

TARGETING ADENOSINE, DOPAMINE, AND GLUTAMATE HETEROMERIC
RECEPTOR COMPLEXES TO TREAT RESTRICTED, REPETITIVE BEHAVIOR

By

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To Jim Dufek – your success is inspiring, your love encouraging, your friendship
priceless, and your spirit refreshing.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CDPPB	3-cyano-N-(1,2 diphenyl-1H-pyrazol-5-yl)bezamide
CNS	Central nervous system
DMSO	Dimethyl sulfoxide
GABA	Gamma-aminobutyric acid
GPe	External segment of the globus pallidus
GPI	Internal segment of the globus pallidus
NAM	Negative allosteric modulator
PAM	Positive allosteric modulator
SNC	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata

Abstract of Thesis Presented to the Graduate School
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TARGETING ADENOSINE, DOPAMINE, AND GLUTAMATE HETEROMERIC
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Restricted repetitive behaviors are extremely common in neurodevelopmental disorders. The deer mouse model of repetitive behavior is a particularly useful animal model because repetitive behavior develops early, persists through much of the lifetime of the animal, and occurs spontaneously. We've found that the repetitive behaviors exhibited by deer mice are a result of a neurobiological imbalance of activation between the direct and indirect pathways of the basal ganglia. The imbalance between the direct and indirect pathways in the deer mice is caused by decreased activation of the indirect pathway that allows direct pathway activation to over-excite the cortex. There are particular heteromeric complexes of receptors on neurons of the direct and indirect pathways. These receptor complexes include dopamine D2, adenosine A2A, and glutamate mGluR5 receptors on indirect pathway neurons in the striatum. Activation of A2A and/or mGluR5 receptors reduces the functioning of D2 receptors. We hypothesized that activation of A2A and mGluR5 receptors and antagonism of D2 receptors could reduce the expression of repetitive behavior by enhancing the functioning of the indirect striatopallidal neurons. In a series of pharmacological studies we found that individually these drugs were each ineffective at reducing repetitive

behavior; however, the triple combination of these drugs significantly lessened the expression of repetitive behavior. These data further suggest that decreased indirect pathway activation mediates repetitive behavior and that targeting these heteromeric receptor complexes on the indirect pathway neurons of the striatum may offer pharmacotherapeutic benefit for individuals with neurodevelopmental disorders.

CHAPTER 1 INTRODUCTION

Repetitive behaviors are present in many different neurodevelopmental, neurological, and psychiatric disorders (e.g., autism, Fragile X syndrome, Rett syndrome, dementias, obsessive compulsive disorder, Tourette syndrome, and schizophrenia). These behaviors are classified as highly repetitive, invariant, and without obvious function. In neurodevelopmental disorders specifically, there is a wide array of repetitive behaviors exhibited by these populations, ranging from rhythmic hand flapping or head banging to circumscribed interests and insistence of sameness (Bodfish et al., 2000). This wide range of behavior has been statistically categorized into two clusters or factors (Turner, 1999; Cuccaro et al., 2003; Szatmari et al., 2006; Mooney et al., 2009; Bishop et al., 2013). Lower-order repetitive behaviors consist of sensory-motor behaviors such as whole body stereotypies, object stereotypies, and self-injury. The higher-order repetitive behaviors consist of compulsions, insistence on sameness, and restricted or circumscribed interests and behaviors.

Repetitive behaviors have a negative impact on both the individual and their family. The presence of these rigid and inflexible behaviors can impede treatment of other phenotypic traits of the disorder (Pierce & Courchesne, 2001; Scahill et al., 2012), become the genesis of mood and other behavioral problems (Green et al., 2006), and are a source of parental stress (Bishop et al., 2007). Unfortunately, we have no proven, effective pharmacological treatments for these maladaptive behaviors. Finding treatments that specifically target the neuropathology that mediates repetitive behavior is our best strategy for elucidating effective pharmacotherapy.

The wide range of repetitive behavior phenotypes and the spectrum of disorders associated with repetitive behavior suggest that the particular molecular pathophysiology that mediates repetitive behavior may vary between people and populations. This implies that the common dysfunction across these populations is altered output of complex neuronal circuitry. The circuitry predominantly associated with repetitive behavior is the cortico-basal ganglia circuitry. The neuroanatomy of the human cortico-basal ganglia circuitry generally consists of cortical inputs to the striatum (caudate, putamen, and nucleus accumbens), which project to the internal segment of the globus pallidus (GPi) and substantia nigra pars reticulata (SNr). These two nuclei, the GPi and SNr, are functionally equivalent and are usually grouped together when describing striatal output regions. The outputs from the striatum to the GPi/SNr take one of two paths. One path is direct from the striatum to the GPi/SNr and is termed the “direct pathway.” The other path is termed the “indirect pathway.” The indirect pathway includes relay projections to the external segment of the globus pallidus (GPe) and on to the subthalamic nucleus. From the GPi/SNr, projections go to the thalamus and then to the cortex.

