotics often includes antagonizing D_2 and 5-HT_{2A} receptors. Additional pharmacological mechanisms such as D_2 antagonist with rapid dissociation or 5-HT partial agonist action are also involved with the main goal to decrease EPS. Atypical antipsychotics target specific 5-HT receptors that modulate DA projections to prevent D_2 receptor up-regulation. Although atypical antipsychotics are a significant improvement from typical antipsychotics, refinements are still needed. Specifically, antipsychotic drugs are effective at reducing positive symptoms; however, the attenuation of cognitive deficits is less effective (Greengard, 2001). The majority of antipsychotics still produce side effects, such as lethargic feelings, which results in the discontinuation of medication generally within 6 months (Stroup et al., 2009). These side

effects and lack of complete alleviation of all symptoms demonstrates a need for a novel therapeutic approach for the treatment of schizophrenia.

As compared to the traditional D_2 antagonist property of typical and atypical antipsychotics, a new third generation antipsychotic is unique in that it has D_2 partial agonist actions. In 2002, the first D_2 partial agonist drug that was approved for treatment of schizophrenia was aripiprazole. Aripiprazole also has higher affinity for D_2 receptors than endogenous dopamine, and has reduced efficacy within the neuron as compared to typical and atypical antipsychotic (Nordquist et al., 2008). Therefore, the characteristics of partial agonists make aripiprazole ideal for targeting the hyperactivity in one region and the hypoactivity in another region without producing an upregulation of receptors in the nigrostriatal pathway. In clinical trials, patients report fewer side effects following aripiprazole administration as compared to other antipsychotics and the drug has been somewhat effective at treating negative and cognitive symptoms (Fleischhacker, 2005).

Antipsychotic pharmacological profiles are constantly being revised to increase effectiveness and decrease side effects. Each drug improves upon previous generations; however some patients still report side effects and lack of effectiveness. This novel antipsychotic mechanism and resulting positive clinical findings support the theory that a new pharmacological approach is needed in treatment of schizophrenia. Furthermore, using a new pharmacological approach and examination of the resulting behavioral and intracellular changes may give us new insight into the underlying mechanism of schizophrenia. The most widely used tool for investigating the underlying mechanisms of schizophrenia is the use of animal models of the disorder.

Creating a Valid Model of Schizophrenia

Animal models are currently the most efficient and ethical way to examine the underlying mechanisms responsible for schizophrenia. However, schizophrenia is an idiopathic disease with a multitude of behavioral and pathophysiological factors thus making it difficult to replicate the entire disorder in an animal (Jentsch & Roth, 1999). Also, the diagnostic categories in psychiatry are constantly evolving; therefore producing an animal that is similar in the underlying mechanism and behavioral symptoms associated with the disease is extremely difficult. Researchers that claim to have a complete animal model are constantly justifying the lack of similarities (i.e., face validity) between their model and the endogenous disorder. The simplest approach is the development of animal models that mimic only specific symptoms associated with the disorder, rather than attempting to generate the entire disorder. Multiple criteria have been used to examine the strength of an animal model. When using the above approach with the goal to examining the underlying mechanism of the human condition the model must satisfy two criteria in order to establish its value in basic neurobiological research: reliability and predictive validity (Markou & Geyer, 1995).

Reliability refers to the consistency and stability with which the variables of interest are observed. In animal models, this means the ability to manipulate the independent variable with a high degree of precision and the ability to measure the dependent variables objectively. Also, the ability to reproduce the effects of the manipulations in similar conditions must be satisfied by the model (Markou et al., 1993). There are many types of validity including: predictive, construct, convergent, etiological, and face validity. Depending on the purpose of the test the experimenter wishes to

validate, different types of validity are relevant. For the purpose of basic neurobiological research, predictive validity and construct validity are generally used to validate an animal model. An animal model has predictive validity in that it allows one to make predictions based on the performance of the model. Construct validity is the accuracy with which the test measures what it is intended to measure, however in animal models of schizophrenia, exactly what a test is supposed to measure is constantly changing as the scientific field is being updated (Markou & Geyer, 1995).

There are multiple types of animal models of schizophrenia including but not limited to genetic, pharmacological, and non-pharmacological models. For the purpose of this paper, we will focus on the models most relevant to the included experiments.

NMDAR Antagonist Animal Model of Schizophrenia

Noncompetitive antagonists of the NMDAR, including PCP and ketamine, seem to be able to induce both positive and negative symptoms of schizophrenia, which include cognitive dysfunction in normal humans (Javitt & Zukin, 1991). These drugs also exacerbate both positive and negative symptoms of schizophrenia (Itil et al., 1967). Other pharmacological animal models, such as the psychostimulant and hallucinogen models, are effective at mimicking the positive symptoms of schizophrenia and some of the underlying mechanisms. These models have high reliability and some predictive validity to the human disorder; however they are not as widely used because they lack the ability to induce negative or cognitive symptoms. From these findings, many researchers believe PCP administration is the most complete pharmacological model of schizophrenia because it induces analogous positive, negative, and cognitive symptoms in the rat (Jentsch & Roth, 1999). PCP-or ketamine-induced animal models of schizophrenia have

high reliability and face validity in that acute PCP or ketamine induces behaviors in rats that mimic behaviors observed in schizophrenic patients, such as social withdrawal, sensorimotor gating deficits, and lack of motivation (Sams-Dodd, 1999; Swerdlow, Bakshi, & Geyer, 1996). Furthermore, NMDAR antagonism induces glutamate and DA changes that are believed to be the same in schizophrenic patients (Jentsch & Roth, 1999). The predictive validity of this model is interesting in that typical antipsychotics, such as haloperidol, generally do not alleviate PCP or ketamine induced behavioral responses; however atypical antipsychotics, such as clozapine, consistently alleviate these behaviors in clinically relevant doses (Swerdlow et al., 1996; Swerdlow, Bakshi, Waikar, Taaid, & Geyer, 1998).

DA Receptor Agonist Animal Model of Schizophrenia

Psychostimulants, such as amphetamine or cocaine, are the most widely used pharmacological model of schizophrenia. This model was first used after observations in humans that developed psychosis after acute or chronic exposure to these drugs (Segal, Geyer, & Schuckit, 1981). In animals, psychostimulants increase dopamine neurotransmission into the striatum; however too much dopamine release into the dorsal striatum can results in stereotypic behaviors. This behavior was once believed to be mimicking the negative symptoms of schizophrenia, however it is now believed the behavior is more similar to the side effects of antipsychotic medication (Geyer & Moghaddam, 2002). Another potential drawback in using this model is the development of tolerance after repeated administration and high dosages that are needed to induce behavioral changes. These results can affect the face validity of the model because they are not observed in schizophrenic patients. However, the model is still widely used and

other more specific DA agonists, such as apomorphine, are often used to induce deficits to test antipsychotic efficacy (Geyer & Moghaddam, 2002).

Sensorimotor Gating Deficits

There are several behavioral tests that are used to exam the validity of an animal model. The specific behavioral task used is dependent on the question being examined. Some behaviors, such as locomotion, do not correspond to symptoms of schizophrenia, but do measure pharmacological stimulation or anti-dopaminergic activity of neuroleptics (Geyer & Moghaddam, 2002). However, other behavioral measures, such as disruption of prepulse inhibition (PPI) or impaired attentional set-shifting, mimic schizophrenia behaviors (Geyer & Moghaddam, 2002).

A common observation in schizophrenic patients is the inability to filter or gate the majority of sensory and cognitive stimuli they perceive (McGhie & Chapman, 1961; Neale & Cromwell, 1970). Several sensorimotor gating mechanisms converge to allow an individual to successfully gate out irrelevant stimuli in order to attend to relevant stimuli (Braff & Saccuzzo, 1985). Impairments in sensorimotor gating can lead to sensory overload and cognitive fragmentation (Braff, Geyer, M.A., 1990). The theory of sensorimotor gating has been operationalized in both humans and animals using PPI and gating of P50 event-related potentials (ERPs). The ability to conduct very similar tests in humans and animals strengthens the generalizability and face validity of this model.

Prepulse Inhibition of the Startle Response

The PPI paradigm is based on the finding that a weak pre-stimulus presented 30-500 msec prior to a startling stimulus reduces or "gates" the amplitude of the startle response. PPI is a robust phenomenon with high reliability because PPI is observable

within and between multiple sensory stimuli using a variety of parameters and does not require learning or comprehension of instructions (Braff & Geyer, 1990). Furthermore, PPI deficits have been observed in schizophrenic, schizotypal, and psychosis-prone people (Cadenhead, Geyer, & Braff, 1993). Individuals with disorders that are believed to have sensorimotor gating deficits, such as Tourette's Syndrome, Obsessive Compulsive Disorder (OCD), and Attention Deficit Hyperactivity Disorder (ADHD), as evidenced by more individuals with these disorders also display PPI deficits as compared to the general population (Swerdlow, 2001, 2005). PPI deficits are significantly correlated with deficits in habituation (Geyer & Braff, 1987), distractibility (Karper et al., 1996) and thought disorder (Perry, Geyer, & Braff, 1999). These findings support the theory that sensorimotor gating deficits are related to cognitive deficits observed in patients with schizophrenia.

In rats, direct or indirect DA or noncompetitive NMDAR antagonists can produce PPI deficits (Geyer, Swerdlow, Mansbach, & Braff, 1990). DA agonist-induced deficits are alleviated by typical and atypical antipsychotics (Geyer et al., 1990). Furthermore strong correlations have been found between clinical efficacy of antipsychotic drugs in schizophrenic patients and their ability to attenuate DA agonist induced PPI deficits in rats (Swerdlow et al., 1994).

The neural circuitry that regulates PPI involves more regions and pathways than a simple brain stem startle response. The brainstem loop quickly connects the incoming stimulus to the outgoing motoric response and is mediated by inhibitory projections from the ventral pallidum (VP) and NAc. GABAergic projections from the NAc project directly to the pedunculopontine nucleus (PPTg) and the VP. Before sending GABA to

the PPTg and closing the "gate", the NAc receives glutamatergic projections from the hippocampus, PFC, amygdala, and cingulate gyrus as well as dopamine projections from the VTA. These findings have led researchers to label the NAc the "hub" in PPI regulation due to its connecting forebrain and limbic structures (Swerdlow, Geyer, & Braff, 2001). This is supported by PPI disruption after NAc lesions or direct infusion of a D_2 receptor agonist (Wan & Swerdlow, 1993). The PPI brain circuit overlaps with key brain regions involved in the underlying mechanisms of schizophrenia and antipsychotic treatment. Taken together, the NAc is a significant brain area in PPI regulation, symptoms of schizophrenia, D_2 receptor localization, and antipsychotic target.

PPI is the objective and quantitative assessment of sensorimotor gating. As compared to a comprehensive model of schizophrenia, PPI deficits observed in animals after DA agonist treatment mimics a specific symptom associated with the disorder. This model is robust and reliable across different stimulus, parameters, and species. PPI deficits are also observed in patients with schizophrenia and antipsychotics block these deficits, thus contributing to the face and predictive validity of the model. Therefore, PPI is the optimal tool to investigate different pharmacological approaches and the resulting behavior and underlying mechanisms.

Repeated D_2-like Receptor Agonist Treatment

In experimental animals, DA agonists in the NAc can produce PPI deficits in rodents and is a useful model to investigate the underlying mechanisms of schizophrenia (Swerdlow, Caine, & Geyer, 1992). Repeated exposure to cocaine and amphetamine can induce behavioral sensitization via increases of DA neurotransmission in the NAc (Kalivas & Stewart, 1991) and has been used to model neuroadaptive changes of

schizophrenia (Robinson & Becker, 1986). Based on these previous findings, it can be hypothesized that repeated cocaine administration, an indirect DA receptor agonist, would exacerbate PPI deficits, however an alleviation of previous PPI deficits was observed (Byrnes & Hammer, 2000). Neurons in the NAc containing D_2-like receptors are believed to mediate PPI as evidenced by direct infusion of D_2-like receptor agonist into the NAc disrupted PPI as compared to no effect after infusion of D_1 receptor agonist (Wan & Swerdlow, 1993). Therefore, we believe "PPI recovery" is D_2-like receptor mediated and repeated treatment with D_2-like receptor agonists, quinpirole or ropinirole, produced PPI recovery (Culm & Hammer, 2004). These findings are significant because they suggest a pharmacological approach alternative to D_2 receptor antagonism that can alleviate PPI deficits.

PPI recovery is observed 28 days after D_2-like agonist treatment (Berger et al., 2011). This behavioral change in the absence of the drug suggests the prior repeated treatment of D_2-like receptor agonist initiated a long-lasting neuroadaptation that persists independent of drug stimulation. It is critical to determine the underlying mechanism responsible for PPI recovery because D_2-like receptor containing neurons within the NAc are involved in the treatment of schizophrenia and determining possible molecular changes could have therapeutic implications.

Underlying Mechanism of PPI Recovery

Our previous observation that rats exhibit recovery of sensorimotor gating after repeated treatment with quinpirole or ropinirole compels us to explore the underlying mechanisms that may be responsible for this behavioral effect. A plausible mechanism for PPI recovery could be a compensatory effect, specifically an increase or decrease in

the number of receptors to regain homeostasis. Another possible mechanism could be tolerance or desensitization of D_2-like receptor function. However, there was no significant change found in the amount of G-protein in the NAc or change in G-protein function after repeated D_2-like receptor agonist treatment (Culm & Hammer, 2004). These findings suggest a change in intracellular mechanisms may contribute to PPI recovery.

As mentioned previously, acute stimulation of D_2-like receptors results in the inhibition of adenylate cyclase, however repeated stimulation of these receptors increase cAMP, known as sensitization of adenylate cyclase (Watts & Neve, 2005). This sensitization of adenylate cyclase can activate the cAMP-signaling transduction cascade thus resulting in down-stream intracellular activation or modifications. An important transcription factor, cAMP response element binding (CREB) protein, is activated via phosphorylation at Serine-133 by several signal transduction cascades, including cAMP activated protein kinase A (PKA). Increases in CREB in the NAc were observed after chronic psychostimulants and opiate administration (Carlezon et al., 1998; Konradi, Cole, Heckers, & Hyman, 1994). CREB binds to the cAMP response element (CRE) in many gene promoters, including several growth factors, enzymes, and structural proteins, as well as other transcription factors (Lonze & Ginty, 2002). In the NAc, CREB and downstream targets are implicated in neural plasticity after chronic drug administration as evidenced by modulation of self-administration and relapse (McClung & Nestler, 2008). Therefore, PKA activity and CREB phosphorylation may induce neural plasticity as evidenced by PPI recovery.

Repeated D_2-like agonist treatment results in increased PKA activity and phosphorylation of CREB (Culm et al., 2004). Furthermore, blocking phosphorylation of CREB with a dominant negative mutant CREB virus infused into the NAc prevented PPI recovery (Berger et al., 2011). These findings were observed immediately after 28 days of repeated D_2-like agonist treatment. Phosphorylation of CREB was not observed 28 days after repeated treatment, therefore phosphorylation of CREB contributes to PPI recovery during treatment, but not PPI recovery after treatment. It is plausible to hypothesize that and additional longer lasting intracellular component may be involved in the mechanism responsible for PPI recovery

ΔFosB: Possible Regulator of Persistent PPI Recovery

In contrast to the transient expression of CREB phosphorylation observed after repeated D_2-like agonist treatment, ΔFosB has a significantly longer half-life and remains present for weeks to months after induction (Nestler, 2008). ΔFosB is a truncated splice variant, lacking the C-terminal end of the full-length FosB protein, which is a member of the Fos family proteins including c-fos, FosB. ΔFosB is a product of the *fosB* gene and slowly accumulates in the cell over repeated stimulation as compared to the rapid induction and decline of c-fos and FosB (Nestler, 2008). The possible mechanism underlying the high stability property of ΔFosB may be due to the lack of the C-terminus that may be involved in the destabilization of the full-length protein (Carle, et al., 2007). All Fos family proteins are transcription factors in which they bind with other proteins to deoxyribonucleic acid (DNA) and control the transcription of genetic information from DNA to messenger RNA (mRNA). Due to its rapid activation, c-Fos is generally used as biological marker of acute stimulation as compared to ΔFosB that is often used as a

marker of repeated stimulation because of its long half-life. However, it will be discussed later that ΔFosB may be more than a biological marker and may induce long-lasting cellular plasticity and adaptations in the brain.

As mentioned previously, ΔFosB is encoded by the *fosB* gene and is detected within the nucleus of the neuron, currently there is no findings of ΔFosB outside the nucleus as compared to other transcription factors such as CREB (Damez-Werno, et al., 2012). ΔFosB is measurable in the striatum and the suprachiasmatic nucleus within the hypothalamus under basal conditions (McClung, et al., 2004). However, after chronic stimuli exposure such as stress, drugs of abuse, antidepressants, and antipsychotics, ΔFosB increased expression is located in brain regions including: NAc, PFC, VTA, amygdala, hippocampus, dorsal striatum, locus coeruleus, and septum (McClung, et al., 2004). Within the NAc, ΔFosB expression after chronic stimuli has been detected in medium spiny neurons (MSN) including dopamine D_1 and D_2 containing neurons (Grueter, et al., 2013). Therefore, ΔFosB can be induced in many different regions in the brain, however areas most affected after repeated stimulation seem to be areas involved in the dopamine pathways, however no direct link between dopamine and ΔFosB under basal conditions has been determined (McClung, et al., 2004).

Although ΔFosB expression is a useful biological marker of repeated activation, through its possible function it may also be involved in cellular and behavioral plasticity. The Fos family of transcription factors preferentially dimerize with Jun family proteins (c-Jun, JunB, and JunD) to form the activator protein (AP-1) complex, which then binds to the AP-1 binding site (Hope et al., 1994). ΔFosB can bind to all Jun family members,

however the predominant binding partner in vivo appears to be JunD (McClung, et al., 2004). Unlike ΔFosB, JunD can form a homodimer with itself to form the AP-1 complex and often does because it is a constitutively active protein and at higher concentrations as compared to ΔFosB (Harlan & Garcia, 1999).

Upon binding with the Jun family of proteins, ΔFosB appears to act as a transcriptional repressor with short-term stimulation and then as a transcriptional activator as it accumulates with chronic stimulations (McClung & Nestler, 2003). Similar to CREB, ΔFosB is implicated in neural plasticity, specifically synaptogenesis as evidenced by increased dendritic spines on D_1 receptor-containing neurons in the NAc after repeated cocaine or sexual experiences (Pitchers et al., 2013; Vialou et al., 2012). A sequence of intracellular events connecting ΔFosB activation with increased dendritic spines has not yet been determined. However, plausible mechanisms include the involvement of other transcription factors or proteins important in cellular growth and plasticity, such as GluR2, nuclear factor kappa B (NFκB), or cyclin-dependent kinase 5 (Cdk5) (Nestler, 2008). There is limited research on the mechanisms required for ΔFosB expression in the NAc after repeated stimuli. After repeated cocaine administration, CREB and serum response factor (SRF) are both needed for ΔFosB in mice (Vialou et al., 2012); however, there is no research on the mechanism required for ΔFosB expression after repeated D_2-like agonist or antagonist treatment is currently unknown.

As mentioned previously, ΔFosB is an important protein because it is a biological marker of long-term stimulation; it is also a useful transcription factor and may be an underlying mechanism for persistant behavioral changes related to drug abuse. However,

little is known about ΔFosB and antipsychotics. Repeated typical antipsychotic treatment significantly increases ΔFosB labeling in both dorsal and ventral areas of the striatum (Rodriguez, Garcia, Nakabeppu, & Pickel, 2001). However, repeated atypical antipsychotic treatment increases ΔFosB labeling only in ventral striatum (Vahid-Ansari & Robertson, 1996). The effect of D_2-like agonist treatment, acute or repeated, on ΔFosB is currently unknown. Furthermore, the majority of studies examining the characteristic, induction, or effects of ΔFosB are done in reference to drugs of abuse, typically with cocaine. This emphasizes a gap in the literaure that could be benificial to schizophrenia research.

Repeated D_2-like receptor agonist treatment alleviates prior PPI deficits, termed a PPI recovery (Culm & Hammer, 2004). The underlying mechanisms for PPI recovery involve phosphorylatoin of CREB in the NAc (Berger et al., 2011). However, this does not fully explain the long-lasting properties of PPI recovery. The long lasting characteristics and involvement in neural plasticity suggests ΔFosB may be involved in the presistant behavioral change. If ΔFosB is involved in the mechanisms of PPI recovery, it would be benificial to determine the process through which it is induced. Currently, there is limited research on the pathway(s) responsible for its expression.

Summary

The goal of this project is to determine the intracellular signaling mechanism by which NAc neurons cause persistent recovery of sensorimotor gating after repeated D_2-like agonist treatment in rats. This intracellular mechanism could provide future therapeutic targets to treat cognitive symptoms and possibly avoid side effects. To test the therapeutic relevance of repeated D_2-like receptor agonist treatment, we will compare

previous behavioral and intracellular findings with a novel antipsychotic that has a similar pharmacological profile. Results from these findings will give us a more complete picture of the dynamic properties of neurons vital to schizophrenia behavior and treatment.

Chapter 2

REPEATED QUINPIROLE TREATMENT INDUCES RECOVERY OF PREPULSE INHIBITION AND ΔFOSB LABELING IN NUCLEUS ACCUMBENS IN RATS

Abstract

Prepulse Inhibition of the acoustic startle response (PPI) is an operational measurement of sensorimotor gating, which is disrupted in patients with schizophrenia. Previous research determined that acute administration of dopamine D_2-like receptor agonist, quinpirole, disrupts PPI while repeated treatment alleviated prior PPI deficits, termed a PPI recovery. Also, cyclic adenosine monophosphate (cAMP) responsive element binding (CREB) protein phosphorylation in the nucleus accumbens (NAc), as is also observed after antipsychotic administration. We examined the effect of repeated treatment with quinpirole (0.1 mg/kg,) on PPI at 1, 14, and 28 days in adult Sprague Dawley rats. PPI was significantly reduced by acute quinpirole, but PPI deficits were gradually alleviated after 28 days of treatment. Furthermore, we investigated the role of the long-lasting transcription factor, ΔFosB as a possible catalyst for these behavioral changes. Stereological analysis of immunohistolochemical labeling revealed a significant increase of ΔFosB expression in the NAc core and shell of rats repeatedly treated with quinpirole as compared to vehicle-treated subjects. By contrast, ΔFosB labeling in the dorsal striatum did not differ significantly. These behavioral data replicate previous findings from our laboratory, while increased ΔFosB labeling localized to the NAc suggests the presence of long-term intercellular changes that could underlie PPI recovery after repeated treatment with D_2-like agonists.