The neurochemistry of the cortico-basal ganglia circuitry consists mainly of glutamate, gamma-aminobutyric acid (GABA), and dopamine. Descending cortical projections to the striatum are glutamatergic. The direct pathway neurons from the striatum to the GPi/SNr are GABAergic, as are the indirect pathway neurons from the striatum to the GPe. The indirect pathway neurons from the GPe release GABA onto the relay neurons of the subthalamic nucleus, and those neurons that project from the subthalamic nucleus to the GPi/SNr are glutamatergic. The output from the GPi/SNr to

the different nuclei of the thalamus is GABAergic, whereas the neurons that project from the thalamus to the cortex release glutamate. This neuroanatomical and neurochemical organization is responsible for differing effects of the direct and indirect pathways on cortical activation. Activation of the direct pathway causes disinhibition of the thalamic neurons and results in excitation of the cortex. Likewise, activation of the indirect pathway increases the inhibition of the thalamic neurons, which results in a reduction of glutamate signaling to the cortex, and therefore, decreased activation of the cortical neurons.

The cortico-basal ganglia circuitry can be modulated by dopamine signaling from neurons originating in the substantia nigra pars compacta (SNc). Dopamine has different effects on direct and indirect pathway neurons of the striatum, which is mediated by the different dopamine receptor subtypes. Direct pathway neurons in the striatum contain mostly D1 dopamine receptors. Dopamine binding to these receptors is excitatory. Indirect pathway neurons in the striatum contain mostly D2 dopamine receptors. Dopamine binding to these receptors is inhibitory. Furthermore, basal ganglia function can also be modulated by other neurotransmitters (e.g., serotonin and acetylcholine) and neuropeptides. The direct and indirect pathways are parallel and complementary pathways and proper, adaptive expression of basal ganglia-mediated behaviors depends on the appropriate balance of activity from these two antagonistic pathways - activation of the direct pathway leads to motor activation whereas activation of the indirect pathway leads to motor inactivation.

Most of the neuroanatomical studies investigating the dysfunction that mediates repetitive behavior in individuals with neurodevelopmental disorders have involved the

striatum. These studies can be challenging because the heterogeneous patient populations, but have consistently found volume differences between the patient populations and neurotypical controls (Casanova et al., 1991; Wong et al., 1996; Subramaniam et al., 1997; Harris et al., 1998; Eliez et al., 2001; Hoefft et al., 2008). In addition, several studies have found a statistical correlation between caudate volume and repetitive behaviors in Fragile X and autistic patients (Reiss et al., 1995; Sears et al., 1999; Hollander et al., 2005; Rojas et al., 2006; Gothelf et al., 2008; Langen et al., 2009; Wolff et al., 2013). All of these studies except Sears et al. (1999) found a positive correlation between caudate volume and some measure of repetitive behavior. These studies further implicate cortico-basal ganglia circuitry dysfunction in the patient populations that exhibit repetitive behavior but do not elucidate the exact pathology that may mediate repetitive behavior.

Studies using animal models are more suitable for tracking the particular molecular pathophysiology that may induce the expression of repetitive behavior. These animal models of repetitive behavior can be categorized into three domains: genetic or CNS insults, pharmacological models, and restricted environments or experience (Lewis et al., 2007). Investigations of the genetic basis of several neurodevelopmental disorders using gene knockout mice have described the expression of repetitive behaviors (e.g., MECP2, GABRB3, Ts65Dn). Pharmacological models of repetitive behavior typically involve dopamine or glutamate agonists that alter basal ganglia functioning (Lewis et al., 2007). Restricted environments or experience also induce repetitive behaviors in a wide variety of species (e.g. mice, birds, dogs, monkeys) in a wide range of environments (e.g., farms, zoos, laboratory housing, and

even human households). Our laboratory uses inbred (including C58 mice) and outbred (deer mice) strains that exhibit spontaneous restricted repetitive behavior as a result of normal laboratory housing.

The behavioral phenotype of C58 mice has been characterized as part of a long-standing effort to find relevant mouse models of autism (Moy et al., 2008, Ryan et al., 2009; Muehlmann et al., 2012). These studies have revealed that C58 mice have several characteristics that are similar to the core symptoms of autism, including low sociability and persistent repetitive behaviors. The repetitive motor behaviors include both jumping and backward somersaulting. Furthermore, we have begun to characterize the dysfunction of cortico-basal ganglia circuitry in C58 mice and find a specific downregulation of indirect pathway activity in C58 mice, compared to C57Bl/6 mice, which don't exhibit repetitive jumping and backward somersaulting (Muehlmann et al., 2013).

Deer mice, when housed in standard laboratory cages, also exhibit high rates of repetitive behavior consisting of vertical jumping and/or backward somersaulting. Several lines of evidence suggest that the cause of repetitive behavior is an imbalance of activation of the direct and indirect pathways of the basal ganglia. Pharmacological studies have identified that by reducing dopamine D1 receptor activation, and thereby dampening the tone of the direct pathway, repetitive jumping in the deer mice is reduced (Presti et al., 2003). In addition to differences in dopamine receptor expression, the direct and indirect pathways also differ by the neuropeptides that they contain. The direct pathway contains dynorphin peptide; the indirect pathway contains enkephalin peptide. Our lab has found that striatum enkephalin levels of mice with high rates of

repetitive behavior are much lower than that of mice that exhibited only low rates of repetitive behavior, whereas dynorphin levels were equal between the two groups (Presti & Lewis, 2005). This suggests that the functioning of the direct pathway is equal in the two groups of mice, but that the indirect pathway of the mice that exhibit high rates of repetitive behavior is dysregulated, and may be the cause of the increased repetitive motor behavior. Another study to suggest that the indirect pathway is hypofunctioning in deer mice with high rates of repetitive behavior used an assay of long lasting neuronal activity, cytochrome oxidase staining, in the subthalamic nucleus. This nucleus is innervated by the indirect pathway and not the direct pathway. Neuronal activation, as measured by cytochrome oxidase staining, was significantly lower in mice with high rates of repetitive behavior compared to mice with low rates of repetitive behavior. In addition, the intensity of cytochrome oxidase staining was negatively correlated with stereotypy rates (Tanimura et al., 2011). This further suggests that decreased indirect pathway function, which causes less neuronal activation in the subthalamic nucleus, is involved in the repetitive behavior exhibited by deer mice. Our lab has also shown previously that housing these mice in an enriched environment reduces the prevalence and frequency of repetitive behavior (Turner et al., 2002; Turner & Lewis, 2003; Turner et al., 2003). This decrease in stereotypic behavior by the environment is due to changes in activation of basal ganglia circuitry (Turner et al., 2002), dendritic branching in the motor cortex and striatum (Turner et al., 2003), and from increased levels of brain derived neurotrophic factor in the striatum (Turner & Lewis, 2003).

Taken together these data suggest that repetitive behavior in our mouse models is mediated by hypofunctioning of the indirect basal ganglia pathway. We hypothesized that specific drug targeting of the indirect basal ganglia pathway cells in the striatum could increase indirect pathway function and reduce repetitive behavior in the deer mice. These striatal cells express a few different neurotransmitter receptors that are either only expressed in those cells and no others in the brain, or form heteromeric complexes with other receptors and those complexes are not found anywhere else in the brain (Fuxe et al., 2003; Cabello et al., 2009). These receptors include the dopamine D2 receptor (Camus et al., 1986), the adenosine A2a receptor (Schiffmann et al., 1991), and the glutamate mGluR5 receptor (Tallaksen-Greene et al., 1998). It has been shown repeatedly that adenosine A2a receptor and glutamate mGluR5 receptor agonism synergistically reduces dopamine binding at the D2 receptor (Ferre et al., 1999; Rimondini et al., 1999; Popoli et al., 2001) and that these agonists increase GABA release from striatopallidal cells (Diaz-Cabiale et al., 2002). This is an effect that is mediated by synergistic activation of cell signaling cascades (e.g., protein kinase A, ERK1/2 and DARPP-32 phosphorylation) and immediate early gene expression (Fuxe et al., 2003; Schiffmann et al., 2007; Dell'anno et al., 2013).

Consistent with our hypothesis, we have found that acute administration of a cocktail of drugs comprising a dopamine D2 receptor antagonist, an adenosine A2a receptor agonist, and a glutamate mGluR5 positive allosteric modulator (PAM), selectively reduces repetitive behavior in deer mice. These drugs did not selectively reduce repetitive behavior either alone or in double combinations. In addition, the triple cocktail of drugs continued to reduce repetitive behavior in deer mice throughout a

seven day, sub-chronic administration protocol. Finally, we were able to demonstrate that a converse drug cocktail (an adenosine A2a receptor antagonist, a dopamine D2 receptor agonist, and a glutamate mGluR5 negative allosteric modulator; NAM) formulated to further reduce striatal indirect pathway cell function significantly increased repetitive behavior in deer mice.

CHAPTER 2 METHODS

Experiment 1: Acute Administration of Drug Cocktail in Saline Vehicle to Reduce Repetitive Behavior

Animals

One hundred and five adult male deer mice were used in experiment 1. They were acquired from our established breeding colony, wherein they were weaned at 21 days and housed with up to seven other male mice in a standard cage (29 x 18 x 13 cm). Room temperature was maintained within a range of 70-75°F, and a 12:12 light:dark cycle, with lights off at 10AM. Food and water were available *ad lib* and two Nestlet squares were provided for nest construction. All procedures were performed in accordance with the guidelines set forth in the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Florida Institutional Animal Care and Use Committee.

Drugs

The dopamine D2 receptor antagonist, L-741,626, was purchased from Tocris Bioscience and suspended in 25% DMSO and saline at either 0.3, 1, or 3 mg/mL and injected at either 3, 10, or 30 mg/kg (respectively). The solution was sonicated and vortexed repeatedly up until time of injection. The adenosine A2a agonist, CGS21680 hydrochloride, and the glutamate mGluR5 PAM, 3-Cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB), were each acquired through the National Institute of Mental Health's Chemical Synthesis and Drug Supply Program. CGS21680 was dissolved in 25% DMSO and saline at 0.005 mg/mL and injected at 0.05 mg/kg. CDPPB was suspended in 25% DMSO and saline at either 0.3 or 3 mg/mL and injected at 3 or 30

mg/kg (respectively). This solution also required repeated sonication and vortexing up until time of injection. When drugs were given together as double or triple cocktails, a single solution was made and was administered in a single injection.

Drug Treatments

Single drug dose response analyses were conducted to evaluate the efficacy of the dopamine D2 receptor antagonist, L-741,626, and the glutamate mGluR5 PAM, CDPPB, to selectively reduce repetitive behavior in deer mice. We injected separate cohorts of mice at the following doses: 3 mg/kg L-741,626 (n=9), 10 mg/kg L-741,626 (n=9), 30 mg/kg L-741,626 (n=13), 3 and 30 mg/kg CDPPB (n=11). We also tested a single dose of CGS21680 (0.05 mg/kg). We found previously that this dose did not reduce repetitive behavior in deer mice and that any higher doses resulted in nonselective motor suppression (Tanimura et al., 2010).