Introduction

Sensorimotor gating deficits affect the ability to properly process information leading to cognitive deficits and thought disorder in patients with schizophrenia. This can be measured using prepulse inhibition of the acoustic startle response (PPI) in both humans and rats. PPI measures the ability of the subject to inhibit the startle response to a pulse stimulus in the presence of barely detectable prepulse stimulus up to 500 msec earlier. Normally, a complex brain circuit transfers information after the prepulse to inhibit the brain stem startle response to the subsequent pulse stimulus. This circuit is disrupted in patients with schizophrenia and schizotypic individuals resulting in PPI deficit (Swerdlow et al., 2006). Animal models of PPI deficits through ablations or pharmacological manipulations have illuminated brain areas and neurotransmitter systems involved in PPI.

Dopamine induces PPI deficits in rats via D_2-like receptors in the nucleus accumbens (NAc) (Wan & Swerdlow, 1993). Rats display PPI deficits after an acute systematic administration of direct and indirect dopamine agonists, apomorphine and cocaine (Geyer, Krebs-Thomson, Braff, & Swerdlow, 2001) and specific D_2-like agonists, ropinirole and quinpirole (Swerdlow & Geyer, 1998). Specifically, PPI deficits are observed after an acute direct infusion of D_2-like agonists into the nucleus accumbens (NAc) (Wan & Swerdlow, 1993). In contrast to acute treatment, repeated treatment with indirect dopamine agonist, cocaine (Byrnes & Hammer, 2000) and direct dopamine agonists, quinpirole and ropinirole (Culm & Hammer, 2004) alleviate prior PPI deficits, which we term PPI recovery. PPI recovery is observable after repeated ropinirole treatment and 28 days later an acute ropinirole challenge continues to have no effect on

PPI (Berger et al., 2011). Mechanisms underlying this long lasting behavioral effect after repeated D_2-like agonists treatment is not well characterized because most pharmacological studies are done acutely.

Acute stimulation of D_2-like receptors in the NAc reduces adenylate cyclase due to G_i/G_o protein coupling, which in turn decreases cyclic adenosine monophosphate (cAMP) activity (Gilman, 1987). However, repeated stimulation of these D_2-like receptors in the NAc after repeated quinpirole treatment increases adenylate cylcase as evidenced by a transient increase of cAMP-dependent protein kinase (PKA) activity and phosphorylation of cyclic adenosine monophosphate (cAMP) response element-binding (CREB) in the NAc (Culm & Hammer, 2004). Furthermore, expression of a dominant negative mutant CREB (mCREB), which inhibits phosphorylation of CREB, in the NAc, prevented PPI recovery (Berger et al., 2011). These findings suggest that the possible heterologous sensitization of these D_2-like receptors is critical for PPI recovery. However, it is unknown how these transient cellular changes listed above are involved in the previously observed long-lasting PPI recovery 28 days after drug treatment. Therefore, we hypothesize that long lasting cellular changes may be involved in PPI recovery after repeated D_2-like agonist treatment.

ΔFosB is a truncated splice variant of FosB protein, which is a member of the Fos family proteins. ΔFosB heterodimerizes with the Jun family of proteins to form the activator protein-1 (AP-1) complexes that bind to the AP-1 binding site in order to regulate transcription (Hope et al., 1994). Fos proteins are observed rapidly in the NAc after acute administration of addictive drugs, such as cocaine, amphetamines, and nicotine (Nestler et al., 2001). ΔFosB is an effective marker for long-term use and

withdrawal of addictive drugs because it remains present several weeks after the last drug administration (Nestler et al., 2001). Repeated treamtent with typical antipsychotic haloperidol, D_2 receptor antagonist, significantly increases ΔFosB labeling in both dorsal and ventral areas of the striatum (Rodriguez et al., 2001). However, repeated atypical antipsychotic clozapine treatment increased ΔFosB labeling in ventral areas of the striatum (Vahid-Ansari & Robertson, 1996). ΔFosB labeling is an excellent marker for neuronal activation that has not been examined after repeated D_2 -like agonist treatment.

This study examined the effects of repeated D_2 -like agonist, quinpirole, on ΔFosB expression in the NAc as a possible underlying mechanism of PPI recovery. We hypothesize that repeated quinpirole treatment would cause rats to exhibit PPI recovery as observed in previous studies (Culm & Hammer, 2004). Furthermore, we believe that repeated quinpirole treatment will increase ΔFosB expression in the NAc, which may be involved in the underlying mechanism of long-lasting PPI recovery.

Materials and Methods

Animals and Drug Treatment

Animals were provided with food and water *ad libitum* while housed in a climate-controlled facility with 12-hour reverse light/dark cycles (lights off at 9:00 AM). All experiments were approved by the Arizona State University and University of Arizona Institutional Animal Care and Use Committee, and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (Charles River Laboratories, Hollister, CA) weighing 250-300g upon arrival were housed three to a cage. Animals were allowed to acclimate to the

laboratory 7 days prior to handling. During this acclimation, animals were habituated to handling and subcutaneous (SC) saline injection.

(-) Quinpirole hydrochloride (Sigma, St. Louis, MO) was dissolved in sterile saline (0.9%). Starting after PPI baseline testing, animals were treated once daily for 28 consecutive days with quinpirole (0.1 mg/kg, SC) or 0.9% saline vehicle (1.0 ml/kg).

Prepulse Inhibition Testing

After acclimation to the laboratory, animals were placed into a Startle Monitor Behavioral Testing chamber (Kinder Scientific, Poway, CA) with 70dB ambient noise for 5 min each day for 2 days prior to baseline testing. Treatment groups were normalized by the mean acoustic startle response observed on two days of baseline testing. After baseline testing, animals were tested on 1, 14, and 28 day of treatment.

PPI startle response was determined using the Startle Monitor behavioral testing system. All PPI testing was conducted during the dark phase (900 –1300 h). Each animal was placed in a PPI chamber and exposed to 70 dB ambient noise for 5 min, followed by a PPI baseline or test session. A PPI baseline session consisted of four consecutive pulse trials (120 dB, 40-msec pulses), a randomized presentation of 8 no stimulation, 16 pulse, and 15 prepulse (10 each of 73, 76, and 82 dB, 20 msec prepulses, followed 100 msec later by a pulse) trials, and ending with four pulse trials. A PPI test session consisted of four consecutive pulse trials (120 dB, 40-msec pulses), a randomized presentation of 10 no stimulation, 16 pulse, and 30 prepulse (10 each of 73, 76, and 82 dB, 20 msec prepulses, followed 100 msec later by a pulse) trials, and ending with four pulse trials. The inter-trial interval averaged 15 sec (range: 8–22 sec). Percent PPI was calculated as: 100-[(mean prepulse response/mean pulse response) × 100].

Perfusion and Tissue Preparation

Animals were euthanized 10 days after last drug treatment to ensure that residual quinpirole had metabolized and that immunohistochemical labeling would reflect the presence of persistent ΔFosB protein. ΔFosB labeling is detectable only 4-12 after induction (McClung & Nestler, 2003). Animals were anesthetized with an overdose of sodium pentobarbital (100 mg/kg) and perfused transcardially with 10 mL of 10% heparin in 0.1M phosphate-buffered saline (pH 7.4) followed by 225 mL of 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were removed, post-fixed for 2h in the same fixative at 4°C. Coronal sections (20μm) were taken on a sliding microtome from the level of 2.7-0.7 mm anterior to bregma for the NAc (Paxinos & Watson, 2007). Sections were collected in 0.1M phosphate buffer (pH 7.4 at 4°C), mounted onto glass slide (Superfrost Plus; Fisher; Waltham, MA), dried and stored at -35°C until the time of processing.

FosB/ΔFosB Immunohistochemistry

Slides were washed three times in 0.05 M potassium phosphate-buffered saline (KPBS, pH 7.4), and incubated in 5% normal goat serum/0.05 m KPBS/0.4% Triton X for 60 min at room temperature. To determine ΔFosB labeling, primary antibody raised against the N-terminal region of FosB (SC-48; Santa Cruz Biotechnology, Santa Cruz, California) was used at a dilution of 1: 5,000 in 5% normal goat serum/0.05M KPBS/0.4% Triton X. This antibody recognizes 32–37 kDa proteins corresponding to the molecular weight of ΔFosB-like proteins and full-length FosB (Perrotti et al., 2004).

Following incubation for 48 h at 4°C, sections were washed three times in 0.05M KPBS and processed using avidin–biotin–peroxidase (Vectastain ABC Elite kit, Vector

Laboratories, Burlingame, California). Sections were developed with 3, 3'-diaminobenzidine (DAB) for 5 min, dehydrated and coverslipped at room temperature. Control procedures included preabsorption of the primary antibody using FosB peptide, and conducting these procedures in the absence of the primary antibody. There was no detectable labeling after any of the procedures (not shown).

Immunohistochemical Analysis

Tissue sections were examined for the presence of a dark grey reaction product. Labeled profiles were obtained from 3-4 sections from each rat. Selected areas of 400 μm^2 within the NAc core, shell, and caudate putamen were captured and digitized using a color digital video camera interfaced to a Zeiss Axioskop microscope with a 20X objective. Labeled profiles within the areas of interest were determined using StereoInvestigator (MicroBrightField Biosciences, Inc., Willston, VT, USA). A systematic random sampling approach using a stereological grid, consisting of 16 equal counting frames (100 X 100μm each) ensured randomization of identification within the area. A profile was considered labeled if its pixel luminance was more than two standard deviations different from the background luminance as calculated by Stereo Investigator software. The average labeled profiles in an area were determined for each animal and these data were transformed to express the number of labeled profiles per mm^2.

Statistical Analysis

Startle response (120dB) and response in the presence of ambient noise alone, were analyzed using a repeated measures analysis of variance (ANOVA). Percent PPI (mean ± SEM) are mean values collapsed across three prepulse intensities. PPI responses within a single day were analyzed using a student's T-test. PPI responses across multiple

days were analyzed using two-way repeated-measures ANOVA with day of testing as within subject factor and drug treatment as between subject factor. ΔFosB labeled profiles were analyzed using two-way repeated measures ANOVA with brain area as within subject factor and drug treatment as between subject factor. Post-hoc comparisons were made using Fisher's least squares difference (LSD). All data are presented as mean ± SEM and PPI responses are presented collapsed across prepulses 3, 6, and 12 dB above ambient noise level (70dB).

Results

PPI Recovery Following Repeated Quinpirole

This experiment demonstrates that acute treatment with quinpirole significantly, disrupted PPI, as previously observed (Figure 2) (Berger et al., 2011; Culm & Hammer, 2004). Specifically, quinpirole (0.1 mg/kg) reduced PPI by 49% compared to vehicle treatment on day 1. Rats did not display PPI deficits after repeated quinpirole treatment when compared to controls on day 28 of testing. Furthermore, repeated quinpirole treatment significant increased in PPI performance from day1 to day 28 of testing; quinpirole (0.1 mg/kg) increased PPI performance by 41% from Day 1 to Day 28. There was also no significant effect of drug treatment on startle (120 dB) or no stimulus (70 dB) ambient noise response on day 28 of PPI testing.

ΔFosB Expression in the NAc Following Repeated Quinpirole

Modified stereological analysis revealed a significant increase of ΔFosB positive cells 10 days after repeated quinpirole treatment (Figure 3). This effect was visible in both the NAc core and shell, but not in the CPu. Labeling density increased by 241.72% in the NAc core and by 158.03% in the shell after repeated quinpirole treatment. There

was also a significant increase in total labeled cells in the NAc core and shell as compared to the CPu.

Discussion

Recovery of Sensorimotor Gating Deficits after Repeated D_2-like Agonist

Acute administration of D_2 -like receptor agonist in the NAc disrupts PPI, a model of sensorimotor gating deficits (Wan & Swerdlow, 1993). Antipsychotics given prior to acute D_2-like receptor agonist challenge can alleviate deficits and are believed to relate to the clinical efficacy of the drug (Swerdlow et al., 1994). Interestingly, repeated D_2-like agonist treatment can also alleviate PPI deficits (Berger et al., 2011; Culm & Hammer, 2004; Culm et al., 2004). In the current study, acute quinpirole induced PPI deficits and repeated quinpirole treatment alleviated prior deficits, termed a PPI recovery. This supports previous observations of PPI recovery after repeated D_2-like agonist treatment (Berger et al., 2011; Culm & Hammer, 2004; Culm et al., 2004). There are no other studies examining the effect of repeated D_2-like agonist treatment on PPI. Repeated D_2 antagonist antipsychotics alleviate phencyclidine (PCP) induce PPI deficits after 6 days of treatment, however this was only observed after atypical antipsychotic treatment (Li, He, & Volf, 2011). This suggests repeated D_2-like agonist treatment may have some therapeutic relevance.

Repeated D_2-like Agonist Treatment Induces ΔFosB in NAc

PPI recovery is observable 28 days after treatment (Berger et al., 2011). This suggests a sustainable intracellular component may be involved in this persistent behavioral change. Previously, repeated D_2-like agonist treatment increased PKA activity and CREB phosphorylation, however CREB phosphorylation was not observable 28 days

after repeated treatment (Berger et al., 2011). Therefore, we hypothesized that due to its long half-life and association with synaptic plasticity that ΔFosB may be involved in preserving PPI recovery. In the current study, we found a significant increase in ΔFosB in the NAc core and shell 10 days after repeated D_2-like agonist treatment. We believe this induction of ΔFosB may be involved in long lasting PPI recovery.

ΔFosB expression in the striatum is observed after multiple types of repeated stimuli, such as chronic cocaine administration, stress exposure, and antipsychotic treatment. ΔFosB induction after these different stimuli is believed to affect different resulting behaviors, however all the resulting behaviors are persistent and may involve neural plasticity. ΔFosB expression after chronic cocaine increases drug reward, which may be a factor in the propensity for relapse (Nestler et al., 2001). Repeated stress exposure induces ΔFosB and is believed to be involved in anti-depressant like behaviors or resiliency (Perrotti et al., 2004). Repeated typical antipsychotic increased ΔFosB and is believed to be responsible to tardive-dyskinesia-like behaviors in rats (Rodriguez et al., 2001). It should be noted the administration of antipsychotic was for 6 months via oral administration, which may be different from systemic administration. These studies suggest that ΔFosB is induced after repeated stimuli and results in long-lasting behavioral change.

The exact mechanism that ΔFosB produces persistent behavioral change is unknown, but is believed to involved neural plasticity. After chronic cocaine and sexual encounters, increased dendritic spines and ΔFosB were observed in the NAc (Pitchers et al., 2013). These neurons are believed to express D_1 receptors because of their

involvement in the reward pathway (Nestler, 2008). There are currently no studies examining the effect ΔFosB on cellular plasticity after repeated D_2-like agonist treatment, however repeated exposure to stress increases ΔFosB in both D_1 and D_2 containing neurons (Vialou et al., 2012). Although we do not know what cells are expressing ΔFosB after repeated D_2-like agonist treatment, we can assume they are neurons expressing D_2-like receptors because PPI regulation is modulated by NAc indirect pathway, which contains neurons expressing D_2-receptors (Wan & Swerdlow, 1993). Therefore, if ΔFosB is found in D_2-containing neurons and cells that express ΔFosB also have increased dendritic spines, we can hypothesize that repeated D_2-like agonist treatment is inducing ΔFosB and increased dendritic spines resulting in persistent PPI recovery.

Future studies will need to be completed to determine the type of cell that regulates PPI recovery and examine if ΔFosB is affecting the cellular structure or function of the cell. It will also need to be determined the extent to which ΔFosB is involved in the preservation of PPI recovery after drug treatment. These studies elucidate a transcription fact that has been implicated in long lasting behavioral changes and may sustain PPI recovery. This study combined with previous findings of intracellular activation after repeated D_2-like agonist treatment has determined a mechanism for the alleviation of a clinically relevant behavior and may have future potential therapeutic relevance.

Chapter 3

ΔFOSB AND CYCLIC ADENOSINE MONOPHOSPHATE RESPONSE ELEMENT BINDING PROTEIN PHOSPHORYLATION IN NUCLEUS ACCUMBENS UNDERLIES RECOVERY OF SENSORIMOTOR GATING DEFICITS FOLLOWING REPEATED D2-LIKE AGONIST TREATMENT IN RATS

Abstract

Prepulse Inhibition (PPI) is a cross-species operational measurement of sensorimotor gating. PPI deficits are observed in patients with schizophrenia and after acute dopamine (DA) D_2-like dopamine receptor agonist in rats. In contrast, repeated treatment with D_2-like receptor agonists alleviates prior PPI deficits, termed a PPI recovery. We have previously found increases in cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB) phosphorylation immediately after repeated D_2-like agonist treatment and ΔFosB 10 days after treatment in the nucleus accumbens (NAc) of rats. This study examined if phosphorylation of CREB is required for ΔFosB expression and if ΔFosB transcription in the NAc is necessary for persistent PPI recovery after repeated D_2-like receptor agonist ropinirole. In the first study, adeno-associated virus (AAV)-mediated dominant negative mutant CREB (mCREB) prevented PPI recovery and suppressed repeated ropinirole-induced ΔFosB expression in the NAc. In the second study, AAV-induced overexpression of dominant negative ΔJunD, antagonist of ΔFosB, in the NAc prevented PPI recovery. The suppression of repeated ropinirole-induced ΔFosB expression by mCREB suggests phosphorylation of CREB is necessary for ΔFosB expression after repeated D_2-like agonist treatment. The attenuation

of PPI recovery after ΔJunD infusion into the NAc after repeated D_2-like receptor agonist treatment suggests ΔFosB is necessary for PPI recovery. These results suggest CREB phosphorylation and ΔFosB are involved in an underlying intracellular mechanism preserving PPI recovery after repeated D_2-like agonist treatment. This underlying intracellular mechanism of long-lasting PPI recovery may be involved in sensorimotor gating deficits, which contribute to cognitive symptoms in patients with schizophrenia. Therefore, repeated D_2-like agonist function may elucidate factors that contribute to the alleviation of cognitive deficits of schizophrenia.

Introduction

Patients with schizophrenia have difficulty processing information when distracting stimuli are presented in close and rapid succession, termed 'sensory flooding' (Braff & Saccuzzo, 1985). Sensorimotor gating prevents 'sensory flooding' by regulating or inhibiting the transmission of sensory information to a motor system (Braff & Geyer, 1990). Prepulse inhibition of the acoustic startle response (PPI) is a quantifiable, valid, and reliable response that can be measured across species to detect sensorimotor gating deficits (Geyer et al., 2001). PPI is the reduction in startle response to an intense stimulus or "pulse" when preceded, up to 500 ms, by a weaker stimulus or "prepulse". This neurological phenomenon involves forebrain regulation of inhibition on the brain stem startle response. Specifically, the nucleus accumbens (NAc) is an integral brain area connecting forebrain and limbic structures that modulate PPI (Swerdlow, Braff, & Geyer, 1990), and acute local infusion of D_2-like agonists produce PPI disruptions (Wan & Swerdlow, 1993).

Dopamine acting on D_2-like, D_2 and D_3, G protein-coupled receptors in the NAc can disrupt PPI in rats (Wan & Swerdlow, 1993). For example, PPI disruptions are observed after acute systemic treatment (Martinez, Ellison, Geyer, & Swerdlow, 1999) or NAc infusion (Wan & Swerdlow, 1993) with D_2-like receptor agonist. In contrast to acute treatment, repeated treatment with indirect dopamine agonist result in an attenuated PPI disruption in rats (Byrnes & Hammer, 2000; Feifel, Priebe, Johnstone-Miller, & Morgan, 2002). Repeated treatment with more specific D_2-like agonists, quinpirole or ropinirole, completely alleviate previous PPI deficits, termed a PPI recovery (Berger et al., 2011; Culm & Hammer, 2004). Furthermore, this recovery after repeated treatment is long-lasting as rats continue to display PPI recovery four weeks after treatment (Berger et al., 2011).

Repeated D_2-like receptor stimulation produces heterologous sensitization of cyclic adenosine monophosphate (cAMP) signaling, as compared to a reduction of cAMP after acute D_2-like receptor stimulation (Watts & Neve, 2005). Although the underlying mechanism of this sensitization is not well understood, it can affect down stream targets to induce intracellular changes. We have found increases in cAMP-dependent protein kinase (PKA) activity and phosphorylation of cAMP response element binding protein (CREB) after repeated D_2-like agonist treatment (Culm et al., 2004). Furthermore, we have determined that phosphorylation of CREB is necessary for PPI recovery (Berger et al., 2011). Increases in PKA activity and phosphorylation of CREB were detected immediately after repeated D_2-like agonist treatment and were not detected four weeks after treatment when PPI recovery is still observable. ΔFosB, a stable truncated splice variant of FosB protein, was increased after repeated D_2-like agonist treatment and may

underlie the long-lasting characteristic of PPI recovery. ΔFosB and CREB are both transcription factors that have been implicated in behavioral plasticity after long-term drug use (Nestler, Kelz, & Chen, 1999; Newton et al., 2002). However, the direct connection between phosphorylation of CREB and ΔFosB expression after repeated D_2-like agonist treatment has never been examined.

The aim of this study is to determine if CREB phosphorylation is necessary for ΔFosB expression in the NAc after repeated D_2-like agonist treatment and if ΔFosB is necessary for persistent PPI recovery. We hypothesize that bilateral overexpression of dominant negative mutant CREB (mCREB) in the NAc, blocking phosphorylation of CREB, will suppress ΔFosB in the NAc following repeated ropinirole treatment. We also hypothesize that bilateral overexpression of dominant negative ΔJunD, ΔFosB antagonist, will prevent PPI recovery after repeated D_2-like receptor agonist treatment.

Methods and Materials

Animals and Drug Treatment

For all the following experiments, animals were housed under a 12:12 h reverse light-dark cycle and given *ad libitum* access to water and food. Rats were allowed to acclimate for 7 days to the laboratory before handling and habituation to the behavioral testing chambers. All experiments were approved by the Arizona State University and the University of Arizona Institutional Animal Care and Use Committees and were conducted in accordance with the *Guide for the care and Use of Laboratory Animals*.

Adult male Sprague-Dawley rats (Charles River Laboratories, Hollister, California) weighing 260-325 g were habituated to handling and subcutaneous (SC) saline injection and placed into a Startle Monitor behavior testing chamber (Kinder

Scientific, Poway, California) with 70 dB ambient noise for 5 min daily for 2 days before baseline testing. Baseline PPI was assessed as described later starting 10 min after 0.9% sterile saline vehicle injection (1.0 mL/kg, SC) on 2 consecutive days to ensure a reliable mean value. Treatment groups were normalized according to the mean PPI observed during the baseline testing.

Ropinirole hydrochloride (HCl), obtained from National Institute of Mental Health's (NIMH) Chemical Synthesis and Drug Supply Program, was dissolved in a vehicle of saline and administered SC. Vehicle controls were 0.9% sterile saline. Injections took place in home cages.

Prepulse Inhibition Testing

All PPI testing was conducted during the dark phase (900 –1300 h). Each animal was placed in a PPI chamber and exposed to 70 dB ambient noise for 5 min, followed by a PPI baseline or test session. A PPI baseline session consisted of four consecutive pulse trials (120 dB, 40-msec pulses), a randomized presentation of 8 no stimulation, 16 pulse, and 15 prepulse (10 each of 73, 76, and 82 dB, 20 msec prepulses, followed 100 msec later by a pulse) trials, and ending with four pulse trials. A PPI test session consisted of four consecutive pulse trials (120 dB, 40-msec pulses), a randomized presentation of 10 no stimulation, 16 pulse, and 30 prepulse (10 each of 73, 76, and 82 dB, 20 msec prepulses, followed 100 msec later by a pulse) trials, and ending with four pulse trials. The intertrial interval averaged 15 sec (range: 8–22 sec). Percent PPI was calculated as: 100-[(mean prepulse response/mean pulse response) × 100].

AAV Vector Construction and Stereotaxic Injections

Adeno-associated virus (AAV) vectors were produced using Stratagene's helper-free system to add an eGFP tag to the N-terminus of mCREB and ΔJunD as described previously (Wallace et al., 2009; Winstanley et al., 2007).

ΔJunD-eGFP and mCREB-eGFP fusion proteins were created by adding an eGFP tag to the N terminus of ΔJunD and mCREB. Previous studies have indicated that tagging the N terminus of CREB does not interfere with its functional activity (Chao et al., 2002). ΔJunD-eGFP and mCREB-eGFP constructs with a CMV promoter were inserted into the pAAV-MCS plasmid and packaged according to the manufacturer's instructions. For purification, packaging cells were centrifuged at 1500 x g and re-suspended in 8 ml. Cells were subjected to 2 freeze-thaw cycles, and contaminating DNA and RNA was digested with 50 U/ml Benzonase. Lysate was subjected to ultracentrifugation through an iodixanol gradient at 350,000 x g for 120 min at 4°C. The 40% iodixanol layer was removed and placed in a Biomax 100k NMWL filter column for buffer exchange. The virus was rinsed twice with PBS and concentrated to 100 µl; the resulting titer was approximately 10_{11} IU/ml.

Rats were anesthetized and placed into a stereotaxic frame, and a microsyringe (Hamilton; Reno, Nevada) was lowered into the NAc at the following coordinates: +1.2 mm anterior to bregma, ±1.3 mm lateral, and 7.1 mm from the pial surface. The bilateral intracranial infusion of AAV was infused bilaterally at a rate of 0.1 µl/min for 10 min on each side, after which the needle remained in place for 10 min. Rats were allowed 3 weeks for recovery and adequate expression of ΔJunD or mCREB before drug treatment.

Experiment 1 Procedure

Following habituation and normalization of groups, rats under-went stereotaxic surgery for AAV vector-mediating gene transfer of mCREB, in which Ser^{133} is replaced with alanine to prevent phosphorylation, co-expressed with enhanced green fluorescent protein (eGFP) or eGFP alone bilaterally in the NAc. Three weeks after stereotaxic surgery, rats received ropinirole HCl (0.0 or 0.1 m/kg) (NIMH Chemical Synthesis and Drug Supply Program) in 0.9% sterile saline. PPI was assessed on the first and last day of drug administration as described earlier.

Experiment 2 Procedure

Following habituation and normalization of groups, rats under-went stereotaxic surgery for AAV vector-mediating gene transfer of ΔJunD, a truncated mutant of the JunD protein that is devoid of a transactivational domain and thus functions as a dominant negative antagonist of activator protein-1 (AP-1) mediated transcription, coexpressed with eGFP or eGFP alone bilaterally in the NAc. Three weeks after stereotaxic surgery, rats received ropinirole HCl (NIMH Chemical Synthesis and Drug Supply Program) in 0.9% sterile saline. PPI was assessed on the first and last day of drug administration as described earlier.

Immunohistochemistry

Ten days after the last drug challenge, rats were anesthetized and perfused with heparinized saline followed by 4% buffered paraformaldehyde. Brains were post-fixed in the same fixative for 2 h at 4° C then placed in graded sucrose solutions in phosphate buffered saline (PBS) overnight. Striatal brain sections (20 μm) were mounted on slides.

The location of eGFP-labeled neurons were documented in each brain (Neurolucida; MBF Biosciences) to determine the infusion site and the spread of viral infusion.

After eGFP confirmation, slides were washed three times in 0.05 M potassium phosphate-buffered saline (KPBS, pH 7.4), and incubated in 5% normal goat serum/0.05 m KPBS/0.4% Triton X for 60 min at room temperature.

To determine ΔFosB labeling in experiment 1, primary antibody raised against the N-terminal region of FosB (SC-48; Santa Cruz Biotechnology, Santa Cruz, California) was used at a dilution of 1: 10,000 in 5% normal goat serum/0.05 m KPBS/0.4% Triton X. This antibody recognizes 32–37 kDa proteins corresponding to the molecular weight of ΔFosB-like proteins and full-length FosB (Perrotti et al., 2004).

To determine CREB labeling in experiment 1, primary antibody raised against the amino terminus, not the Ser^{133}, of CREB (48H2; Cell Signaling Technology, Inc., Danvers, Massachusetts) was used at a dilution of 1: 12,000 in 5% normal goat serum/0.05 m KPBS/0.4% Triton X. This antibody does not recognize the Ser^{133}, therefore it will detect both endogenous CREB and mCREB (Berger et al., 2011).

To determine JunD labeling in experiment 2, primary antibody raised against the C-terminus region of JunD (SC-74; Santa Cruz Biotechnology, Santa Cruz, California) was used at the dilution of 1:20,000 in 5% normal goat serum/0.05 m KPBS/0.4% Triton X. This antibody recognizes the C-terminus of the JunD protein that is shared with the truncated ΔJunD protein (Peakman et al., 2003; Zachariou et al., 2006).

Following incubation for 48 h at 4°C, sections were washed three times in 0.05M KPBS and processed using avidin–biotin–peroxidase (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, California). Sections were developed with 3, 3'-

diaminobenzidine (DAB) for 5 min, dehydrated and coverslipped at room temperature. Control procedures included pre-absorption of the primary antibody using FosB peptide, and conducting these procedures in the absence of the primary antibody. There was no detectable labeling after any of the procedures (not shown).

Fluorescent immunohistochemistry

Sections were washed in 0.05M potassium phosphate-buffered saline (KPBS), then blocked for 1h in 5% normal goat serum, 0.4% Triton X-100 in 0.05M KBPS. Sections were incubated with a primary antibody of mouse anti-FosB/ΔFosB (SC-48; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:500 in 5% normal goat serum/0.05M KPBS/ 0.4% Triton X for 48 hours in 4°C. Sections were washed three times for 5 min in 0.05M KPBS and then incubated with goat anti-rabbit IgG and Alexa Fluor® 546 goat anti-mouse IgG (Invitrogen, NY, USA) at a dilution of 1:1000 in 5% normal goat serum/0.05M KPBS/ 0.4%Triton X for 1 hr. Slides were cover slipped with Vectashield® hard set mounting medium with 4',6-Diamido-2-Phenylindole, Dihydrochloride (DAPI; Vector Laboratories, Burlingame, CA, USA). Appropriate positive and negative controls were used for both primary and secondary antibodies (not shown).

Immunohistochemical Analysis

Tissue sections were examined for the presence of a dark grey reaction product. Labeled profiles were obtained from 3-4 sections from each rat. Selected areas of 400 μm^2 within the NAc core, shell, and caudate putamen were captured and digitized using a color digital video camera interfaced to a Zeiss Axioskop microscope with a 20X objective. Labeled profiles within the areas of interest were determined using

StereoInvestigator (MicroBrightField Biosciences, Inc., Willston, VT, USA). A systematic random sampling approach using a stereological grid, consisting of 16 equal counting frames (100 X 100μm each) ensured randomization of identification within the area. A profile was considered labeled if its pixel luminance was more than two standard deviations different from the background luminance as calculated by Stereo Investigator software. The average labeled profiles in an area were determined for each animal and these data were transformed to express the number of labeled profiles per mm^2. For JunD labeling analysis, immunohistochemical conditions were optimized to minimize detection of endogenous JunD, which is expressed universally in the brain.

Statistical Analysis

Startle response (120dB) and response in the presence of ambient noise alone, were analyzed using a repeated measures analysis of variance (ANOVA). Percent PPI (mean ± SEM) are mean values collapsed across three prepulse intensities. PPI responses within a single day were analyzed using a one-way ANOVA. PPI responses across multiple days were analyzed using two-way repeated-measures ANOVA with day of testing as within subject factor and treatment (drug and virus) as between subject factor. Labeled profiles were analyzed using two-way repeated measures ANOVA with brain area as within subject factor and drug treatment as between subject factor. Post-hoc comparisons were made using Fisher's least squares difference (LSD). All data are presented as mean ± SEM and PPI responses are presented collapsed across prepulses 3, 6, and 12 dB above ambient noise level (70dB).

Results

mCREB Prevents PPI Recovery after Repeated Ropinirole Treatment

Acute ropinirole challenge significantly reduced PPI as compared to acute saline challenge (Figure 5). After 28 days of ropinirole treatment, PPI was fully restored in rats expressing eGFP alone. In contrast, PPI remained disrupted after repeated ropinirole treatment in rats overexpressing mCREB. There was no effect of virus infusion or repeated ropinirole treatment on pulse (120dB) startle response (Table 2) or no stimulus (70dB ambient noise) response (Table 3). However, pulse (120dB) startle response over time; this has been observed previous (Berger et al., 2011) and was not dependent on repeated drug treatment.

mCREB Prevents Repeated Ropinirole-Induced ΔFosB in the NAc

Repeated ropinirole treatment significantly increased ΔFosB in the NAc core and shell in rats expressing eGFP alone (Figure 6) compared to repeated saline treated animals; this was previously observed (Chapter 2). In contrast, ΔFosB was not significantly increased after repeated ropinirole treatment in rats overexpressing mCREB as compared to repeated ropinirole treatment rats expressing only eGFP (Figure 7, Figure 8). There was an unexpected increase of ΔFosB in the NAc of animals repeatedly treated with saline, although some co-expression with eGFP was detected, ΔFosB labeling was expressed throughout the NAc (Figure 9). Inter-Reliability Score, Avg = 95.67, SD = 0. 577. There was a significant increase in total CREB labeling in rats with mCREB overexpression (Figure 10) as compared to rats with eGFP alone. There was also significantly more total ΔFosB expression in the NAc core as compared to the other areas of the striatum; Inter-Reliability score, Avg = 95.67, SD = 0. 577. There was no

significant difference in the amount of adeno-associated virus for gene transfer infused into the different treatment groups (Table 4).

ΔJunD Prevents PPI Recovery After Repeated Ropinirole Treatment

Acute ropinirole challenge significantly reduced PPI as compared to acute saline challenge (Figure 11). After 28 days of ropinirole treatment, PPI fully recovered in rats expressing eGFP alone. In contrast, PPI remained disrupted after repeated ropinirole treatment in rats overexpressing ΔJunD (ΔFosB antagonist). There was no effect of virus infusion on pulse (120dB) startle response (Table 5) or no stimulus (70dB ambient noise) response (Table 6). There was an effect of repeated ropinirole on no stimulus (70dB ambient noise) response as compared to repeated saline treated animals regardless of virus infusion. Pulse (120dB) startle response over time; this has been observed previous (Berger et al., 2011) and was not dependent on repeated drug treatment.

ΔJunD Infusion Increased JunD in the NAc Core

JunD labeling was significantly increased in the NAc core as compared to other areas of the striatum (Figure 12) in rats after ΔJunD infusion in the NAc core; intra-reliability score, Avg = 98.5, SD = 2.12. There was no significant difference in the amount of AAV for gene transfer infused into the different treatment groups (Table 7).

Discussion

Recovery of Sensorimotor Gating Deficits after Repeated D_2-like Receptor Agonist Treatment

In order to examine sensorimotor gating deficits in rodents, our laboratory and others have determined acute systemic administration of D_2-like receptor agonist, ropinirole, induces PPI deficits (Berger et al., 2011; Swerdlow, Taaid, Oostwegel,

Randolph, & Geyer, 1998). In contrast to acute administration, repeated D_2-like agonist treatment alleviates prior deficits (Berger et al., 2011; Culm & Hammer, 2004; Culm et al., 2004). In the current studies, acute ropinirole (0.1 mg/kg) challenge caused PPI deficits and repeated treatment alleviated the previous deficits, thus replicating our earlier findings. This previously observed behavioral change suggests the underlying mechanisms for the change may be a tolerance or adaptation in the brain after repeated treatments. Repeated D_2-like agonist treatment failed to change in the amount of G protein levels or change G protein function in the NAc after repeated activation of D_2-like receptors (Culm & Hammer, 2004), thus suggesting an intracellular mechanism may be regulating PPI recovery. Repeated D_2-like receptor agonist ropinirole significantly increase CREB phosphorylation and ΔFosB supporting they theory that intracellular adaptations may underlie PPI recovery (Chapter 2) (Berger et al., 2011; Culm et al., 2004).

CREB Activation in the NAc is Necessary for PPI Recovery and ΔFosB After Repeated D_2-like Agonist Treatment

Previously, we observed an increase in CREB phosphorylation after repeated D_2-like agonist treatment and blocking CREB phosphorylation with a dominant negative mCREB virus infused into the NAc prevented PPI recovery (Berger et al., 2011; Culm et al., 2004). We also observed an increase in ΔFosB in the NAc after repeated D_2-like agonist treatment (Chapter 2) that may underlie the long-lasting property of PPI recovery. The selective expression of CREB phosphorylation and ΔFosB in the NAc and the involvement of these transcription factors in behavioral plasticity led us to hypothesize

that the phosphorylation of CREB was required for ΔFosB in the NAc after repeated D_2-like agonist treatment.

To determine if ΔFosB induction requires CREB phosphorylation in the NAc, we infused an AAV-mediated gene transfer to produce prolonged overexpression of mCREB in the NAc and assessed PPI and ΔFosB expression. We found that mCREB overexpression prevented PPI recovery, supporting prior work (Berger et al., 2011). Mutant CREB overexpression in the NAc also suppressed ropinirole-induced ΔFosB expression in the NAc. We also observed increased CREB expression, which confirmed the function of the mCREB virus function to manufacture mCREB thus increasing the amount of CREB labeled cells.

We found an unexpected significant increase of ΔFosB in the NAc of animals that received mCREB virus and repeated saline treatment. The limited florescent overlay of eGFP, used to confirm mCREB infusion, and ΔFosB displayed some cells that contain co-expression, however most ΔFosB labeled cells were not in the infusion area or co-labeled with GFP. A similar study did not find increased ΔFosB after using a viral-mediated Cre-recombinase knock out of CREB, however only eGFP positive neurons were analyzed, therefore it is unknown if cells outside the infusion site expressed ΔFosB (Vialou et al., 2012). In order to examine the effect of repeated D_2-like treatment and mCREB on the NAc, the current experiment did not require ΔFosB positive cells to also express eGFP in order to be counted. Therefore, cells may be counted that were not infused with the virus, future analysis will determine if only counting eGFP containing cells yields different results. There are two theories that may explain the unexpected

increase of ΔFosB. The first theory is that cells infected with mCREB may become dysregulated and affect other nearby medium spiny neurons (MSN) resulting in an elevation of ΔFosB. We believe these are MSN because this cell type comprises approximately 95 percent of the NAc (Meredith, 1999). Projection neurons from the NAc arborizing locally before projecting out of the accumbens (Meredith, Pennartz, & Groenewegen, 1993) and approximately 10 percent of MSN are interneurons (Meredith, 1999), thus supporting that if a dysregulation occurred after mCREB infusion it could affect nearby cells in the NAc. The second theory suggests that additional chromatin modifications at the fosB gene are being affected by mCREB and may result in an increased expression of ΔFosB (Vialou et al., 2012). Future studies will test the validity of these theories; until then, we conclude from these results that mCREB is necessary for ΔFosB after repeated ropinirole treatment. Therefore, phosphorylation of CREB may not be necessary for ΔFosB expression, however after repeated D_2-like agonist treatment CREB phosphorylation is necessary for ΔFosB and PPI recovery.

CREB and ΔFosB have been implicated in behavioral plasticity after chronic cocaine administration (Vialou et al., 2012). Both transcription factors regulate gene expression, however this regulation is dependent on length of drug exposure. CREB and serum response factor (SRF) are necessary for ΔFosB expression in the NAc after repeated cocaine exposure (Vialou et al., 2012). It should be noted these behavioral effects after cocaine exposure generally coincide with alteration in dynorphin expression thus suggesting these changes are occurring in D_1-like containing neurons in the NAc. However, PPI is regulated by D_2-like receptor containing neurons and PPI recovery is

produced by D_2-like receptor agonist treatment, suggesting D_2-like receptor containing neurons are preferentially involved in PPI recovery. No other study has examined the connection between phosphorylation of CREB and ΔFosB after repeated D_2-like agonist treatment. SRF may also be involved in ΔFosB expression after repeated D_2-like agonist treatment, however in the current study the blockage of CREB phosphorylation significantly decreased ropinirole-induced ΔFosB in the NAc, suggesting CREB phosphorylation is necessary for ΔFosB in the NAc after repeated D_2-like agonist treatment.

ΔFosB Function in the NAc is Necessary for Persistent PPI recovery

Previously, we observed PPI recovery 28 days after repeated D_2-like agonist treatment (Berger et al., 2011). Due to the long half-life and stability of ΔFosB, we hypothesized it was critical for persistent PPI recovery. Previously, repeated D_2-like agonist treatment increased ΔFosB in the NAc after repeated D2-like agonist treatment (Chapter 2); however, it was unknown if ΔFosB was required for long-lasting PPI recovery. In the current study, we determined ΔJunD overexpression blocked ΔFosB function and prevented PPI recovery. Overexpression of ΔJunD increased JunD expression in the NAc confirming the virus over expressed ΔJunD, which was detected with the JunD antibody.

ΔFosB has been associated long-term behavioral plasticity (Nestler et al., 2001). Within the NAc, long term ΔFosB expression leads to enhanced food and drug-reinforcement, and voluntary wheel running (McClung & Nestler, 2008). These believed mechanism for these long-lasting behavioral changes include ΔFosB regulation of gene

expression resulting in increased synaptogenesis (McClung & Nestler, 2008; Pitchers et al., 2013). Although these effects are believed to be occurring in NAc D_1-like receptor containing neurons, there is evidence of co-expression of different types of dopamine receptors that may be involved in PPI recovery after repeated D_2-like agonist treatment (Pou, Mannoury la Cour, Stoddart, Millan, & Milligan, 2012). Neurons containing D_2-like receptors in the NAc may be modified via ΔFosB expression after repeated D_2-like agonist treatments to cause PPI recovery. Further investigations will characterize these NAc cells that regulate PPI recovery

Repeated D_2-like receptor agonist treatment produces PPI recovery in rats (Berger et al., 2011; Culm & Hammer, 2004; Culm et al., 2004). To determine the underlying intracellular mechanisms, we determined that CREB phosphorylation was necessary for PPI recovery and repeated ropinirole-induced ΔFosB expression in the NAc. ΔFosB in the NAc was also necessary for PPI recovery after repeated D_2-like agonist treatment, suggesting it may be vital for the preservation of PPI recovery. Collectively, these findings are a causative connection between CREB phosphorylation and ΔFosB after repeated D_2-like receptor agonists, suggesting these factors are involved in an intracellular underlying mechanism regulating sensorimotor gating deficits. These findings elucidate vital factors that control gene regulation within a specific brain region that is involved in a clinically relevant behavioral deficit observed in patients with schizophrenia.

Chapter 4

REPEATED ARIPIPRAZOLE TREATMENT REVERSES QUINPIROLE-INDUCED PREPULSE INHIBITION DEFICITS AND INDUCES ΔFOSB IN THE RAT NUCLEUS ACCUMBENS

Abstract

Prepulse inhibition (PPI) of the startle response is an operational measurement of sensorimotor gating, which is disrupted in patients with schizophrenia. Dopamine D_2-receptor partial agonist aripiprazole alleviates positive, negative, and to some degree cognitive symptoms of schizophrenia. Our laboratory has shown that repeated treatment with D_2-like dopamine agonists, such as quinpirole, produces long-lasting recovery of PPI deficits and induces phosphorylation of cAMP response element-binding protein (CREB) and ΔFosB labeling in the rat nucleus accumbens (NAc). Although most antipsychotics act as D_2-receptor antagonists, we hypothesize that the D_2-receptor agonist efficacy of aripiprazole will produce persistent PPI recovery and induce ΔFosB labeling in the NAc. We examined the effect of acute aripiprazole treatment prior to a quinpirole challenge on PPI in male Sprague Dawley rats. Acute aripiprazole treatment significantly attenuated quinpirole-induced PPI deficits. We also examined the effect of repeated aripiprazole treatment for 28 days on PPI. Quinpirole challenge significantly reduced PPI prior to aripiprazole treatment, however quinpirole-induced PPI deficits were not observed 7 days after aripiprazole treatment. Immunohistochemical labeling and stereological analysis revealed a significant increase of ΔFosB expression in the striatum, especially NAc core, of aripiprazole treated rats as compared to vehicle-treated subjects. The reversal of PPI deficits by repeated treatment with quinpirole and aripiprazole

suggests that agonist properties of aripiprazole might underlie PPI recovery. Furthermore, the induction of ΔFosB in the striatum of aripiprazole treated rats indicates the presence of long-term intracellular activity resulting from repeated D_2-receptor agonist treatment.

Introduction

Sensorimotor gating, the regulation of transmission of sensory information to a motor system, is disrupted in patients with schizophrenia and manifests as sensory overload and cognitive fragmentation (Braff & Geyer, 1990). Prepulse inhibition of the acoustic startle response (PPI) is a quantifiable, valid, and reliable response that can be measured across species to detect sensorimotor gating deficits (Geyer et al., 2001). PPI is the reduction in startle response to an intense stimulus or "pulse" when preceded, up to 500 ms, by a weaker stimulus or "prepulse". This neurological phenomenon involves forebrain regulation of inhibition on the brain stem startle response. Specifically, the nucleus accumbens (NAc) is an integral brain area connecting forebrain and limbic structures that modulate PPI (Swerdlow et al., 1990).