Our investigations of double drug combinations were also run in separate cohorts of mice. We selected the highest dose of the individual drugs that were run in the single drug experiments: L-741,626 at 30 mg/kg, CGS21680 at 0.05 mg/kg, and CDPPB at 30 mg/kg. We injected separate cohorts of mice with the following double drug combinations: L-741,626 + CGS21680 (n=12), L-741,626 + CDPPB (n=12), and CGS21680 + CDPPB (n=13). Finally, a separate cohort of deer mice received a triple combination of drugs at the individual doses used in the double drug cocktail (n=12). All of these studies were run in a random crossover design, wherein each mouse was also administered a vehicle injection (25% DMSO, saline) on a test day separated by at least a week from the drug challenge. All injections were administered at 4:00 PM (six hours following lights off), leaving two hours of the dark cycle remaining to study the effects of the drugs on repetitive behavior.

Repetitive Behavior Testing

The stereotypy observed in deer mice consists largely of two response topographies: jumping and backward somersaulting. The former topography involves the animal rearing against the cage wall and engaging in vertical hindlimb jumping. The second topography (backward somersaulting) involves the animal rotating its body such that it starts with all four paws on the cage floor, inverts its ventral surface to the cage top, and returns to the cage floor, upright and on all four paws. As these behaviors involve vertical activity, they were quantified using photobeam arrays which, when interrupted, recorded a count. We routinely video-recorded test sessions in order to identify the topography of stereotypy, insure accuracy of the automated counters, and measure the occurrence of non-stereotyped behavior.

Measurement of stereotypy was done in six standardized test cages using the automated apparatus described. We also employed a video surveillance system that allows digital recording of each automated test cage during the entire 8 hour dark cycle. The system makes use of a DVR capture card (GeoVision) and acquires images at 240 frames per second which provided a high resolution per individual cage (ca. 40 fps). This permitted precise determination of the individual behavior of the animals and precise estimates of the reliability of the automated apparatus. The testing protocol involved removing mice from their home cages, weighing them, and placing them singly in standard testing cages (22 x 15 x 28 cm) one hour prior to the beginning of the dark cycle to allow for habituation. Food and water were provided. Each animal was assessed for the 8 hours of the dark cycle.

Locomotor Monitoring

To assess the selectivity of the motor effects of the triple cocktail of drugs, we injected three deer mice with L-741,626 (30 mg/kg), CGS21680 (0.05 mg/kg), and CDPPB (30 mg/kg) and compared their locomotor responsivity in an open field to three deer mice injected with vehicle. We used video tracking software (Ethovision, Noldus Information Technology) to measure total distance traveled and velocity. Locomotion was tested in individual mice 20 minutes following injection and testing lasted ten minutes.

Data analysis

The dependent measure used for these experiments was a difference score of the number of jumps counted in the 60 minutes following injections minus the number of jumps counted in the 60 minutes preceding injections. These scores were compared for each mouse in the vehicle and drug conditions using paired t-tests. This crossover design was necessitated by the high individual differences between mice and the need to reduce type II error when examining new drug treatments. Data from the locomotor monitoring experiment were compared using unpaired t-tests.

Experiment 2: Acute Administration of an Inverse Drug Cocktail to Increase Repetitive Behavior

Animals

Five male deer mice were used in this experiment to evaluate the effect on repetitive behavior by a drug cocktail made up of an adenosine A2a antagonist, a dopamine D2 receptor agonist, and a glutamate mGluR5 NAM. Mice were weaned and housed as described in Experiment 1.

Drugs

SCH58261 (an adenosine A2a antagonist), quinpirole hydrochloride (a dopamine D2 agonist), and MTEP hydrochloride (a glutamate mGluR5 NAM) were purchased from Sigma-Aldrich. They were dissolved in 10% DMSO and saline at 0.1 mg/mL for SCH58261, 0.3 mg/mL for quinpirole, and 0.5 mg/mL for MTEP. The combined drug cocktail was administered at 1 mg/kg SCH58261, 3 mg/kg quinpirole, and 5 mg/kg MTEP.

Repetitive Behavior Testing

Repetitive behavior was quantified as described in experiment 1. For this experiment we hypothesized that the drug cocktail would increase the expression of repetitive behavior. We injected the drug cocktail at a time during the dark cycle when repetitive behavior counts were at their lowest (unpublished observations), approximately 2:00 PM.

Data Analysis

This experiment was run as a random crossover design wherein injections were separated by at least one week. The number of jumps was recorded for 30 minutes pre-injection and 30 minutes post injection. Pre-injection and post-injection jump frequencies were compared between the vehicle and drug administration days using a 2-way analysis of variance (ANOVA).

Experiment 3: Acute Administration of Drug Cocktail in Oil to Extend the Duration of Action on Repetitive Behavior

Animals

Fifty two male deer mice were used for this experiment to evaluate an oil based formulation of the triple drug cocktail (using a dopamine D2 receptor antagonist, an

adenosine A2a agonist, and a glutamate mGluR5 PAM). Mice were weaned and housed as described in Experiment 1.