Dopamine acting on D_2-like, D_2 and D_3, receptors in the NAc can disrupt PPI in rats (Wan & Swerdlow, 1993). For example, PPI disruptions were observed after acute systemic treatment (Martinez et al., 1999) or direct NAc infusion (Wan & Swerdlow, 1993) with a D_2-like receptor agonist. In contrast to acute treatment, repeated treatment with an indirect dopamine agonist resulted in an attenuated PPI disruption in rats (Byrnes & Hammer, 2000; Feifel et al., 2002). Repeated treatment with more specific D_2-like agonists, quinpirole or ropinirole, completely alleviate previous PPI deficits, termed a PPI recovery (Berger et al., 2011; Culm & Hammer, 2004). Furthermore, this recovery after

repeated treatment is long-lasting as rats continue to display PPI recovery 28 days after treatment (Berger et al., 2011).

The transcription factor ΔFosB is a truncated splice variant of FosB protein and with longer stability compared to c-Fos and FosB. It is implicated in long-term behavioral adaptation and cellular plasticity (Nestler et al., 2001). ΔFosB was significantly increased in the NAc of rats 10 days after repeated D_2-like agonist treatment (Chapter 2) and blocking the function of ΔFosB prevented PPI recovery (Chapter 2). Therefore, we believe that ΔFosB is involved in the preservation of PPI recovery after repeated D_2-like agonist treatment. This alleviation of a clinically relevant behavior and induction of a transcription factor association with behavioral plasticity suggests repeated D_2-like agonist action may be a therapeutically relevant pharmacological approach to treatment for patients with schizophrenia. In order to examine the clinical relevance of repeated D_2-like agonist action, behavioral and pharmacological results must be compared with an effective antipsychotic with a similar pharmacological profile.

Aripiprazole, an atypical antipsychotic, is a D_2-receptor partial agonist with moderate affinity for several serotonin receptors (Kikuchi et al., 1995). Typical and atypical antipsychotics are D_2-receptor antagonists and can cause side effects such as extrapyramidal symptoms (EPS). In clinical trials, aripiprazole significantly decreased reported side effects and patients with first episode psychosis remained on the drug longer as compared to older conventional antipsychotics, suggesting adequate treatment of positive symptoms (Crespo-Facorro et al., 2013). In early clinical trials aripiprazole was suggested to be more effective than older antipsychotics at treatment of cognitive and negative symptoms, however these trials had few participants and should be

interpreted cautiously (Fleischhacker, 2005; Sato, Yoshimura, Yamashita, Okamoto, & Yamawaki, 2012). Larger studies of patients with schizophrenia have found significant improvements in working memory (Kim et al., 2012) and verbal memory and fluency (Bervoets et al., 2012) after aripiprazole treatment. A Prospective, Multicenter, Open-Label Study to Evaluate the Effectiveness and the Effect on Cognitive Function of a Treatment with Aripiprazole in a Broad Range of Schizophrenia Patients (ESCAPE) concluded that switching to or initiating aripiprazole treatment resulted in improvement of verbal cognitive functioning (Bervoets et al., 2012). However, other studies have found only marginal improvements in cognitive functioning as compared to traditional antipsychotics (Yasui-Furukoir, Kaneda, Sugawara, Tomita, & Kaneko, 2012). In human and animal research, aripiprazole is efficient at decreasing EPS, however treatment of cognitive and negative symptoms of schizophrenia seem hopeful, yet not a cure.

The possible increased effectiveness and decreased side effects of aripiprazole may be due to the unique D_2-partial agonist function that results in 'dopamine stabilization' in the schizophrenic brain (Burris et al., 2002). This stabilization is due to the partial agonist's ability to exert an agonist or antagonist effect on dopamine D_2-receptor containing neurons, thus increasing dopamine effect in hypofunctioning regions and decreasing DA effect in hyperfunctioning regions (Kilts et al., 2002; Lawler et al., 1999). In animal models of schizophrenia, acute aripiprazole treatment attenuates apomorphine, a non-specific dopamine receptor agonist, induced PPI deficits, although the exact mechanism is unknown (Auclair, Kleven, Besnard, Depoortere, & Newman-Tancredi, 2006; Nordquist et al., 2008). The effect of acute aripiprazole treatment on D_2-like receptor agonist induced PPI deficits has never been examined, although,

aripiprazole is a D_2-receptor partial agonist. Furthermore, the effect of repeated aripiprazole treatment on D_2-like agonist-induced PPI deficits has never been examined and the underlying mechanism is unknown.

Unlike D_2 receptor antagonists, we hypothesize the D_2 receptor agonist efficacy of aripiprazole will alleviate PPI deficits after treatment. Furthermore, we believe aripiprazole's ability to have a D_2 agonist effect on neurons in the NAc will mimic our previous behavioral and intracellular results after repeated D_2-like agonist treatment. We predict acute and repeated aripiprazole treatment will alleviate quinpirole-induced PPI deficits. Furthermore, if repeated aripiprazole treatment produces PPI recovery after treatment, we predict NAc ΔFosB may contribute to the behavioral change.

Materials and Methods

Animals and Drug Treatment

All animals were housed under a 12:12 h reverse light-dark cycle and given *ad libitum* access to water and food. Rats were allowed to acclimate for 7 days to the laboratory before handling and habituation to the behavioral testing chambers. All experiments were approved by the Arizona State University and the University of Arizona Institutional Animal Care and Use Committees and were conducted in accordance with the *Guide for the care and Use of Laboratory Animals*.

Adult male Sprague-Dawley rats (Charles River Laboratories, Hollister, California) weighing 260-325 g were habituated to handling and subcutaneous (SC) saline injection and placed into a Startle Monitor behavior testing chamber (Kinder Scientific, Poway, California) with 70 dB ambient noise for 5 min daily for 2 days before baseline testing. Baseline PPI was assessed as described later starting 10 min after 0.9%

sterile saline vehicle injection (1.0 mL/kg, SC) on 2 consecutive days to ensure a reliable mean value. Treatment groups were normalized according to the mean PPI observed during the baseline testing.

Aripiprazole, obtained from National Institute of Mental Health's (NIMH) Chemical Synthesis and Drug Supply Program, was dissolved in a vehicle of saline and 0.3% Tween 80 and administered intraperitoneal (IP). Injections were use due to poor bio-availability of the drug after oral administration (Nordquist et al., 2008) and dosages of 1.0 and 10.0 mg/kg were based on previous findings of these dosages were successfully blocked apomorphine-induced hyperlocomotion and PPI deficits (Auclair et al., 2006; Nordquist et al., 2008). Quinpirole HCl (Sigma-Aldrich, St. Louis, Missouri) was dissolved in vehicle of 0.9% sterile saline and administered SC; dosage was based on previous finding of PPI recovery with this dosage (Berger et al., 2011). Vehicle controls were 0.9% sterile saline during quinpirole challenges or 0.9% sterile saline dissolved in 0.3% Tween 80 during repeated aripiprazole treatment. Injections took place in home cages.

Prepulse Inhibition Testing

All PPI testing was conducted during the dark phase (900 –1300 h). Each animal was placed in a PPI chamber and exposed to 70 dB ambient noise for 5 min, followed by a PPI baseline or test session. A PPI baseline session consisted of four consecutive pulse trials (120 dB, 40-msec pulses), a randomized presentation of 8 no stimulation, 16 pulse, and 15 prepulse (10 each of 73, 76, and 82 dB, 20 msec prepulses, followed 100 msec later by a pulse) trials, and ending with four pulse trials. A PPI test session consisted of four consecutive pulse trials (120 dB, 40-msec pulses), a randomized presentation of 10

no stimulation, 16 pulse, and 30 prepulse (10 each of 73, 76, and 82 dB, 20 msec prepulses, followed 100 msec later by a pulse) trials, and ending with four pulse trials. The intertrial interval averaged 15 sec (range: 8–22 sec). Percent PPI was calculated as: 100-[(mean prepulse response/mean pulse response) × 100].

Acute Aripiprazole Experiment

Following habituation and baseline normalization of groups, rats received a dose of aripiprazole (1.0 or 10.0 mg/kg, (IP) or saline vehicle (1.0 mL/kg)) followed by PPI testing. Five days later, rats received a dose of aripiprazole (1.0 or 10.0 mg/kg, IP) or saline vehicle (1.0 mL/kg) 30 min prior to a dose of quinpirole (0.1 mg/kg, SC) or saline vehicle (1.0 mL/kg) followed by PPI testing.

Repeated Aripiprazole Experiment

A separate cohort of rats than the acute aripiprazole experiment, following habituation and baseline normalization of groups, received a challenge dose of quinpirole (0.1 mg/kg, SC) or saline vehicle (1.0 mL/kg) followed by PPI testing. The following day, rats received daily dose of aripiprazole (1.0 or 10.0 mg/kg, IP) or saline vehicle (1.0 mL/kg) for 28 consecutive days. Seven days after repeated treatment, rats were given another challenge dose of quinpirole (0.1 mg/kg, SC) or saline vehicle (1.0 mL/kg) followed by PPI testing.

FosB/ΔFosB Immunohistochemistry

Seven days after the last drug challenge, rats were anesthetized and perfused with heparinized saline followed by 4% buffered paraformaldehyde. Brains were post-fixed in the same fixative for 2 h at 4° C then placed in graded sucrose solutions in phosphate buffered saline (PBS) overnight. Striatal brain sections (20 μm) were mounted on slides,

washed three times in 0.05 M potassium phosphate-buffered saline (KPBS, pH 7.4), and incubated in 5% normal goat serum/0.05 m KPBS/0.4% Triton X for 60 min at room temperature. Primary antibody raised against the N-terminal region of FosB (SC-48; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1: 10,000 in 5% normal goat serum/0.05 m KPBS/0.4% Triton X. This antibody recognizes 32–37 kDa proteins corresponding to the molecular weight of ΔFosB-like proteins and full-length FosB (Perrotti et al., 2004). Following incubation for 48 h at 4°C, sections were washed three times in 0.05M KPBS and processed using avidin–biotin–peroxidase (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA, USA). Sections were developed with DAB for 5 min, dehydrated and coverslipped at room temperature. Control procedures included preabsorption of the primary antibody using FosB peptide, and conducting these procedures in the absence of the primary antibody. There was no detectable labeling after either of the procedures (not shown).

Immunohistochemical Analysis

Tissue sections were examined for the presence of a dark grey reaction product. Labeled profiles were obtained from 3-4 sections from each rat. Selected areas of 400 μm^2 within the NAc core, shell, and caudate putamen were captured and digitized using a color digital video camera interfaced to a Zeiss Axioskop microscope with a 20X objective. Labeled profiles within the areas of interest were determined using StereoInvestigator (MicroBrightField Biosciences, Inc., Willston, VT, USA). A systematic random sampling approach using a stereological grid, consisting of 16 equal counting frames (100 X 100μm each) ensured randomization of identification within the area. A profile was considered labeled if its pixel luminance was more than two standard

deviations different from the background luminance as calculated by Stereo Investigator software. The average labeled profiles in an area were determined for each animal and these data were transformed to express the number of labeled profiles per mm^2.

Statistical Analysis

Acute aripiprazole PPI, startle response (120dB) and response in the presence of ambient noise alone, were analyzed using a one-way analysis of variance (ANOVA). Percent PPI (mean ± SEM) are mean values collapsed across three prepulse intensities. PPI responses within a single day were analyzed using a one-way ANOVA. PPI responses across multiple days were analyzed using two-way repeated-measures ANOVA with day of testing as within subject factor and drug treatment as between subject factor. ΔFosB labeled profiles were analyzed using two-way repeated measures ANOVA with brain area as within subject factor and drug treatment as between subject factor. Post-hoc comparisons were made using Fisher's least squares difference (LSD). All data are presented as mean ± SEM and PPI responses are presented collapsed across prepulses 3, 6, and 12 dB above ambient noise level (70dB).

Results

Startle Response and PPI After Acute Aripiprazole Treatment

This experiment demonstrates that aripiprazole treatment does not affect startle response or PPI. There was no significant difference in startle response (120dB) after acute aripiprazole (1.0 or 10.0 mg/kg) treatment (Figure 13). There was also no significant difference in PPI response after acute aripiprazole (1.0 or 10.0 mg/kg) treatment (Figure 14).

Acute Aripiprazole Treatment Prior to Attenuates Quinpirole-Induced PPI Deficits

This experiment demonstrates that acute treatment with aripiprazole can attenuate quinpirole induced PPI deficits**.** There was a significant PPI disruption by acute quinpirole challenge. However, acute aripiprazole (1.0 or 10.0 mg/kg) treatment 30 min prior to quinpirole challenge attenuated quinpirole-induced deficits as compared to control rats. Only one dose of acute aripiprazole treatment (10.0 mg/kg) completely alleviated the quinpirole-induced deficit as compared to control rats (Figure 15). Neither mean acoustic startle (120dB) response nor response in the presence of ambient noise alone were altered by acute aripiprazole or quinpirole challenge.

Repeated Aripiprazole Treatment Alleviates Quinpirole-Induced PPI Deficits

This experiment demonstrated that repeated aripiprazole treatment was able to attenuate quinpirole-induced PPI deficits. A significant interaction was detected between day of quinpirole challenge and drug treatment; this suggests the effect of drug treatment is dependent on the day of quinpirole challenge. Further investigation revealed initial quinpirole challenge, prior to aripiprazole treatment, significantly reduced PPI as compared to control rats. Quinpirole significantly disrupted PPI in rats after repeated vehicle treatment; however, repeated aripiprazole (1.0 or 10.0 mg/kg) treatments 7 days prior to quinpirole challenge significantly attenuated quinpirole-induced PPI deficits treatment (Figure 16). There was no significant effect of aripiprazole treatment on PPI (Day 7, 14, 21, 28) during repeated treatment (Figure 17). There was a significant effect of drug treatment on response in the presence of ambient noise alone, specifically repeated saline animals moved more during no stimulation trials, however there was no significant effect of drug on startle (120dB) response on Day 35 of PPI testing.

ΔFosB Expression in the Striatum Following Repeated Aripiprazole

Modified stereological analysis revealed the higher dose of aripiprazole (10.0 mg/kg) increased ΔFosB in the striatum 10 days after repeated treatment as compared to repeated vehicle treatment (Figure 18). Specifically, ΔFosB labeling in animals after repeated aripiprazole (10.0 mg/kg) treatment increased 215% in the NAc core and 140% in the NAc shell as compared to repeated vehicle treatment (Figure 19). The lower dose of aripiprazole (1.0 mg/kg) failed to increase ΔFosB expression in any region in the striatum as compared to vehicle treatment**.** Furthermore, overall ΔFosB expression was increased in the NAc as compared to the CPu and the highest amount of ΔFosB was in the NAc core as compared to other sub-regions examined within the striatum.

Discussion

Acute Aripiprazole Prevents Quinpirole-Induced PPI Disruption

Acute administration of selective D_2-like receptor agonists reduces PPI, an indication of sensorimotor gating deficits (Culm & Hammer, 2004; Swerdlow, Taaid, et al., 1998). In the current study, acute aripiprazole treatment attenuated these deficits in a dose response manner and one dose, 10.0 mg/kg, restored quinpirole-induced PPI deficits to the response of vehicle treated animals. The ability of acute aripiprazole to attenuate PPI deficits has been observed after apomorphine-induced PPI deficits (Auclair et al., 2006; Nordquist et al., 2008); however in this study, aripiprazole was not able to restore PPI to the response of vehicle treated animals. The ability of acute aripiprazole (10.0 mg/kg) to completely alleviate D_2-receptor agonist-induced deficits and attenuate non-specific dopamine receptor agonist-induced deficits suggests aripiprazole may be more effective at targeting the D_2-like receptor and preventing PPI deficits through neurons

containing these receptors. Acute aripiprazole did not induce any increased movement or startle response in rats, possibly reflecting its ability to induce fewer side effects in humans (Fleischhacker, 2005).

Recovery of Sensorimotor Gating Deficits Following Repeated Aripiprazole

In the current study, repeated aripiprazole (1.0 or 10.0 mg/kg) treatment attenuated D_2-like agonist induced PPI deficits, which was also observed after acute treatment. Furthermore, the quinpirole challenge was 7 days after repeated treatment; therefore, aripiprazole was no longer in the system. This finding suggests aripiprazole may stimulate similar mechanisms as repeated D_2-like agonist treatment resulting in a persistent change of a clinically relevant behavior.

Repeated aripiprazole failed to significantly disrupt startle response, however there was a decrease in activity during no stimulus trials among all non-control groups. This measurement is not a component of the percentage of PPI; therefore it does not directly affect the results. The doses used in this study have not been found to produce motor problems (Nordquist et al., 2008) and there was no significant affect detected in startle response, which would detect a lack of movement.

The alleviation of PPI deficits following repeated treatment with quinpirole or aripiprazole suggests that D_2-like receptor agonist properties of aripiprazole might underlie PPI recovery. We believe this because quinpirole challenge dose 7 days after repeated treatment failed to disrupt PPI (Berger et al., 2011). Similar behavioral results after both drugs allow us to reason that the common D_2-like agonist pharmacological action is involved in long-lasting behavioral change in the absence of treatment.

D_2 partial agonists are different than D_2-like agonists as evidenced after the acute administration of the drug. Acute D_2-like agonist treatment produced PPI deficits, whereas D_2-like partial agonist prevented quinpirole-induced deficits. Acute aripiprazole is able to block quinpirole induced disruption because it has a high affinity for the D_2-like receptor (Nordquist et al., 2008); therefore, it has an antagonizing action at the receptor by blocking quinpirole and having less efficacy within the cell resulting in a normal PPI response. Therefore, the acute action is very different between D_2-like agonist and D_2 partial agonist. Interestingly, when given repeatedly, repeated quinpirole and aripiprazole produce the same behavioral effects, possibly by exerting action on the same underlying intracellular mechanism involved in preserving a behavioral change.

ΔFosB in the Striatum Following Repeated Aripiprazole

To determine if repeated aripiprazole treatment is modifying the same intracellular mechanism affected after repeated D_2-like agonist treatment, we investigated the expression of ΔFosB after repeated D_2-like agonist treatment. In the present study, we observed ΔFosB expression in the striatum of rats after repeated aripiprazole treatment (10.0 mg/kg). ΔFosB expression was not observed in the lower aripiprazole (1.0 mg/kg) dose and suggests the partial agonist property of the drug may require a larger dose for ΔFosB expression as compared to lower doses of quinpirole (0.1 mg/kg) (Chapter 2). However, the lower dose of aripiprazole was able to attenuate PPI deficits. This suggests ΔFosB was expressed, but possibly at lower levels than observed with repeated D_2-like agonist treatment or our procedure for detecting ΔFosB expression is not sensitive enough to detect a slight induction.

Repeated typical antipsychotic treatments also increase ΔFosB in the striatum; however, dense labeling was found throughout the striatum especially in the dorsal striatum (Rodriguez et al., 2001). Atypical antipsychotic treatment failed to increase ΔFosB expression as compared to typical antipsychotics and labeling was slightly more ventral than dorsal (Rodriguez et al., 2001). From these findings, it was suggested that dense labeling of ΔFosB in the dorsal striatum after repeated typical antipsychotic treatment induced tardive dyskinesia-like behaviors in rodents. In the current study, ΔFosB expression was not increased in the dorsal striatum, but was increased in the ventral striatum. This suggests repeated aripiprazole treatment is affecting the NAc as compared to the dorsal striatum and is supported by the lack of disruption in startle response, activity during ambient noise, or PPI after acute aripiprazole. Therefore, we believe the novel antipsychotic pharmacological profile of aripiprazole it is more effective at targeting brain regions implicated in treatment, such as the NAc, and less effective at targeting regions that may induce side effects, such as the dorsal striatum in rats.

ΔFosB expression has been associated with synaptic plasticity in NAc neurons after repeated stimuli (Grueter, Robison, Neve, Nestler, & Malenka, 2013). This synaptic plasticity may be stimulated after repeated D_2-like agonist treatment and aripiprazole treatment and contribute to long-lasting changes that prevent pharmacological disruptions, such as quinpirole. The similar intracellular findings of ΔFosB expression in both aripiprazole and repeated D_2-like agonist treatment suggests repeated D_2-like agonist treatment is activating a mechanism we observe to alleviate PPI deficits, however it may also be involved in other symptoms of schizophrenia due to the success of aripiprazole at

treating some cognitive symptoms (Sato et al., 2012). Future experiments will test the involvement of this underlying intracellular mechanism in regulating cognitive deficits, however these similarities suggest factors that contribute to long lasting behavioral change after repeated D_2-like agonist treatment and aripiprazole may be of potential treatment interests.

The main finding of the current study was that repeated aripiprazole treatment attenuated quinpirole-induced PPI deficits and increased ΔFosB labeling in the striatum especially in the NAc core. Acute aripiprazole treatment also attenuated quinpirole-induced deficits and acute or repeated aripiprazole treatment had no effect on startle (120dB) response or PPI. These findings combined with previous findings of PPI recovery after repeated D_2-like agonist treatment (Berger et al., 2011; Culm & Hammer, 2004; Culm et al., 2004) suggests the D_2 receptor agonist properties of aripiprazole may underlie sensorimotor gating recovery, a clinically relevant behavior. Furthermore, ΔFosB expression in the striatum of aripiprazole treated rats indicates the presence of long-term intracellular activity resulting from an antipsychotic and repeated D_2-receptor agonist treatment. These behavioral and intracellular similarities between an effective antipsychotic and repeated D_2-like agonist treatment suggests D_2-like receptor agonist action and factors involved in persistent behavioral change may of clinical interest for treatment of cognitive deficits of schizophrenia.

Chapter 5

SUMMARY AND DISCUSSION

Summary of Experiments

Repeated D_2-like agonist treatment alleviates prior prepulse (PPI) deficits in rats, termed a PPI recovery, and is observable 28 days after treatment (Berger et al., 2011; Culm & Hammer, 2004; Culm et al., 2004). The aim of the current project was to illuminate the underlying mechanism for long lasting PPI recovery and determine the clinical relevance of repeated D_2-like agonist treatment. Our results revealed a significant increase in ΔFosB in the nucleus (NAc) 10 days after repeated D_2-like agonist treatment. Additionally, we investigated if ΔFosB was necessary for long-lasting PPI recovery and discovered that a bilateral infusion of dominant-negative ΔJunD (ΔFosB antagonist) prevented PPI recovery after repeated D_2-like agonist treatment. To further develop the underlying mechanism of PPI recovery, we observed that dominant negative mutant cyclic adenosine monophosphate (cAMP) response element binding (CREB) prevented repeated D_2-like agonist-induced ΔFosB expression in the NAc. We then compared our previous behavioral and intracellular findings to the results of repeated aripiprazole, a novel D_2-like partial agonist antipsychotic, to determine if repeated D_2-like receptor agonist action is a clinically relevant pharmacological approach. As compared to previous PPI recovery and ΔFosB expression after repeated D_2-like agonist treatment, we found similar PPI recovery and ΔFosB expression after repeated aripiprazole treatment in rats. We can conclude a great deal from these results, therefore critical examination and appropriate interpretations are necessary to determine their contribution to already established and future schizophrenia research.

Discussion

Underlying Intracellular Mechanism of PPI Recovery after Repeated D_2-like Agonist Treatment

Results of the current experiments demonstrated that ΔFosB in the NAc was required for persistent PPI recovery after repeated D_2-like agonist treatment. We also demonstrated ΔFosB induction was mediated by CREB phosphorylation. These findings supplement previous findings of increased PKA activity and phosphorylation of CREB after repeated dopamine (DA) D_2-like agonist treatment (Berger et al., 2011; Culm et al., 2004). Together, our results detail an intracellular process regulating PPI recovery.

We believe this intracellular cascade begins with D_2-like receptor activation and repeated stimulation does not modify the receptor function (Culm et al., 2004); however, the subsequent intracellular components are altered over time. As previously discussed, D_2-like receptors are G*i* coupled protein receptors, in which stimulation of these receptors inhibits adenylate cylase. In contrast, repeated D_2-like receptor activation results in an increase of cAMP through sensitization of adenylate cylcase (Watts & Neve, 2005). Increases of cAMP initiates a signal transduction cascade including protein kinase A (PKA) and CREB (McClung & Nestler, 2008), We therefore believe activation of the cAMP pathway promotes the observed phosphorylation of CREB and increased ΔFosB. We believe CREB phosphorylation precedes ΔFosB expression because previous examinations observed CREB phosphorylation only immediately after repeated D_2-like agonist treatment, while ΔFosB expression is observed 10 days after treatment (Berger et al., 2011). Increased gene expression after acute DA stimulation is more dependent on CREB-dependent whereas increased gene expression following repeated DA stimulation

is ΔFosB-dependent (McClung & Nestler, 2003). These results can be explained as CREB being constitutively expressed in neurons as compared to ΔFosB, which requires time to accumulate after repeated stimulations (Nestler, 2001).

Additional support of a relationship between CREB and ΔFosB in the NAc is derived from experiments examining chronic cocaine administration or stress exposure. After chronic cocaine administration, CREB and serum response factor (SRF) are required for ΔFosB induction, whereas only SRF is required after chronic stress in resilient mice (Vialou et al., 2012). These results do not negate the current findings, but present a theory that suggests distinct transcriptional mechanisms underlie ΔFosB induction after different repeated stimulus. There are currently no studies examining the role of SRF after repeated D_2-like agonist or antagonist treatment; therefore, it is unknown if SRF contributes to ΔFosB after D_2-like agonist treatment. Regardless, there are alternative explanations why CREB phosphorylation is required for ΔFosB induction after repeated D_2-like agonist treatment and not for chronic cocaine or stress.

Repeated D_2-like agonist treatment may target different cells and intracellular pathways than chronic cocaine or stress. ΔFosB expression is found in D_1 containing neurons after chronic cocaine and D_1 and D_2 containing neurons after chronic stress (Kelz et al., 1999; Perrotti et al., 2004). We do not believe repeated D_2-like agonist treatment is affecting D_1 containing neurons because NAc D_1 receptor-mediated direct pathway does not regulate PPI behavior as evidenced by direct infusion of D_1 agonist into the NAc failing to affect PPI (Wan & Swerdlow, 1993). It is unknown what cells are targeted by repeated D_2-like agonist treatment. SRF may be required for ΔFosB for in D_1 containing

neurons; however, SRF may not have the same function in D_2-like receptor containing neurons regulating PPI recovery. Also, repeated D_2-like agonist treatments selectively target D_2-like receptors as compared to cocaine and stress, which indirectly increase overall DA levels in the NAc (Abercrombie, Keefe, DiFrischia, & Zigmond, 1989; Hernandez & Hoebel, 1988). Therefore, repeated selective D_2-like receptor activation might selectively increase CREB phosphorylation and ΔFosB expression as compared to indirect DA targeting multiple receptors and signaling cascades resulting in other transcription factors, such as SRF, being critical for ΔFosB induction. Also, these different repeated stimuli might activate different chromatin modifications, which influence the production of ΔFosB after SRF, CREB or other factors (Vialou et al., 2012).

After repeated D_2-like agonist treatment, we believe that ΔFosB induction in the NAc is required for persistent PPI recovery, observable 28 days after treatment. In the current studies, we found ΔFosB expression 10 days after treatment and blocking ΔFosB activity in the NAc prevented PPI recovery. This finding further elucidates the role of ΔFosB in long-term behavioral change after repeated D_2-like agonist treatment, in addition to its already established role in chronic stress, drugs of abuse, or natural rewards.

In terms of drug addiction, virtually every drug of abuse administered repeatedly induces a long-lasting accumulation of ΔFosB in the NAc (Nestler, 2008). ΔFosB increases the rewarding properties of drugs of abuse and may cause the propensity to relapse as evidenced by increased sensitization and drug seeking after a period of abstinence in bitransgenic mice specifically overexpressing ΔFosB in NAc dynorphin

containing neurons (Nestler, 2008). Repeated stress also increases ΔFosB in dynorphin and encephalin containing neurons in the NAc and may represent a positive coping mechanism as evidenced by ΔFosB overexpression exerting lasting antidepressant-like responses (Perrotti et al., 2004). ΔFosB induction after repeated stimulation, such as stress or drugs of abuse, can result in the preservation of a behavioral change. This supports the conclusion from the current experiments that ΔFosB is necessary for persistent PPI recovery.

The mechanism underlying the ability of ΔFosB to produce long lasting behavioral effects is still being investigated, however it has been determined that this mechanism involves an increase in dendritic spines and silent synapses, which create an optimal condition for long-term potentiation (LTP) (Grueter et al., 2013). These changes in dendritic morphology and possible function are believed to be occurring in D_1 containing neurons and affecting the NAc direct pathway. Overexpression of ΔFosB in dynorphin containing neurons in mice produced synaptogenesis and silent synapses, which was not observed in enkephalin containing neurons (Grueter et al., 2013). Also, blocking these changes blocked preference for cocaine, which suggests these adaptations are occurring in the reward pathway. We do not believe repeated D_2-like agonist treatments are targeting the same cells involved in the NAc D_1 direct pathway; however, synaptogenesis after ΔFosB induction could still be a possible component of long lasting PPI recovery. There are two reasons ΔFosB induced plasticity in the NAc may be involved in persistent PPI recovery although we do not believe we are targeting the same neurons as chronic cocaine.

First, PPI is differentially regulated in mice versus rats (Ralph-Williams, Lehmann-Masten, Otero-Corchon, Low, & Geyer, 2002). In mice, neurons containing D_1 receptors are believed to be more involved in PPI regulation than D_2 containing neurons, which is opposite in the rat (Ralph-Williams, Lehmann-Masten, & Geyer, 2003). In the previous study using mice, cells containing D_1 receptors that are involved in PPI recovery may be displaying changes in dendritic morphology after ΔFosB overexpression. Therefore, these same neurons in rats, which contain D_2-like receptors, have to potential to display changes in dendritic morphology after ΔFosB induction, as in PPI recovery. Increased dendritic morphology has been found in rats after an increase of ΔFosB in the NAc, however the cell types have not been characterized (Pitchers et al., 2013). Second, the characterization of D_1 or D_2 containing neurons based on dynorphin and enkephalin expression does not correctly characterize all cells in the NAc. In the NAc, dynorphin and D_1 are co-expressed and enkephalin and D_2 are co-expressed. However, a subset of neurons co-expresses dynorphin, D_1, and D_3 receptors, but not enkephalin (Curran and Watson, 1995). Therefore, studies using transgenic mice that overexpress ΔFosB in dynorphin-containing neurons may be overexpressing ΔFosB in cells that also express D_2-like receptors. Furthermore, we have evidence this subset of neurons in the NAc that co-express dynorphin, D_1, and D_3 receptors may be involved in maintaining PPI recovery.

NAc Neurons Involved in Preserving PPI Recovery

Repeated D_2-like agonist treatment increases PKA activity, CREB phosphorylation, and ΔFosB selectively in the ventral striatum (Berger et al., 2011; Culm et al., 2004). D_1 and D_2 containing neurons are located throughout the striatum including the dorsal and ventral regions, however D_3 containing neurons are mainly located in the

ventral striatum (Curran & Watson, 1995). Quinpirole and ropinirole have both been used to generate PPI recovery in rats and have high affinity for both D_2 and D_3 receptors, with slightly higher affinity for the D_3 receptor (Kvernmo, Hartter, & Burger, 2006; Levant, Grigoriadis, & DeSouza, 1992). Therefore, this suggests that the intracellular changes after repeated D_2-like agonist treatment are possibly stimulating D_3 receptors that are co-expressed on D_1 containing neurons. As mentioned previously, a small population of neurons in the NAc expresses D_1, D_3 and dynorphin, but not enkephalin (Curran & Watson, 1995). Also, there is growing evidence that D_1 /D_3 receptors assemble into functional heterodimers expressing binding and coupling profiles different from their respective monomer profile (Maggio, Aloisi, Silvano, Rossi, & Millan, 2009). In an attempt to characterize neurons modulating PPI recovery after repeated D_2-like agonist treatment, we labeled tissue from rats after repeated D_2-like agonist treatment for ΔFosB and enkephalin (Figure 20) and distinguished regions expressing enkephalin from ΔFosB labeled cells. Due to the difficulty of accurately characterizing dopamine cells based on receptor expression, there is limited research on neurons expressing D_3 receptors cellular function. However, we hypothesize they are vital to the regulation of PPI recovery after repeated D_2-like agonist treatment.

In order to validate the hypothesis that a unique population of neurons in the NAc regulates PPI recovery, several follow-up studies are needed. First, cells expressing ΔFosB after repeated D_2-like agonist treatment would need to be characterized. One way to characterize these cells is by *in situ* hybridization for D_3 receptor mRNA. In order to conclude D_3 containing cells are involved in PPI recovery, the same cell would need to contain D_3 mRNA and express ΔFosB after repeated D_2-like agonist treatment. Therefore,

in situ hybridization would be combined with immunohistochemistry to make this detection. Characterization of neurons affected after repeated D_2-like agonist treatment may also be possible in mice with transgenic eGFP expression in D_1 or D_2 containing neurons, however currently it is unknown if repeated D_2-like agonist treatment has the same effect in mice. If co-labeling were detected, the next step would be to examine function of these cells in PPI recovery. This would be very difficult in the rat; however, viral vectors may provide the necessary selectivity for targeting D_3 containing neurons and expressing a dominant negative for ΔFosB that would block its function. This would test if ΔFosB in D_3 containing neurons is necessary for PPI recovery. If this was concluded, it would suggest that neurons co-expressing D_1 and D_3 receptors might be involved in PPI recovery.

Future studies are also needed to characterize the progression of events between ΔFosB expression and PPI recovery. Experiments examining the presence of dendrite morphology and silent synapses after repeated D_2-like agonist treatment would determine if cellular structure is altered after repeated treatment. Silent synapses can be detected by the increased presence of AMPA receptors as compared to NMDA receptors in a given area. These amounts can be detected by immunohistochemistry, in situ hybridization, or the electrophysiological behavior of the receptor. Furthermore, electrophysiology studies would be beneficial to determine if cells that regulate PPI recovery function differently after repeated treatments and if this contributes to PPI recovery. For example, the detection of excitatory postsynaptic potential (EPSP) or inhibitory postsynaptic potential (IPSP) would provide information on the function of the neuron. If repeated D_2-like agonist treatment strengthens synaptic connections in the nucleus accumbens indirect

pathway, it would suggest that synaptic plasticity is possible in neurons containing D_2-like receptors and as a result the indirect pathway regulating PPI recovery may be more efficient. Until this hypothesis is validated, we can speculate on how the intracellular mechanisms observed in the current studies affect PPI recovery through the PPI brain circuitry.

Integration of the Underlying Mechanism of PPI Recovery into the PPI Circuit

The intracellular changes observed in the NAc after repeated D_2-like agonist treatment may strengthen synaptic connections from excitatory projections, such as PFC, amygdala, or hippocampus; thereby increasing gamma-Aminobutyric acid (GABA)ergic activity in the VP via projections from the NAc to the VP (Figure 21). Loss of DA function in the NAc following direct infusion of D_2-like agonist results in PPI deficits (Wan & Swerdlow, 1993). This loss of DA function in the NAc results in decreased GABA activity in the VP, which also disrupts PPI, as observed after direct infusion of GABA antagonist into the VP (Swerdlow et al., 1990). These projection influences this PPI circuitry by exerting a negative modulation of downstream targets by inhibiting the outgoing startle response in PPI or closing the "gate". When this pathway is disrupted, the lack of inhibition results in increased startle responses and a PPI deficit. Repeated D_2-like treatments might enhance projections from the NAc to the VP, creating long-lasting modifications in the pathway that becomes resilient to future pharmacological disruptions. This long-lasting resilience is demonstrated by the inability of quinpirole to produce PPI deficits 29 days after repeated D_2-like agonist or aripiprazole treatment (Berger et al., 2011).

Possible Clinical Relevance of Repeated D_2-like Receptor Agonist Action

In the current studies we investigated the underlying intracellular mechanism of PPI recovery following repeated D_2-like agonist treatment. We speculated on how this intracellular mechanism may produce long lasting modification to brain circuitry regulating PPI. PPI is a reliable and valid measurement of sensorimotor gating (Markou & Geyer, 1995). Patients with schizophrenia exhibit sensorimotor gating deficits, which are associated with cognitive symptoms of the disorder (Swerdlow et al., 1994). Therefore, these findings are related to schizophrenia and the underlying mechanism of PPI recovery may be of clinical relevance. In the current studies, we examined the clinical effectiveness of repeated D_2-like receptor agonist function by comparing our previous results to an antipsychotic with a similar pharmacological profile, specifically the D_2-like partial agonist aripiprazole. Aripiprazole alleviated prior PPI deficits 10 days after repeated treatment and induced ΔFosB expression in the NAc, similar to repeated D_2-like agonist treatment. We concluded that repeated D_2-like agonist activation might be of potential therapeutic interest. However, it is important to examine how repeated D_2-like agonist treatment compares to antipsychotic drugs, which are D_2 antagonists.

In rats, PPI is a used as a test of clinical efficacy of antipsychotics because of the strong correlation between the ability of an antipsychotic to alleviate DA induced PPI deficits and later clinical efficacy (Swerdlow et al., 1994). These tests are usually after acute antipsychotic administration and do not examine PPI alleviation over repeated treatments, therefore we cannot make direct comparisons between our model and antipsychotics other than aripiprazole. One experiment did examine the effect of repeated antipsychotic treatment on phencyclidine (PCP) induced PPI deficits over 6 days and

found atypical antipsychotics were more successful at continuing alleviation as compared to typical antipsychotics (Li et al., 2011). Repeated typical antipsychotic administration via oral administration significantly increased ΔFosB throughout the striatum as compared to limited labeling following repeated atypical antipsychotic administration (Rodriguez et al., 2001). The authors concluded that increased ΔFosB expression in the dorsal striatum was responsible for antipsychotic side effects because only typical antipsychotic treated animals displayed vacuous chewing movements, the animal equivalent of tardive dyskinesia in humans. From the limited research on repeated antipsychotic treatment in rats, it seems repeated D_2-like agonist treatment produces behaviors similar to behavioral results after atypical antipsychotics. Although typical antipsychotic treatment and repeated D_2-like agonist treatment both increased ΔFosB labeling in the striatum, we only observed ΔFosB in the ventral striatum and did not observe any tardive dyskinesia-like behaviors. The similarity between repeated D_2-like agonist treatment and atypical antipsychotics might reflect the ability of atypical antipsychotics to some extent treat cognitive deficits as compared to typical antipsychotics (Stahl, 2007).

Although clinical effectiveness of antipsychotic drugs can be determined by their ability to alleviate DA induced PPI deficits in rats (Swerdlow et al., 1994), the ability of antipsychotics to alleviate sensorimotor gating deficits in patients with schizophrenia is unclear. Drug naïve patients consistently display PPI deficits, however there is little evidence of typical antipsychotics alleviating PPI deficits as compared to some findings with atypical antipsychotics (Braff et al., 2001). Interestingly, aripiprazole did not alleviate PPI deficits in schizophrenic patients, however it attenuated the deficits

significantly more than typical or atypical antipsychotics (Kishi et al., 2010). The alleviation of PPI deficits in rodents does not directly translate to humans because DA and PCP are used to induce rodent deficits and it is unknown what induces PPI deficits in humans. However these findings suggest that aripiprazole may be closer to alleviating sensorimotor gating and possibly cognitive deficits in humans.

Atypical antipsychotics are more effective at treatment of negative and cognitive symptoms of schizophrenia compared to typical antipsychotics, but still produce side effects. National Institute of Mental Health (NIMH) Clinical Antipsychotic Trails of Intervention Effectiveness (CATIE) study found that side effects were the most reported reason for discontinuing medication. During this study, 3/4 of patients stopped medication before 18 months (Lieberman et al., 2005). This finding suggests antipsychotics need to be improved and possibly a new pharmacological approach is needed to avoid the unwanted side effects and compliance issues.

Aripiprazole's unique pharmacological profile as a D_2-partial agonist is a novel pharmacological approach as compared to D_2 antagonist. Clinical trials suggest the drug produces significantly less side effects as compared to typical and atypical antipsychotics (Hirose et al., 2004). In the current studies, we believe acute aripiprazole is creating an antagonist-like effect at the D_2-receptor by binding and blocking quinpirole, however after repeated treatment we believe it has an agonist effect at the receptor because it prevents PPI disruption, mimicking PPI recovery after repeated D_2-like agonist treatment. Due to the similar PPI behavior and ΔFosB labeling after a clinically effective antipsychotic and repeated D_2-like agonist treatment, we can extrapolate that repeated D_2-like agonist treatment might be a possible therapeutic approach.

An alternative conclusion to repeated D_2-like receptor activation stimulating an alleviation of sensorimotor gating deficits, could be that repeated D_2-like receptor treatment may be inducing an anxiolytic effect in rats that indirectly alleviates sensorimotor gating deficits. This conclusion would also be supported by our findings of repeated aripiprazole treatment also alleviating sensorimotor gating deficits because aripiprazole is also prescribed as an adjunctive therapy in major depressive disorder in humans (Spielmans, et al., 2013). In the previous studies, no assessment of stress was conducted, however rats were habituated to handling and injections to control for this confounding variable. Sensorimotor gating deficits are found in disorders other than schizophrenia, however it is not generally found in patients with generalized anxiety or depressive disorders (Kohl, Heekeren, Klosterkotter, & Kuhn, 2013); therefore PPI would not be the ideal test to detect anxiety or depressive behaviors in rodents. It should be noted that stress paradigms, such as maternal separation, can cause PPI deficits in rodents, however these models are also used to model sensorimotor gating deficits and other behaviors related to symptoms of schizophrenia (Ellenbroek, van den Kroonenberg, & Cools, 1998). Also, if repeated D_2-like receptor agonist treatment was producing an anxiolytic effect, we would believe that rats after treatment would perform better than controls, which is not the case with repeated D_2-like receptor agonist or aripiprazole treatment. The definitive method for determining if repeated D_2-like agonist treatment is having an anxiolytic effect would be to obtain corticosterone levels from rats during the experiment, however due to PPI not detecting depressant or anxiety like behaviors and our treatment groups not performing better than controls, we believe at this time repeated

D_2-like agonist treatment alleviated sensorimotor gating deficits due to its effects associated with the cognitive deficits of schizophrenia.

Although repeated D_2-like receptor activation seems like an attractive therapeutic option, dosages of such treatment should always be considered. Comparable doses of repeated D_2-like agonist treatment used in these studies in humans would not be an effective treatment for schizophrenia. Patients with schizophrenia have sensorimotor gating deficits and assumed dysregulation of neurotransmitter systems, as compared to rats that display normal sensorimotor gating before treatment. Acute D_2-like agonist treatment induces deficits before PPI recovery; therefore, giving an acute treatment to attain repeated treatments and intracellular activation would theoretically exacerbate the current deficits. Initial low doses that escalate over time, may overcome the acute D_2-like agonist exacerbation, where eventual stimulation of the underlying intracellular mechanism would result in alleviation. This is similar the partial agonist action of aripiprazole that has less intracellular effects than D_2 agonist or antagonist, however when given repeatedly it can cause intracellular change, as observed in the current studies. Although repeated D_2-like agonist treatment is not a viable treatment for schizophrenia, components of the underlying intracellular pathway, which regulates PPI recovery, might lead to a potential therapeutic target. The role of ΔFosB in synaptic plasticity makes it an attractive target; however, an intervention to increase expression would have to be cell-and region-specific because ΔFosB can enhance undesirable behaviors. For example, increased expression of ΔFosB in the reward pathway in the NAc could increase drug reward and would not be beneficial for schizophrenic patients (Nestler, 2008). Therapeutically targeting transcription factors in specific neuron

populations is currently not possible in humans; however, scientific advancements in research capabilities, such as the use of specialized viral vectors in animals, suggest this hypothetical therapy may be a future possibility.

The goal of the current studies was to elucidate the underlying intracellular mechanism for persistent PPI recovery after repeated D_2-like agonist treatment and determine if it is a therapeutically relevant pharmacological approach. We determined ΔFosB induction after CREB phosphorylation was necessary for long lasting PPI recovery. Additionally, similar behavioral and intracellular results after aripiprazole treatments suggest repeated D_2-like agonist treatment is a clinically relevant pharmacological approach.

Final Conclusion

These findings are important because they propose an alternative approach to understanding and treating schizophrenia. This work underscores the importance of testing pre-conceived theories a drug's effect on behavior. For instance, D_2-like agonist treatment was expected to exacerbate PPI deficits, but repeated treatments unexpectedly alleviated PPI deficits and uncovered a potential novel therapeutic mechanism for long-lasting PPI recovery. From these results, we believe neurons in the NAc projecting to the VP may have dynamic capabilities for neural plasticity and may influence cognitive functioning. Therefore, further characterization of these neurons may provide valuable insight into the causes of cognitive deficits in patients with schizophrenia. In the future, components of this underlying mechanism could be targeted to alleviate sensorimotor gating deficits, which are not alleviated by current antipsychotic drugs. Continued research into additional factors that sustain this clinically relevant behavior could be used

to develop treatments that would produce long lasting behavioral changes after treatment in the absence of drugs, which would help address noncompliance issues. This work will hopefully aid in the development of effective treatment options that produce fewer side effects in order to give patients with schizophrenia the opportunity to live longer lives with alleviation of their devastating symptoms.

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Table 1

Study 1: Drug Effect on PPI Control Measures

Repeated Drug Treatment	Startle (120dB pulse) (N)	No Stimulus (N)
Saline	0.828 ± 0.574	0.157 ± 0.085
Quinpirole	0.522 ± 0.510	0.106 ± 0.091

Note. $p > 0.05$, no significant increase in startle and no stimulus trials.