Drugs

L-741,626, CGS21680, and CDPPB were acquired as described in Experiment 1. Each drug was suspended in peanut oil and left stirring for at least one hour before injection. L-741,626 was suspended at 0.5 mg/mL, CGS21680 was suspended at 0.03 mg/mL, and CDPPB was suspended at 1.5 mg/mL. When drugs were given together as double or triple cocktails, a single solution was made and was administered in a single injection.

Drug Treatments

Based on the known hydrophylicity of each drug, additional testing of efficacy and nonselective motor suppression was required. Using separate cohorts of mice we tested L-741,626 at 5 mg/kg (n=19), CGS21680 at 0.3 mg/kg (n=11), and CDPPB at 15 mg/kg (n=11). In addition, we ran a crossover experiment comparing the oil vehicle, and double drug combinations, L-741,626 + CGS21680, L-741,626 + CDPPB, and CGS21680 + CDPPB in a separate cohort of mice (n=10). We also ran a comparison of the triple drug cocktail and oil vehicle in a separate cohort of mice (n=10).

Repetitive Behavior Testing

Repetitive behavior was quantified as described in Experiment 1. For the single and double drug analyses, injections were administered at 2:00 PM in order to evaluate the duration of action of any effective combinations. For the triple drug cocktail assessment, injections were given as soon as the lights turned off (10 AM) in order to evaluate the full duration of action.

Data Analysis

For the single and double drug experiments we used a difference score of the total number of jumps counted post-injection (4 hours total) minus the total number of jumps counted pre-injection (4 hours total). The comparison of L-741,626 and vehicle was run with independent groups of mice (drug: n=7, vehicle: n=12) and was compared using an unpaired t-test. The comparisons for CGS21680, CDPPB, and the double drug combinations were run as crossover experiments and were analyzed using paired t-tests (for single drug experiments) or repeated measures ANOVA (for double drug experiment). In a follow-up analysis of the CGS21680 data, we analyzed pre-injection and post-injection jump counts separately using paired t-tests. For the triple drug cocktail experiment, the total number of jumps for the five hours following injections was used as the dependent measure. A paired *t*-test was used for this crossover experiment.

Experiment 4: Sub-chronic Administration of Drug Cocktail in Oil for Long-term Reduction of Repetitive Behavior

Animals

Twenty one male deer mice were used for this experiment. The mice were weaned and housed as described in Experiment 1.

Drugs

L-741,626 (0.5 mg/mL), CGS21680 (0.03 mg/mL), and CDPPB (1.5 mg/mL) were suspended in peanut oil. The suspensions were made up fresh each day and left stirring for at least one hour before injection.

Drug Treatments

Independent groups of deer mice were injected with either the triple drug cocktail (L-741,626 at 5 mg/kg, CGS21680 at 0.3 mg/kg, and CDPPB at 15 mg/kg; n=11) or peanut oil vehicle (n=10). Injections were given at lights out (10 AM) each day for seven days.

Repetitive Behavior Testing

Repetitive behavior testing was conducted as described in Experiment 1, but only on days 1, 4, and 7 of drug administration. On days 2, 3, 5, and 6 each mouse was injected and then immediately returned back to their home cage for the rest of the day.

Data Analysis

The total number of jumps counted throughout the test day was used as the dependent measure. Drug and vehicle comparisons were completed using a two-way repeated measures ANOVA, which analyzed the main effects of treatment and time and their interaction. The significant effects were further analyzed by a Bonferroni post-test.

CHAPTER 3 RESULTS

Experiment 1: Acute Administration of Drug Cocktail in Saline Vehicle to Reduce Repetitive Behavior

In the single drug experiments we evaluated the efficacy of three doses of the dopamine D2 receptor antagonist, L-741,626, and two doses of the glutamate mGluR5 receptor PAM, CDPPB. Repetitive behavior was not significantly reduced by any of these single drug challenges (3 mg/kg L-741,626: $t(12)=0.5573$, $p=0.5876$; 10 mg/kg L-741,626: $t(8)=0.9497$, $p=0.3701$; 30 mg/kg L-741,626: $t(12)=1.084$, $p=0.2997$; 3 mg/kg and 30 mg/kg CDPPB: $F(2,32)=0.6528$, $p=0.5314$; Figs. 3-1 and 3-2). In addition, our previous work found that a single dose of 0.05 mg/kg of the adenosine A2a receptor agonist, CGS21680, also had no significant effect on repetitive behavior (Tanimura et al., 2010).

Our analyses of the double drug combinations revealed much of the same. No significant reduction of repetitive behavior was found with the L-741,626 + CGS21680 combination ($t(11)=0.9446$, $p=0.3652$; Fig. 3-3), nor the L-741,626 + CDPPB combination ($t(11)=1.561$, $p=0.1468$, Fig. 3-4), or the CGS21680 + CDPPB combination ($t(12)=1.974$, $p=0.0719$, Fig. 3-5).

A significant reduction in repetitive behavior was found using the triple drug cocktail of drugs, L-741,626 + CGS21680 + CDPPB ($t(11)=3.985$, $p=0.0021$; Fig. 3-6). This effect was selective for repetitive behavior and was not due to nonselective motor suppression, as revealed by our test of locomotor reactivity in an open field. We found no between groups differences in total distance traveled or velocity in mice treated with either vehicle or the triple drug cocktail (distance: $t(4)=1.811$, $p=0.1443$; velocity: $t(4)=1.692$, $p=0.1659$; Fig. 3-7).