Table 2

Study 2: Ropinirole and mCREB PPI Startle Response

	eGFP		mCREB	
	Day 1	Day 28	Day 1	Day 28
Saline	0.61 ± 0.16	0.75 ± 0.18*	0.35 ± 0.06	0.61 ± 0.10*
Ropinirole	0.44 ± 0.15	0.71 ±0.22*	0.76 ± 0.23	0.93 ± 0.31*

Note. Data are expressed in Newtons (N) as mean ± SEM. There was no significant effect of ropinirole [$F(1,27) = 0.487$, $p > 0.05$] or mCREB [$F(1,27) = 0.032$, $p > 0.05$] on pulse startle response, however there was an effect over time [$F(1,27) = 27.78$, $p < 0.001$] using repeated measures ANOVA, treatment groups increased startle response on Day 28 as compared to Day 1 ($p < 0.05$). * $p < 0.05$ compared to Day 1 startle response within treatment group.

Table 3

Study 2: Ropinirole and mCREB on no Stimulus Response

	eGFP		mCREB	
	Day 1	Day 28	Day 1	Day 28
Saline	0.041 ± 0.006	0.043 ± 0.007	0.055 ± 0.006	0.054 ± 0.008
Ropinirole	0.04 ± 0.006	0.043 ± 0.006	0.036 ± 0.006	0.040 ± 0.006

Note. Data are expressed in Newtons (N) as mean ± SEM. There was no significant effect of ropinirole [$F(1,27) = 1.412$, $p > 0.05$], mCREB [$F(1,27) = 0.575$, $p > 0.05$], or day $F(1,27) = 0.23$, $p > 0.05$] on pulse startle response using repeated measures ANOVA.

Table 4

Study 2: Rostral to Caudal Spread of mCREB Viral Vector

	eGFP		mCREB	
	Left	Right	Left	Right
Saline	0.54 ± 0.11	0.56 ± 0.11	0.86 ± 0.2	0.57 ± 0.17
Ropinirole	0.85 ± 0.17	0.77 ± 0.15	0.56 ± 0.15	0.73 ± 0.16

Note. Data are expressed in mm as mean ± SEM. There was no significant difference between side of hemisphere [$F(1,24) = 0.499$, $p > 0.05$], mCREB [$F(1,24) = 0.062$, $p > 0.05$], or ropinirole $F(1,24) = 1.05$, $p > 0.05$] and control spread of expressing viral vector using repeated measures ANOVA.

Table 5

Study 3: Ropinirole and ΔJunD PPI Startle Response

	eGFP		ΔJunD	
	Day 1	Day 28	Day 1	Day 28
Saline	0.65 ± 0.14	0.80 ± 0.16*	0.53 ± 0.14	0.60 ± 0.14*
Ropinirole	0.88 ± 0.28	1.40 ± 0.42*	0.70 ± 0.13	0.96 ± 0.23*

Note. Data are expressed in Newtons (N) as mean ± SEM. There was no significant effect of ropinirole [$F(1,24) = 3.163$, $p > 0.05$] or ΔJunD [$F(1,24) = 1.597$, $p > 0.05$] on startle response using repeated measures ANOVA. There was a significant effect of day $F(1,24) = 22.99$, $p < 0.001$], treatment groups increased startle response on Day 28 as compared to Day 1 ($p < 0.05$). * $p < 0.05$ compared to Day 1 startle response within treatment group.

Table 6

Study 3: Ropinirole and mCREB on No Stimulus Response

	eGFP		ΔJunD	
	Day 1	Day 28	Day 1	Day 28
Saline	0.058 ± 0.009	0.071 ± 0.01*	0.046 ± 0.01	0.052 ± 0.012*
Ropinirole	0.022 ± 0.006	0.027 ± 0.005$^{*\#}$	0.045 ± 0.06	0.047 ± 0.007$^{*\#}$

Note. Data are expressed in newtons (N) as mean ± SEM. There was no significant effect of ΔJunD [$F(1,24) = 0.129$, $p > 0.05$] on response during ambient background stimulus using repeated measures ANOVA. There was a significant effect of day $F(1,24) = 5.617$, $p < 0.05$], treatment groups increased startle response on Day 28 as compared to Day 1 ($p < 0.05$). There was a significant effect of ropinirole [$F(1,24) = 7.06$, $p < 0.05$], repeated ropinirole treatment decreased response ($p < 0.05$) as compared to controls, however there was no effect of virus ($p > 0.05$) within treatment group. * $p < 0.05$ compared to Day 1 startle response within treatment group. # $p < 0.05$ compared to control treatment on Day 28.

Table 7

Study 3: Rostral to Caudal Spread of ΔJunD Viral Vector

	eGFP		ΔJunD	
	Left	Right	Left	Right
Saline	0.41 ± 0.12	0.50 ± 0.10	0.47 ± 0.12	0.39 ± 0.08
Ropinirole	0.50 ± 0.05	0.56 ± 0.05	0.48 ± 0.13	0.38 ± 0.07

Note. Data are expressed in mm as mean ± SEM. There was no significant difference between side of hemisphere [$F(1,19) = 0.803$, $p > 0.05$], ΔJunD [$F(1,19) = 0.66$, $p > 0.05$], or ropinirole $F(1,19) = 0.005$, $p > 0.05$] and control spread of expressing viral vector using repeated measures ANOVA.

Table 8

Study 4: Effect of Drug Treatment on PPI Control Measurements

Drug Treatment	Startle (120dB pulse) (N)	No Stimulus (N)
S-S-S	0.796 ± 0.622	0.101 ± 0.043[a]
S-S-Q	0.419 ± 0.353	0.064 ± 0.031
Q-A1-Q	0.405 ± 0.253	0.059 ± 0.031
Q-A10-Q	0.599 ± 0.572	0.067 ± 0.035

Note. [a] $p < 0.05$ significant increase in no stimulus trial.

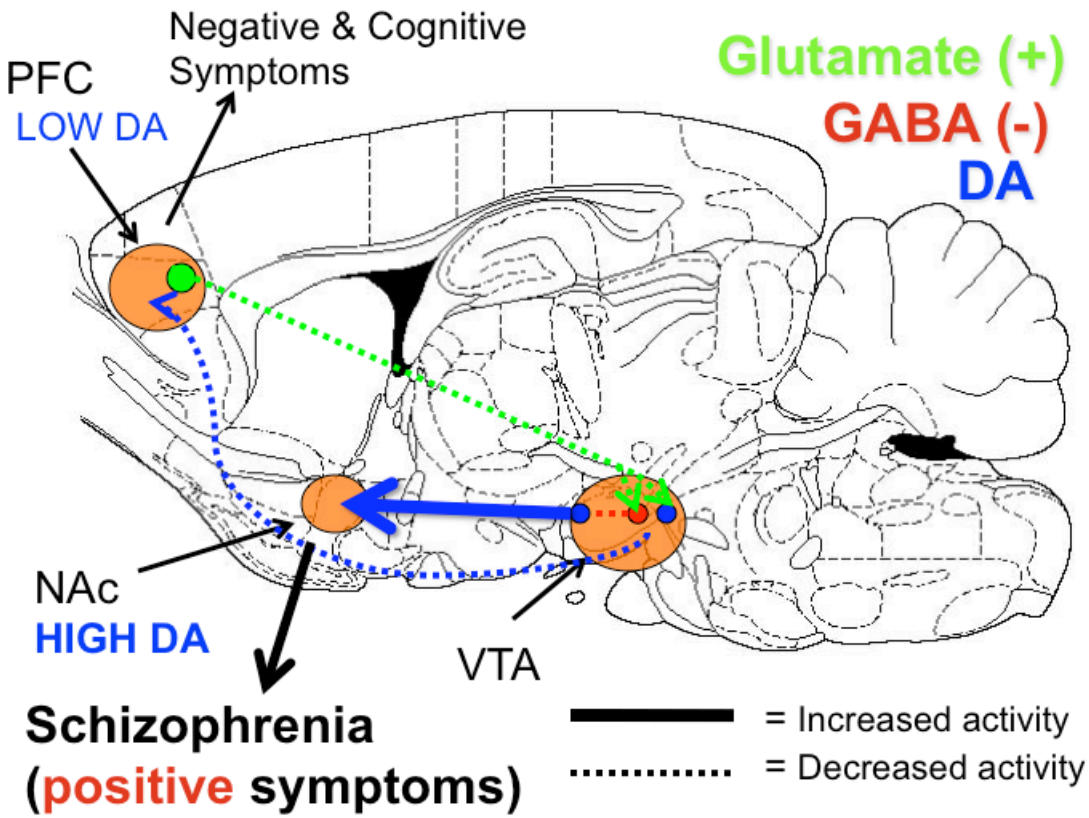

Negative & Cognitive Symptoms
PFC
LOW DA
Glutamate (+)
GABA (-)
DA
NAc
HIGH DA
VTA
Schizophrenia
(positive symptoms)
= Increased activity
= Decreased activity

Figure 1. Diagram of glutamate and dopamine pathways and interactions that contribute to positive and negative symptoms of schizophrenia via GABA connections.

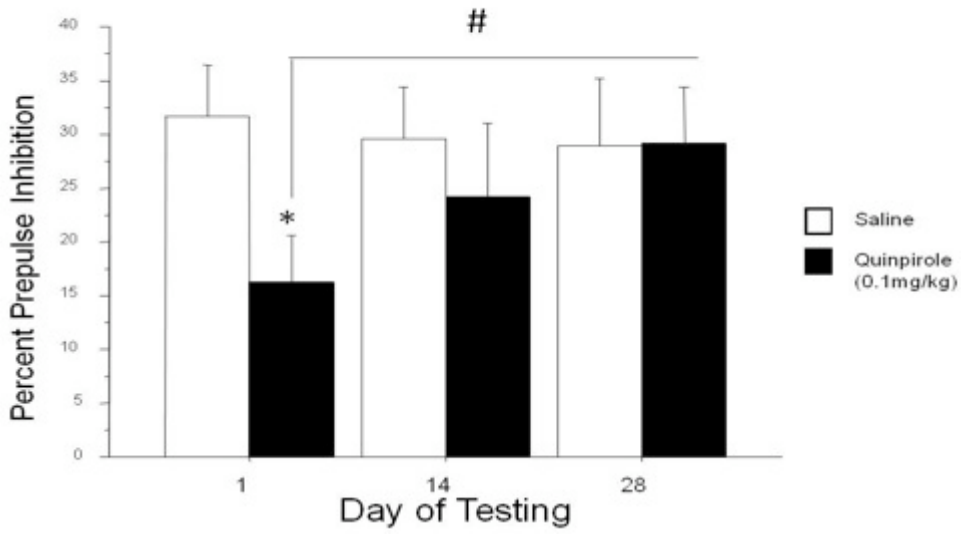

#
*
Percent Prepulse Inhibition
40
35
30
25
20
15
10
5
0
1
14
28
Day of Testing
Saline
Quinpirole
(0.1mg/kg)

Figure 2. Prepulse inhibition (PPI) recovery after 28 days of repeated quinpirole treatment. Animals were treated daily for 28 consecutive days with quinpirole (0.0, n = 12; or 0.1 mg/kg, n = 12). Following baseline testing, PPI was assessed 10 min after drug treatment on Days 1, 14, and 28. Repeated measures analysis of variance (ANOVA) with Sphericity Assumed determined a significant interaction between day of PPI test and drug treatment, $F(1,14) = 4.779$, $p < 0.05$. Day 1, post-hoc analysis revealed a significant effect of quinpirole, ($p < 0.05$), treatment on PPI. Day 35, post-hoc analysis revealed no significant effect of quinpirole, ($p > 0.05$). There was also a significant increase of PPI in quinpirole treated animals over time ($p < 0.05$). Percent PPI (mean ± SEM) data were collapsed across prepulse levels 3,6 or 12 dB above ambient noise level (70 dB). * $p <$ 0.05 compared to same day vehicle group. # $p < 0.001$ compared to Day 1.

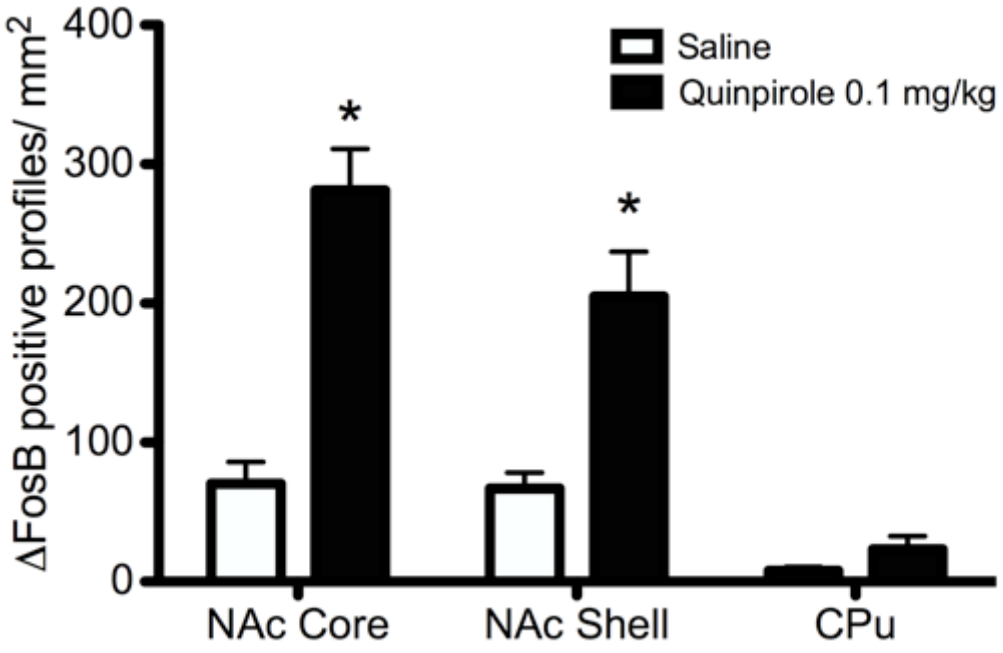

ΔFosB positive profiles/ mm2
400
300
200
100
0
Saline
Quinpirole 0.1 mg/kg
*
*
NAc Core
NAc Shell
CPu

Figure 3. Repeated quinpirole treatment increased ΔFosB in the NAc core and shell. Immunohistochemistry was performed on tissue obtained from rats 7 days after drug treatment. Rats were treated with ropinirole (0.0, $n = 7$; 0.1 mg/kg/day, $n = 7$) for 28 days. Repeated measures analysis of variance (ANOVA) with Sphericity assumed determined a significant effect of drug treatment across brain areas $F(2, 16) = 26.706$, $p < 0.001$, specifically there was a significantly more ΔFosB labeling within the in NAc core and shell ($p < 0.001$) as compared to repeated saline treated animals. There was also a significant main effect of brain region, $F(2, 17) = 76.173$, $p < 0.001$, with the most labeling in the NAc as compared to the CPu ($p < 0.001$). Data are expressed as number of nuclear profiles (mean ± SEM) per mm^2. * $p < 0.001$ compared to same day repeated saline treated animals in same brain region.

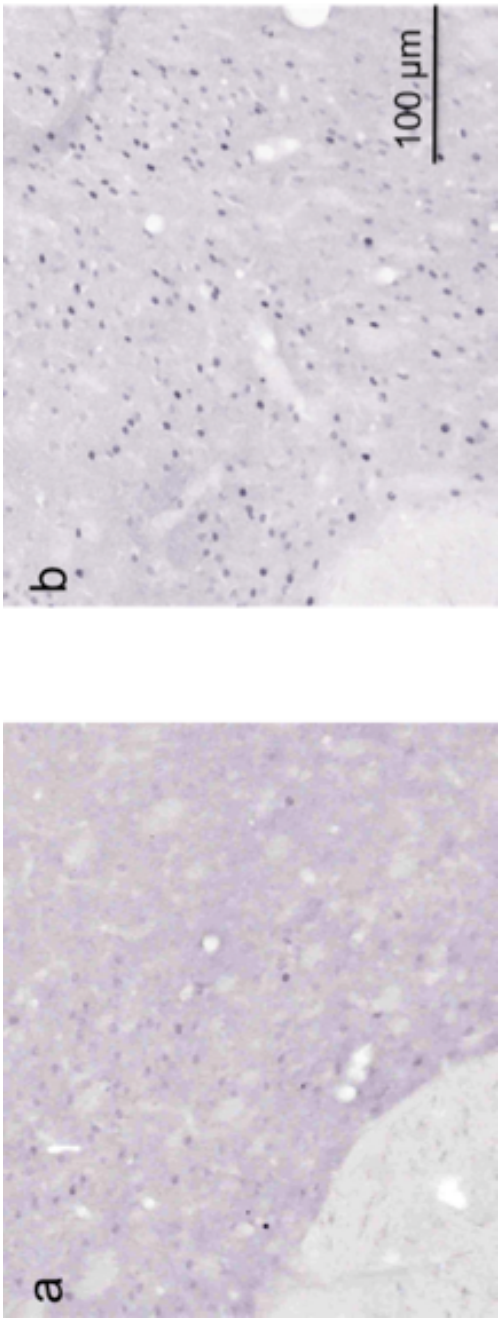

100 µm
b
a

Figure 4. Effect of repeated quinpirole on ΔFosB in the NAc core. (a) ΔFosB positive labeled cells after repeated saline treatment. (b) ΔFosB positive labeled cells after repeated quinpirole treatment. The anterior commissure is shown at the lower left.

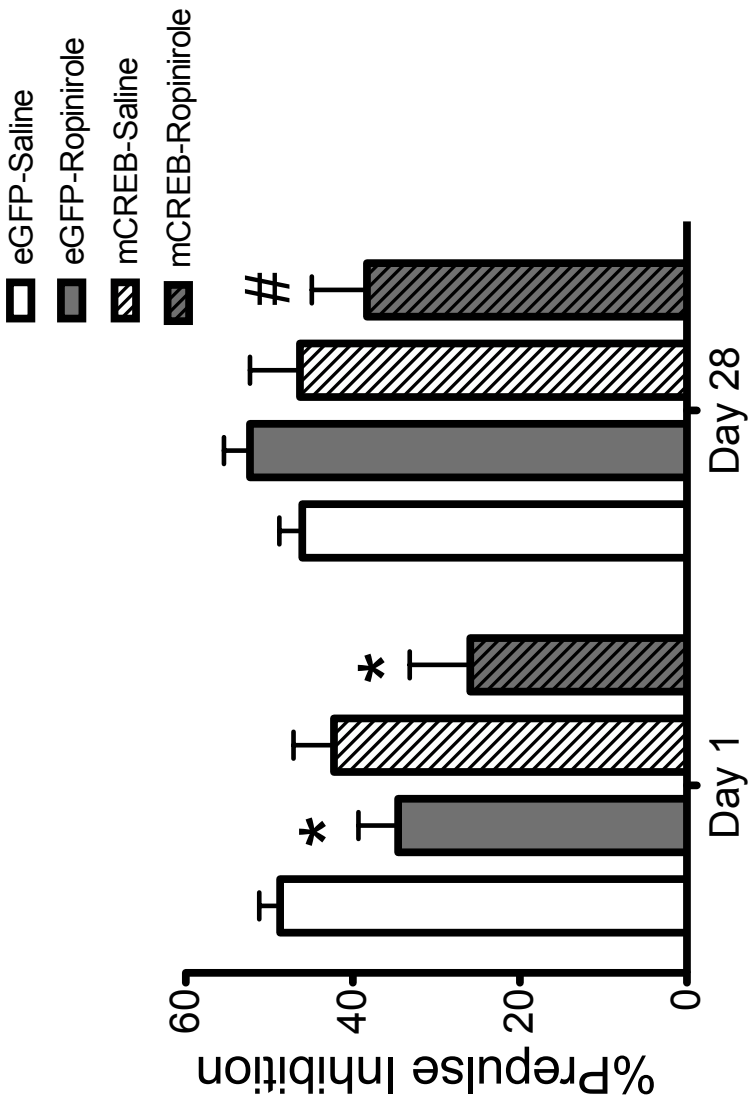
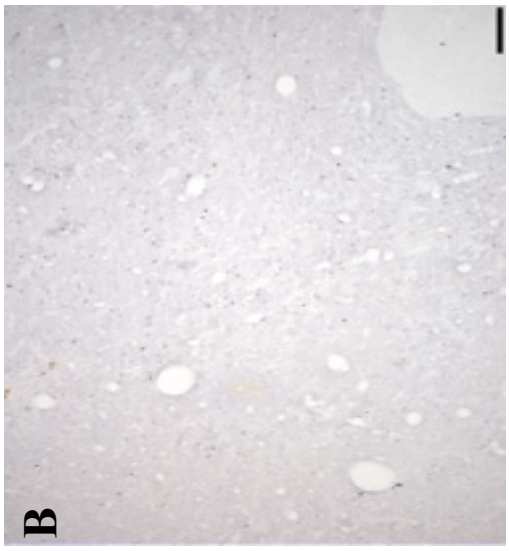

eGFP-Saline
eGFP-Ropinirole
mCREB-Saline
mCREB-Ropinirole
%Prepulse Inhibition
60
40
20
0
Day 1
Day 28
*
*
#

Figure 5. Mutant cyclic adenosine monophosphate response binding protein (mCREB) prevents prepulse inhibition (PPI) recovery induced by repeated ropinirole treatment. PPI was determined three weeks after intracranial infusion of adeno-associated virus for gene transfer of enhanced green fluorescent protein (eGFP) or mCREB-eGFP. Rats were treated with ropinirole (0.0, 0.1 mg/kg/day, $n = 7$ per virus group) for 28 days; PPI was tested the first and last day of treatment. Percent PPI (mean ± SEM) data were collapsed across prepulse levels 3,6 or 12 dB above ambient noise level (70 dB). Repeated measures analysis of variance (ANOVA) with Sphericity Assumed determined a significant interaction between day of PPI testing and virus/drug treatment [$F(3, 54) = 4.55$, $p < 0.01$]. Day 1, post-hoc analysis revealed a significant effect of ropinirole, ($p < 0.05$). Day 28, post-hoc analysis revealed a significant effect of ropinirole, ($p < 0.05$) only in mCREB virus infused animals. * $p < 0.05$ compared to same day saline treated group(s). # $p < 0.05$ compared to eGFP virus infused same drug treatment group.