Experiment 2: Acute Administration of an Inverse Drug Cocktail to Increase Repetitive Behavior

Results from Experiment 1 suggested that only a triple cocktail of drugs targeted to the dopamine D2, adenosine A2a, and glutamate mGluR5 receptors, which are located on the striatal indirect pathway neurons, could reduce repetitive behavior. As a proof of concept we investigated whether a converse combination of drugs targeted to the heteromeric receptors could further decrease striatal indirect pathway neurons and increase repetitive behavior. We found that the dopamine D2 agonist, quinpirole, the adenosine A2a antagonist, SCH58261, and the glutamate mGluR5 NAM, MTEP, significantly increased repetitive behavior in the thirty minutes following injection (Fig. 3-8). The two-way ANOVA revealed no significant main effect of time ($F(1,8)=0.5142$, $p=0.4937$) or drug ($F(1,8)=1.539$, $p=0.25$), but a significant drug x time interaction ($F(1,8)=7.913$, $p=0.0227$). This significant interaction was mediated by a reduction of repetitive behavior in the vehicle group, which we anticipated based on our understanding of the time course of repetitive behavior throughout the dark cycle, and a significant increase in repetitive behavior in the mice administered the converse triple drug cocktail.

Experiment 3: Acute Administration of Drug Cocktail in Oil to Extend the Duration of Action on Repetitive Behavior

Results from Experiment 1 were encouraging but the duration of drug effect was relatively short, lasting only 60 minutes. Drugs suspended in oleaginous solution have a longer duration of action, so we examined whether a peanut oil vehicle could extend the duration of action of the triple drug cocktail. Because the hydrophobicity of the drugs, which changes their solubility in oil relative to aqueous solution, we retested each single

and double drug combination to confirm that there were no nonselective motor effects with the new doses and formulation. A single dose of the dopamine D2 receptor antagonist, L-741,626, did not significantly reduce repetitive behavior ($t(17) = 2.099$, $p=0.051$; Fig 3-9). The paired t -test of the difference score (post-injection minus pre-injection) for the adenosine A2a agonist, CGS21680, revealed a significant difference in repetitive behavior between the vehicle and drug-treated mice ($t(10)=2.701$, $p=0.0223$; Fig. 3-10), though a follow-up analysis of the pre-injection and post-injection jump totals showed that this significant effect was mediated by a difference in pre-injection jump totals ($t(10)=2.254$, $p=0.0478$) and not by any drug-mediated change in post-injection behavior ($t(10)=1.335$, $p=0.2114$). Furthermore, a single injection of the glutamate mGluR5 receptor PAM, CDPPB, also did not significantly reduce repetitive behavior ($t(10)=0.1065$, $p=0.9173$; Fig. 3-11). A crossover study also showed no significant effect of any of the double drug combinations on repetitive behavior ($F(3,39)=1.88$, $p=0.1568$; Fig. 3-12).

Consistent with our finding in Experiment 1, the triple drug combination significantly reduced repetitive behavior ($t(9)=2.705$, $p=0.0242$; Fig. 3-13). This significant reduction in repetitive behavior lasted nearly five hours and an examination of the video recordings suggested no nonselective motor suppression.

Experiment 4: Sub-chronic Administration of Drug Cocktail in Oil for Long-term Reduction of Repetitive Behavior

To examine the long-term efficacy of the triple drug cocktail in oil, we injected independent groups of mice each day for seven days and tested their repetitive behavior on days 1, 4, and 7. A two-way repeated measures ANOVA revealed a significant main effect of drug ($F(1,36)=14.57$, $p=0.0013$) and no significant main effect

of time ($F(2,36)=1.643$, $p=0.2075$) or drug x time interaction ($F(2,36)=0.003$, $p=0.9968$). This shows that the triple drug cocktail continued to stay effective at redacting repetitive behavior over each of the test days and that rates of repetitive behavior did not change within either the drug or vehicle groups across time. The Bonferroni post-test confirmed that the reduction in repetitive behavior by the triple drug cocktail was significant at each time point (Fig. 3-14).

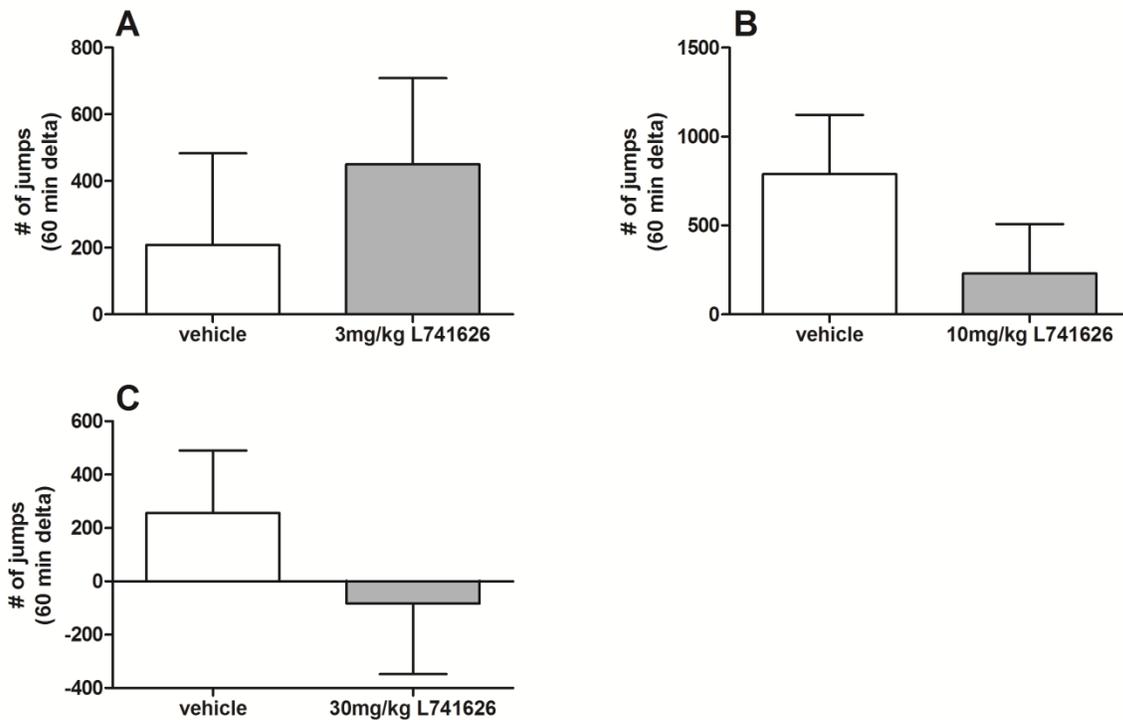


Figure 3-1. Acute administration of a dopamine D2 receptor antagonist in saline. Single doses of the dopamine D2 receptor antagonist, L-741,626, had no significant effect on repetitive behavior.

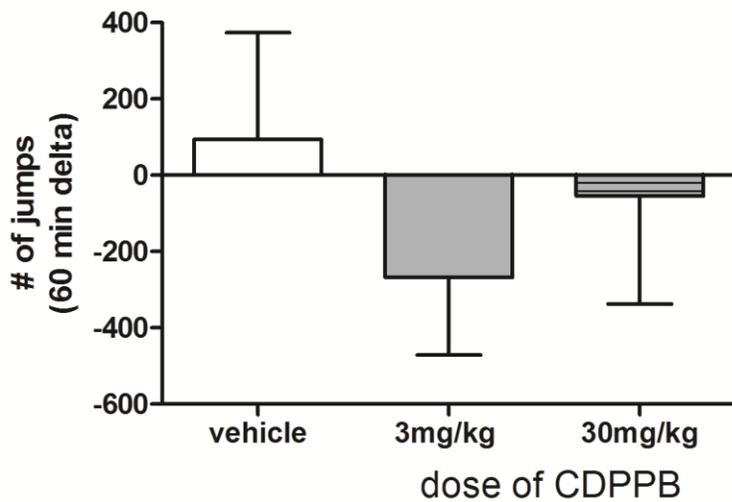


Figure 3-2. Acute administration of a glutamate mGluR5 receptor positive allosteric modulator in saline. Single doses of the glutamate mGluR5 PAM, CDPPB, had no significant effect on repetitive behavior.

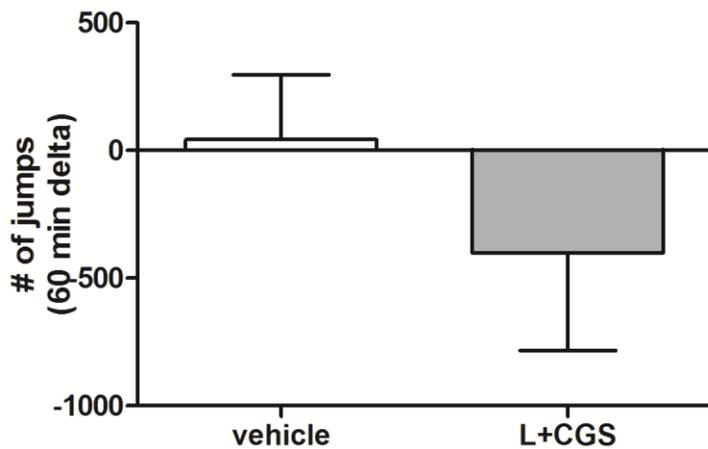


Figure 3-3. Acute administration of a dopamine D2 receptor antagonist and an adenosine A2a receptor agonist in saline. A combination of the dopamine D2 receptor antagonist (L-741,626) and the adenosine A2a receptor antagonist (CGS21680) had no significant effect on repetitive behavior.