ΔFosB Positive Profiles / mm2
0
100
200
300
400
500
eGFP-Saline
eGFP-Ropinirole
mCREB-Saline
mCREB-Ropinirole
*
#
*
#
NAc Core
NAc Shell
CPu

Figure 6. Mutant cyclic adenosine monophosphate response binding protein (mCREB) attenuated repeated ropinirole induced ΔFosB expression in the NAc core and shell. Immunohistochemistry was performed on tissue obtained from rats 7 days after drug treatment. Rats under-went intracranial infusions with adeno-associated virus (AAV) for gene transfer of enhanced green fluorescent protein (eGFP) or mCREB-eGFP 3 weeks prior to treatment. Rats were treated with ropinirole (0.0, 0.1 mg/kg/day, $n = 5$ per virus group) for 28 days. Repeated measures analysis of variance (ANOVA) with Sphericity assumed determined no significant interactions, however there was a significant effect of treatment [$F(3, 21) = 4.618$, $p < 0.05$] in the NAc core. Data are expressed as number of nuclear profiles (mean ± SEM) per mm^2. * $p < 0.05$ compared to same brain region, eGFP virus infused, repeated saline treated animals. # $p < 0.05$ compared to same brain region, mCREB virus infused, repeated saline treated animals.


B

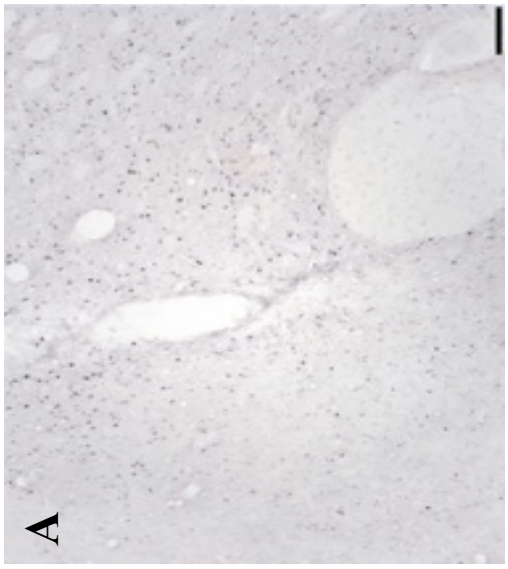

A

Figure 7. Mutant cyclic adenosine monophosphate response binding protein (mCREB) attenuated repeated ropinirole induced ΔFosB expression in the NAc core 10 days after repeated ropinirole treatment and 9 weeks after intracranial infusion with adeno-associated virus for gene transfer of enhanced green fluorescent protein (eGFP) or mCREB-eGFP. Image of ΔFosB labeling in the NAc core after A) eGFP- or B) mCREB-virus infused after 28 days of repeated ropinirole treatment. Anterior commissure is shown at lower right in each illustration. Scale bar: 100 μm.

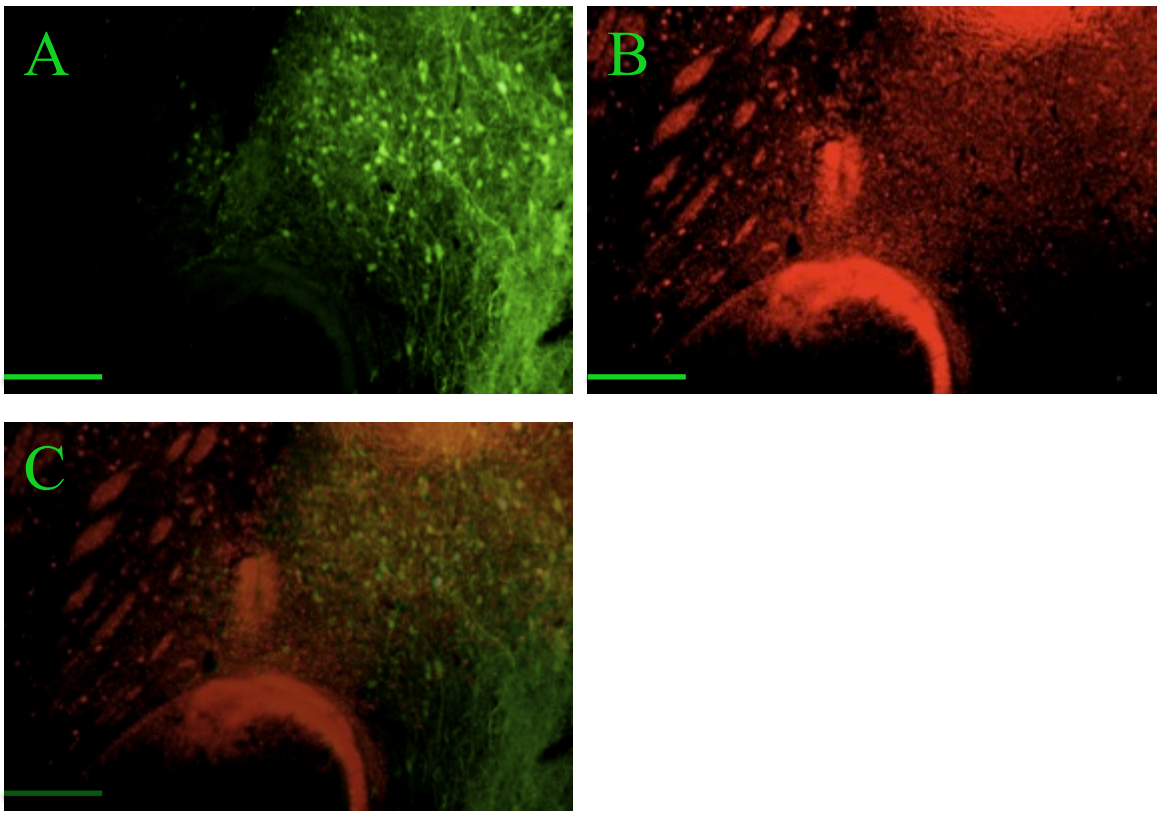

A
B
C

Figure 8. Mutant cyclic adenosine monophosphate response binding protein (mCREB) attenuated repeated ropinirole induced ΔFosB in NAc core. Intracranial infusion with A) mCREB-eGFP 3 weeks prior to repeated ropinirole induced B) ΔFosB is C) not located in the same region within the NAc core. Anterior commissure is shown at lower right in each illustration. Scale bar: 100μm.

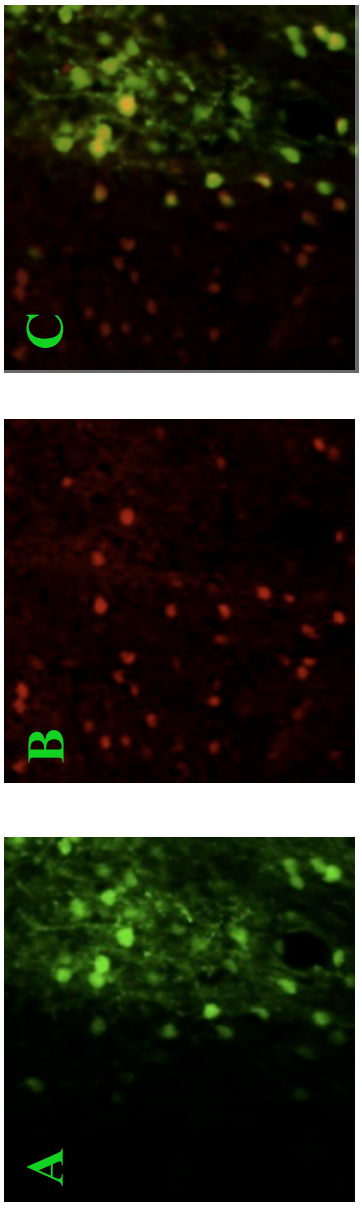

C
B
A

Figure 9. Mutant cyclic adenosine monophosphate response binding protein (mCREB) increased ΔFosB in NAc core. Intracranial infusion with A) mCREB-eGFP 3 weeks prior to repeated saline increased B) ΔFosB within the NAc core, C) co-expression was detected in some, but not all cells.

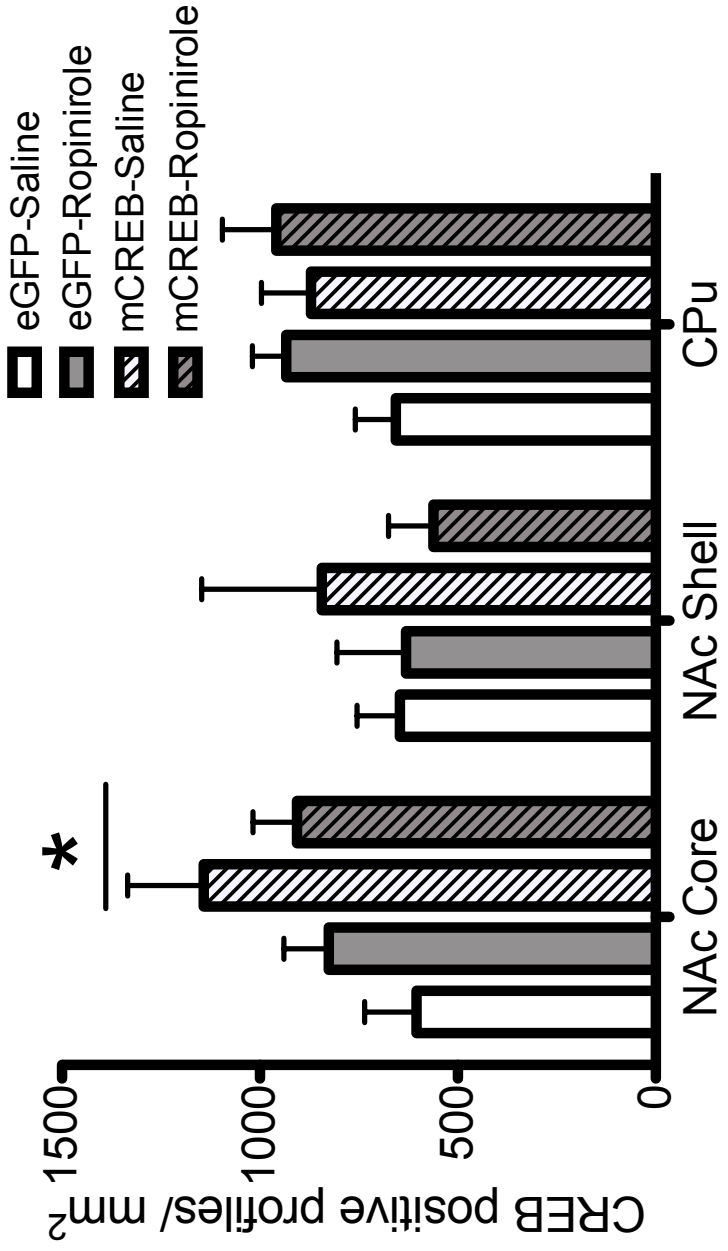

eGFP-Saline
eGFP-Ropinirole
mCREB-Saline
mCREB-Ropinirole
CREB positive profiles/ mm2
1500
1000
500
0
*
NAc Core
NAc Shell
CPu

Figure 10. Mutant cyclic adenosine monophosphate response binding protein (mCREB) increased (CREB) in the NAc core. Immunohistochemistry was performed on tissue obtained from rats 7 days after drug treatment. Rats were intracranial infusion with adeno-associated virus for gene transfer of enhanced green fluorescent protein (eGFP) or mCREB-eGFP 3 weeks prior to treatment. Rats were treated with ropinirole (0.0, 0.1 mg/kg/day, $n = 5$ per virus group) for 28 days. Repeated measures analysis of variance (ANOVA) with Sphericity assumed determined a significant effect of treatment across brain areas [$F(2, 28) = 5.20$, $p < 0.05$], specifically there was a significantly more CREB labeling within the in NAc core ($p < 0.05$) in animals with mCREB virus. Data are expressed as number of nuclear profiles (mean ± SEM) per mm^2. * $p < 0.05$ compared to same day eGFP virus.

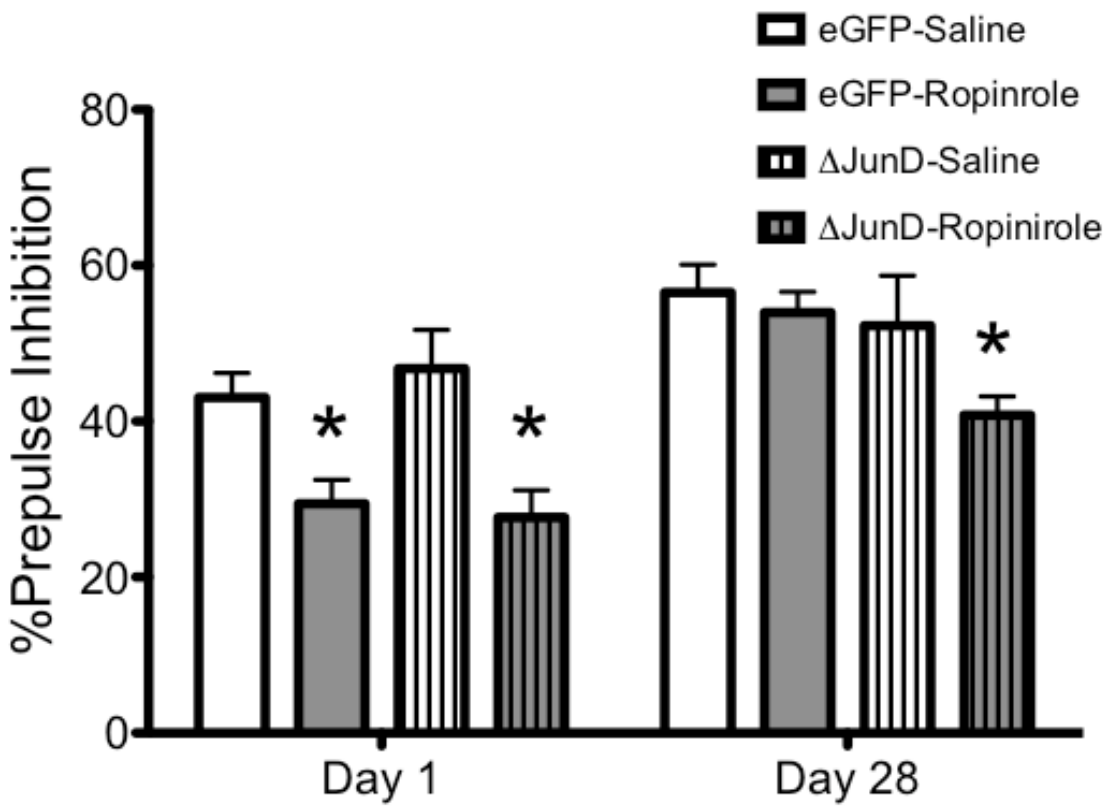

eGFP-Saline
eGFP-Ropinrole
ΔJunD-Saline
ΔJunD-Ropinirole
%Prepulse Inhibition
80
60
40
20
0
*
*
*
Day 1
Day 28

Figure 11. Truncated mutant of the JunD protein (ΔJunD) prevents prepulse inhibition (PPI) recovery induced by repeated ropinirole treatment. PPI was determined three weeks after intracranial infusion of adeno-associated virus (AAV) for gene transfer of enhanced green fluorescent protein (eGFP) or ΔJunD-eGFP 3 weeks prior to treatment. Rats were treated with ropinirole (0.0, 0.1 mg/kg/day, $n = 8$ per virus group) for 28 days; PPI was tested the first and last day of treatment. Percent PPI (mean ± SEM) data were collapsed across prepulse levels 3,6 or 12 dB above ambient noise level (70 dB). Repeated measures analysis of variance (ANOVA) with Sphericity Assumed determined a significant interaction between day and drug treatment [$F(6, 48) = 2.839$, $p < 0.05$]. Day 1, post-hoc analysis revealed a significant effect of ropinirole, ($p < 0.05$). Day 28, post-hoc analysis revealed a significant effect of quinpirole, ($p < 0.05$) only in ΔJunD virus infused animals. * $p < 0.05$ compared to same day vehicle group(s).

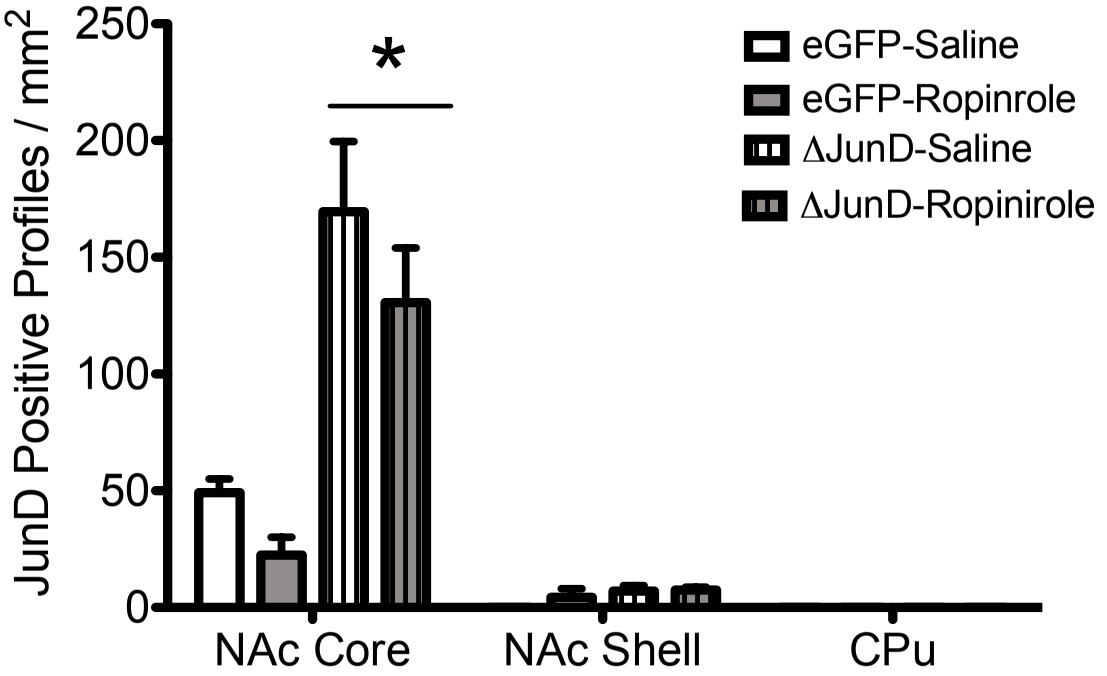

JunD Positive Profiles / mm2
250
200
150
100
50
0
*
eGFP-Saline
eGFP-Ropinrole
ΔJunD-Saline
ΔJunD-Ropinirole
NAc Core
NAc Shell
CPu

Figure 12. Truncated mutant of the JunD protein (ΔJunD) increased ΔJunD in the NAc core. Immunohistochemistry was performed on tissue obtained from rats 7 days after last drug treatment. Rats were intracranial infusion with adeno-associated virus for gene transfer of enhanced green fluorescent protein (eGFP) or ΔJunD-eGFP 3 weeks prior to treatment. Rats were treated with ropinirole (0.0, 0.1 mg/kg/day, $n = 6$ per virus group) for 28 days. Repeated measures analysis of variance (ANOVA) with Greenhouse-Geisser correction determined a significant brain area by treatment interaction [$F(6, 28) = 4.67$, $p < 0.05$], specifically there was a significantly more JunD labeling within the in NAc core ($p < 0.05$) in animals with ΔJunD. Data are expressed as number of nuclear profiles (mean ± SEM) per mm^2.* $p < 0.05$ compared to same day GFP virus.

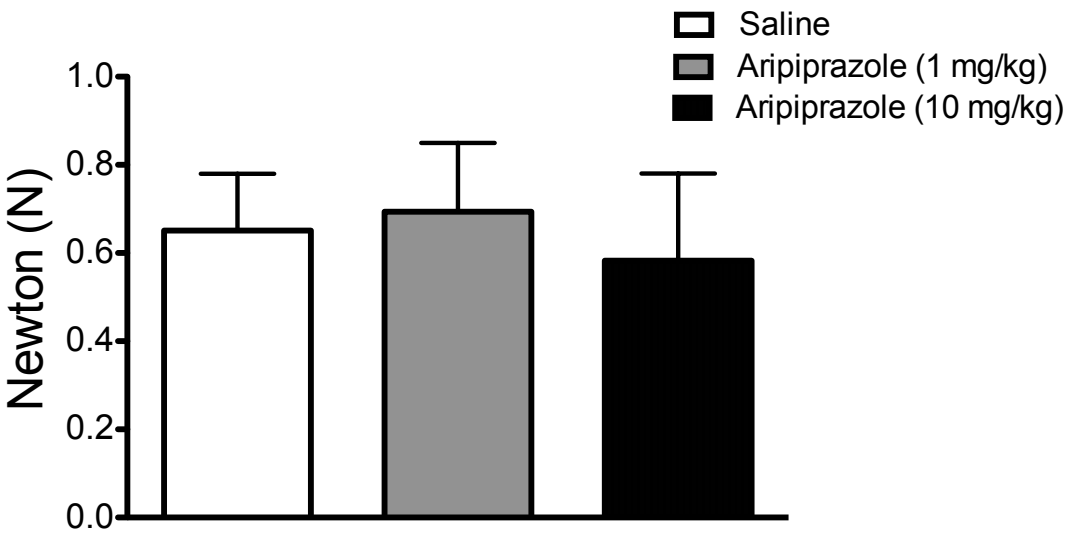

Saline
Aripiprazole (1 mg/kg)
Aripiprazole (10 mg/kg)
Newton (N)
1.0
0.8
0.6
0.4
0.2
0.0

Figure 13. Startle (120dB) response after acute aripiprazole treatment. Animals were treated with aripiprazole (0.0, $n = 18$; 1.0, $n = 8$; or 10.0 mg/kg, $n = 10$) 30 min before PPI testing. There was no significant effect of aripiprazole dose [$F(2,33) = 0.096$, $p > 0.05$] on startle response using one-way analysis of variance (ANOVA).

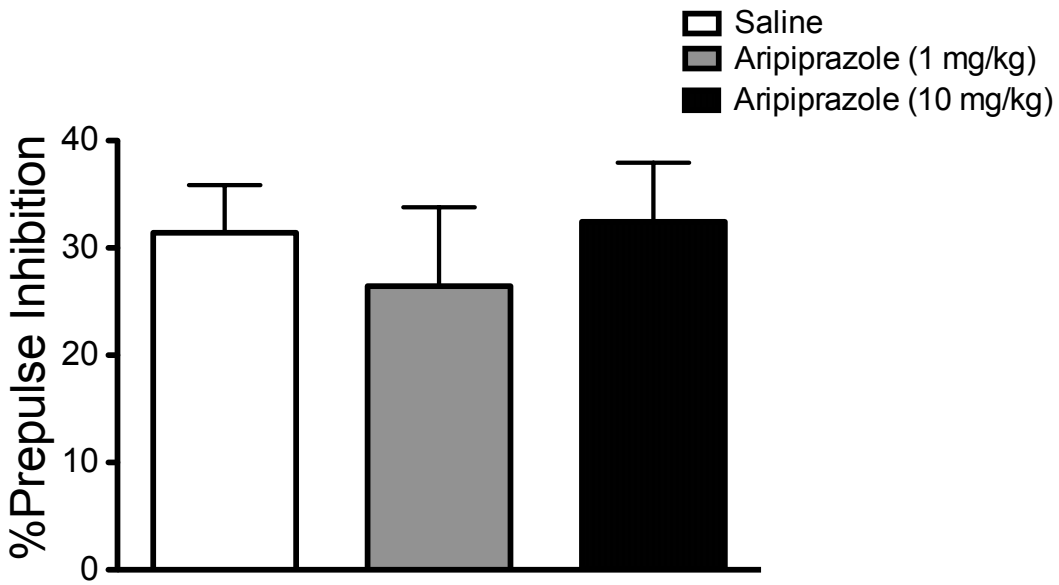

Saline
Aripiprazole (1 mg/kg)
Aripiprazole (10 mg/kg)
%Prepulse Inhibition
40
30
20
10
0

*Figure 14***.** Percent prepulse inhibition (PPI) response after acute aripiprazole treatment. Animals were treated with aripiprazole (0.0, $n = 18$; 1.0, $n = 8$; or 10.0 mg/kg, $n = 10$) 30 min before PPI testing. There was no significant effect of aripiprazole dose on [$F(2, 33) = 0.256$, $p > 0.05$] on PPI.

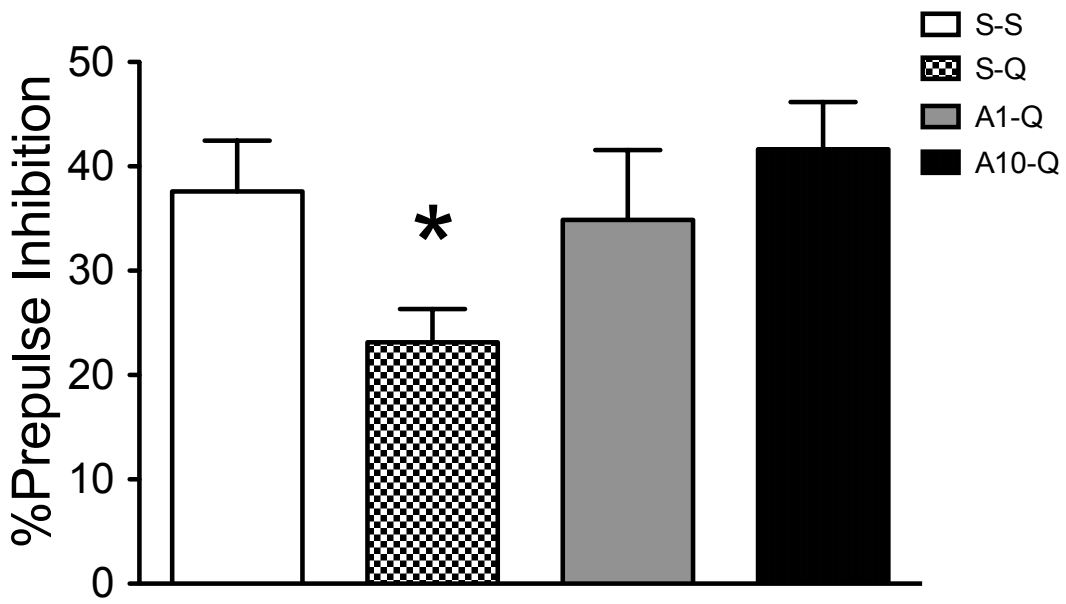

%Prepulse Inhibition
50
40
30
20
10
0
*
S-S
S-Q
A1-Q
A10-Q

*Figure 15***.** Attenuation of quinpirole induced prepulse inhibition (PPI) deficit after acute aripiprazole treatment. Animals were treated with aripiprazole (0.0, $n = 15$; 1.0, $n = 7$; or 10.0 mg/kg, $n = 8$) 30 min before quinpirole (0.0 mg/kg, $n = 7$) (S-S) challenge; (0.1 mg/kg, $n = 7$) (S-Q); aripiprazole 1.0 mg/kg – quinpirole (A1-Q), $n = 7$; aripiprazole 10.0 mg/kg- quinpirole (A10-Q), $n = 7$) before PPI testing. There was a trending effect of drug treatment [$F(3, 26) = 2.514$, $p = 0.08$] on %PPI. Furthermore, a planned contrast between control and acute quinpirole challenge detected a significant effect $t(13)= 2.41$, $p < 0.05$, while there was no significant difference between control and acute aripiprazole challenge. * $p < 0.05$ compared to vehicle animals.

S-S-S
S-S-Q
Q-A1-Q
Q-A10-Q
%Prepulse Inhibition
0
10
20
30
40
50
Day 0
Day 35
*
*

Figure 16. Attenuation of quinpirole induced prepulse inhibition (PPI) deficit after repeated aripiprazole treatment. Animals were challenged with quinpirole (0.0, $n = 30$ or 0.1 mg/kg, $n = 30$) the day prior to repeated aripiprazole (0.0, $n = 30$; 1.0, $n = 15$; or 10.0 mg/kg, $n = 15$), 7 days after repeated treatments; animals were challenged again with quinpirole (0.0, $n = 15$ or 0.1 mg/kg, $n = 45$) before PPI testing. S-S-S, S-S-Q, Q-A1-Q, Q-A10-Q, $n = 15$ each group. Repeated measures analysis (ANOVA) with Greenhouse-Geiesser correction determined a significant interaction between day of quinpirole challenge and drug treatment [$F(3, 89.753) = 3.945$, $p < 0.05$]. Day 0, post-hoc analysis revealed a significant effect of quinpirole, ($p < 0.05$), treatment on PPI. Day 35, post-hoc analysis revealed a significant effect of quinpirole, ($p < 0.05$), in animals that received repeated vehicle treatment and no significant effect of quinpirole in animals that received repeated aripiprazole treatment ($p > 0.05$) as compared to control animals. Percent PPI (mean ± SEM) data were collapsed across prepulse levels 3,6 or 12 dB above ambient noise level (70 dB). * $p < 0.05$ compared to same day vehicle group(s).

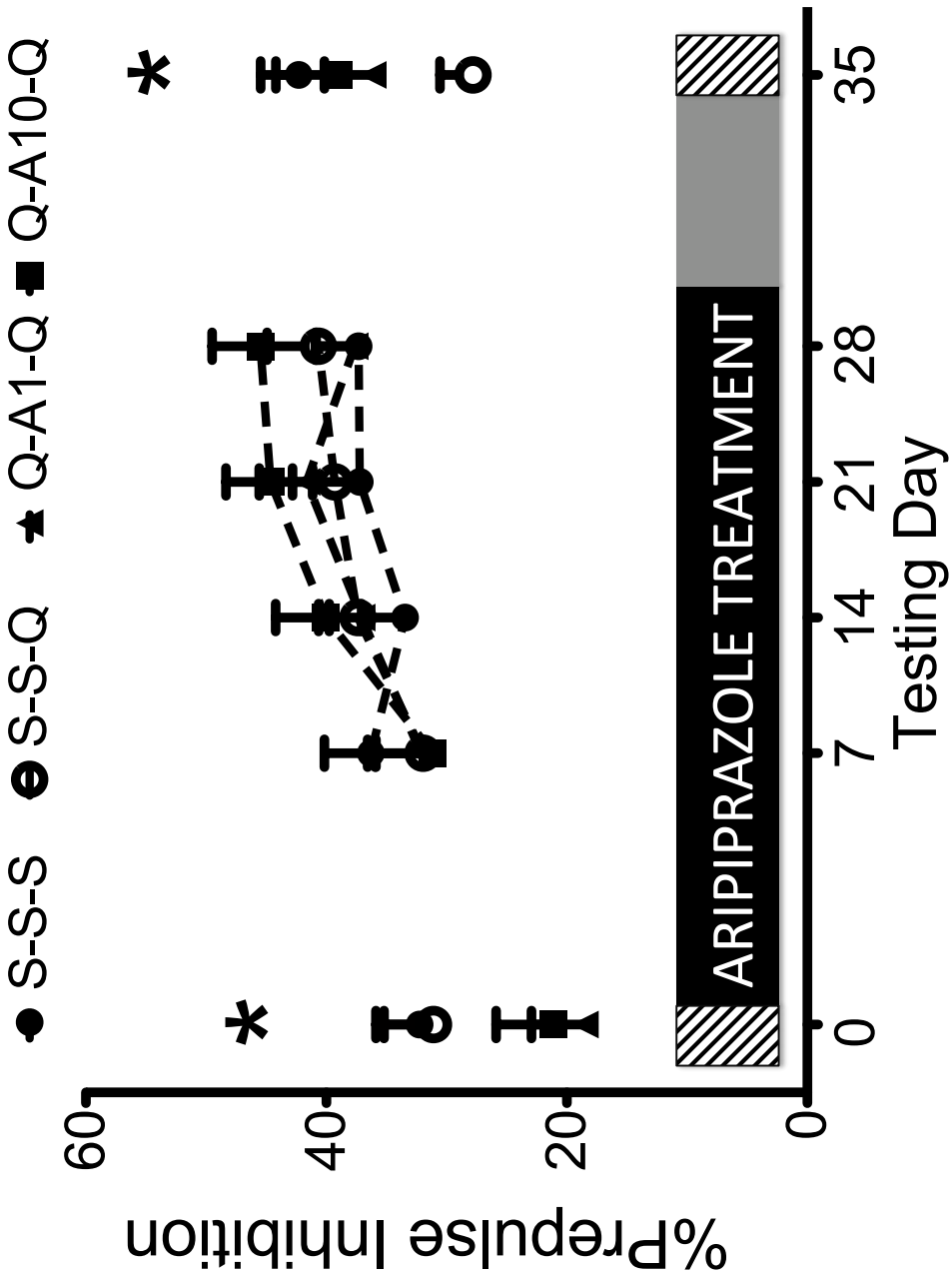

S-S-S
S-S-Q
Q-A1-Q
Q-A10-Q
%Prepulse Inhibition
60
40
20
0
ARIPIPRAZOLE TREATMENT
0
7
14
21
28
35
Testing Day

*Figure 17***.** Attenuation of quinpirole induced prepulse inhibition (PPI) deficit after repeated aripiprazole treatment. Animals were challenged with quinpirole (0.0, $n = 30$ or 0.1 mg/kg, $n = 30$) the day prior to repeated aripiprazole (0.0, $n = 30$; 1.0, $n = 15$; or 10.0 mg/kg, $n = 15$), 7 days after repeated treatments; animals were challenged again with quinpirole (0.0, $n = 15$ or 0.1 mg/kg, $n = 45$) before PPI testing. S-S-S, S-S-Q, Q-A1-Q, Q-A10-Q, $n = 15$ each group. Repeated measures analysis (ANOVA) with Greenhouse-Geiesser correction determined a significant interaction between day of quinpirole challenge and drug treatment [$F(3, 89.753) = 3.945$, $p < 0.05$]. Day 0, post-hoc analysis revealed a significant effect of quinpirole, ($p < 0.05$), treatment on PPI. Day 35, post-hoc analysis revealed a significant effect of quinpirole, ($p < 0.05$), in animals that received repeated vehicle treatment and no significant effect of quinpirole in animals that received repeated aripiprazole treatment ($p > 0.05$) as compared to control animals. There was no significant effect of drug on PPI during repeated treatment. Percent PPI (mean ± SEM) data were collapsed across prepulse levels 3,6 or 12 dB above ambient noise level (70 dB). * $p < 0.05$ compared to same day vehicle group(s).

ΔFosB positive profiles/ mm2
800
600
400
200
0
NAc Core
NAc Shell
CPu
S-S-S
S-S-Q
Q-A1-Q
Q-A10-Q
#
*
#
*

Figure 18. Repeated aripiprazole (10.0 mg/kg) treatment increased ΔFosB in the striatum (nucleus accumbens (NAc) Core, shell, caudatoputamen (CPu)). Immunohistochemistry was performed on tissue obtained from rats treated for 28 days with aripiprazole (0.0, n = 9; 1.0, n = 5; or 10.0 mg/kg, n = 6). Repeated measures analysis of variance (Walsh et al.) with Spericity Asuumed determined a significant interaction between brain area and drug treatment [$F(6, 22) = 3.345$, $p < 0.05$]. There was a significant difference in amount of ΔFosB labeling between the three brain regions [$F(2, 22) = 52.658$, $p < 0.001$]. Post-Hoc analysis revealed a significant increase of ΔFosB labeling in NAc core as compared to other sub-regions ($p < 0.05$) and significant effect of repeated aripiprazole (10.0 mg/kg) treatment ($p < 0.05$) on ΔFosB labeling within each sub-region as compared to repeated vehicle treated animals. Data are expressed as number of nuclear profiles (mean ± SEM) per mm^2. * $p < 0.001$ compared to same day vehicle group(s). # $p < 0.001$ compared to NAc core.

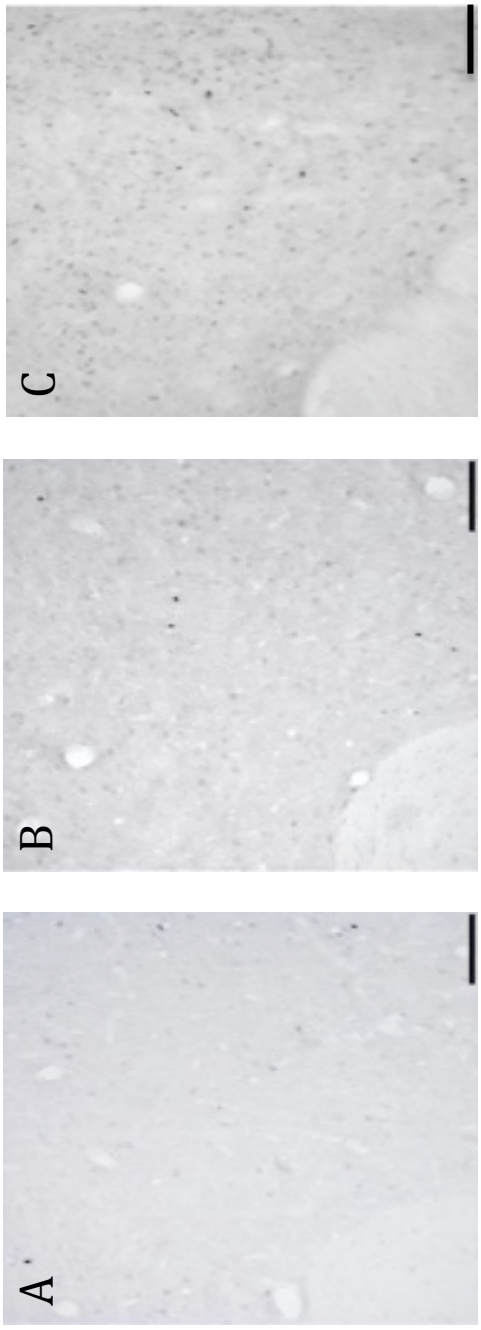

C
B
A

Figure 19. ΔFosB increased in the striatum after repeated aripiprazole (10.0 mg/kg) treatment. Image of ΔFosB labeling in the nucleus accumbens (NAc) core from repeated (A) saline, (B) aripiprazole (1.0 mg/kg), or (C) aripiprazole (10.0 mg/kg) treated rats 10 days after treatment. The anterior commissure is shown at the lower left. Scale bar: 100 μm.

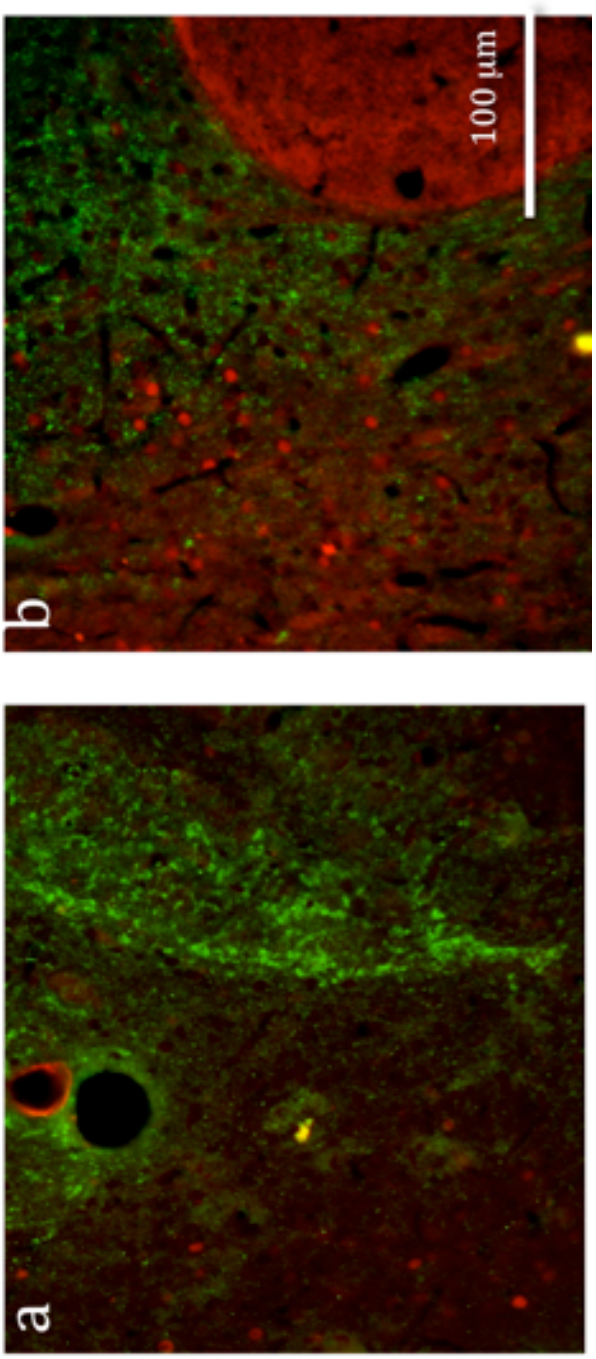

b
100 µm
a

Figure 20. Double labeling of enkephalin (green fluorescence) and ΔFosB-like (red fluorescence) immunoreactivity in cells of the NAc core after repeated ropinirole treatment. Enkephalin and ΔFosB do not seem to be localized in the same areas; a) NAc shell b) NAc core, anterior commissure is shown at the lower right.

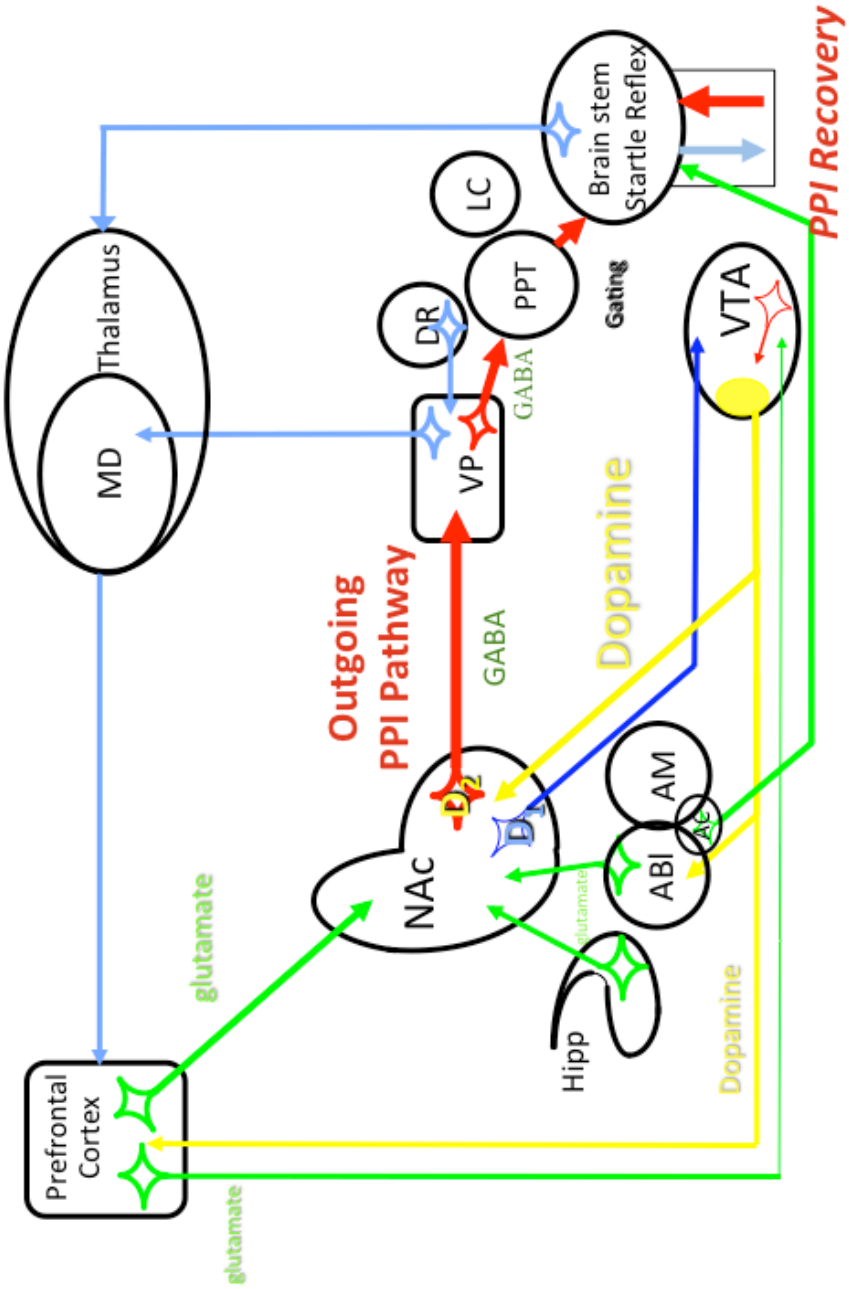

Brain stem
Startle Reflex
PPI Recovery
LC
PPT
Gating
DR
GABA
VTA
Thalamus
MD
VP
Outgoing
PPI Pathway
GABA
Dopamine
D2
D1
NAc
AM
ABl
Ac
glutamate
glutamate
Hipp
Dopamine
Prefrontal
Cortex
glutamate

Figure 21. Schematic diagram illustrating the neural substrates regulating prepulse inhibition of the acoustic startle response in the rat. This figure emphasizes the convergence of dopamine and glutamate projections in the nucleus accumbens (NAc), and indicates that intracellular changes in the NAc may modify the outgoing PPI pathway (GABA) to the ventral pallidum (VP), which regulates the outgoing startle response resulting in PPI recovery following repeated D_2-like agonist treatment.