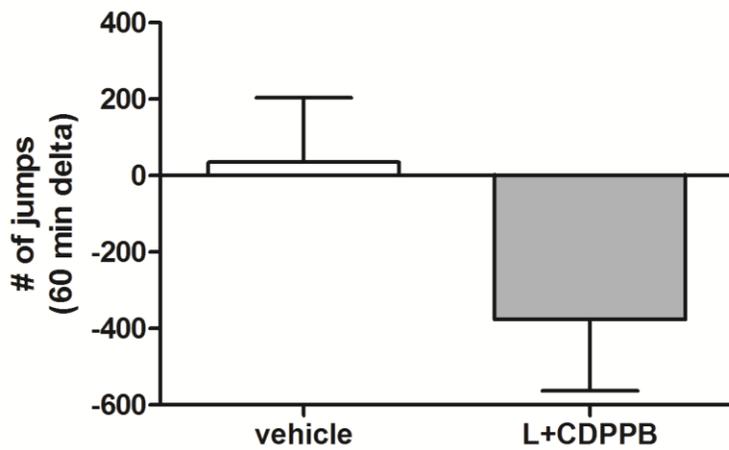


Figure 3-4. Acute administration of a dopamine D2 receptor antagonist and a glutamate mGluR5 receptor positive allosteric modulator in saline. A combination of the D2 receptor antagonist (L-741,626) and the glutamate mGluR5 PAM (CDPPB) had no significant effect on repetitive behavior.

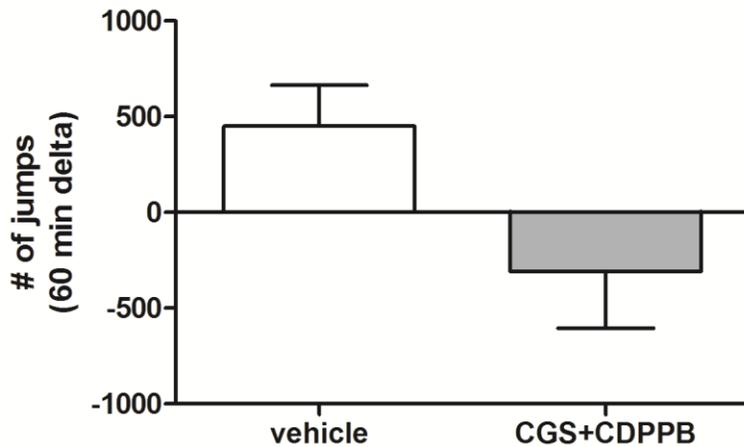


Figure 3-5. Acute administration of an adenosine A2a receptor agonist and a glutamate mGluR5 receptor positive allosteric modulator in saline. A combination of the adenosine A2a receptor agonist (CGS21680) and the glutamate mGluR5 PAM (CGS21680) had no significant effect on repetitive behavior.

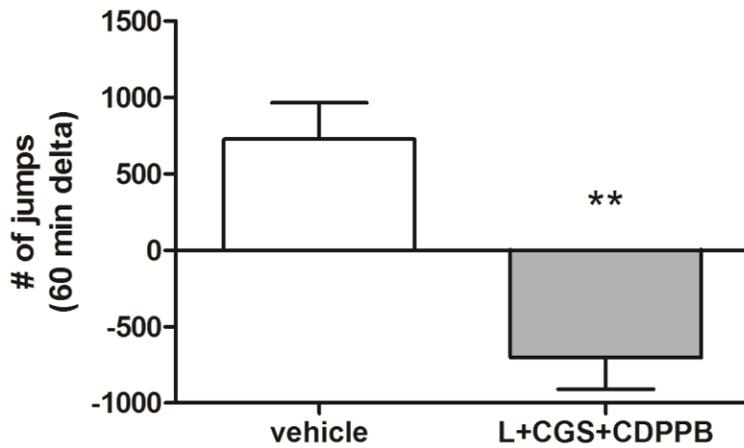


Figure 3-6. Acute administration of a dopamine D2 receptor antagonist, an adenosine A2a receptor agonist, and a glutamate mGluR5 receptor positive allosteric modulator in saline. A triple drug cocktail made up of the dopamine D2 receptor antagonist (L-741,626), the adenosine A2a receptor agonist (CGS21680), and the glutamate mGluR5 PAM significantly reduced repetitive behavior.

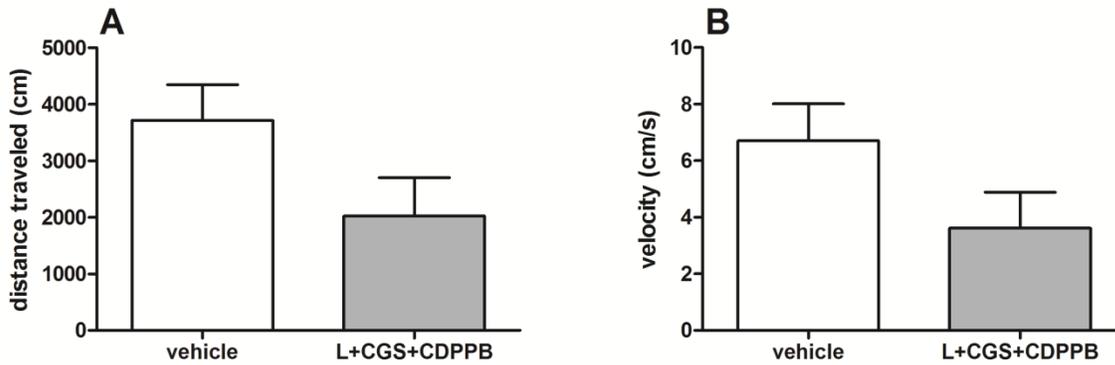


Figure 3-7. Test of locomotor reactivity in an open field. The triple drug cocktail had no significant effect on distance traveled (A) or velocity of locomotion (B), compared to that of vehicle-treated mice.

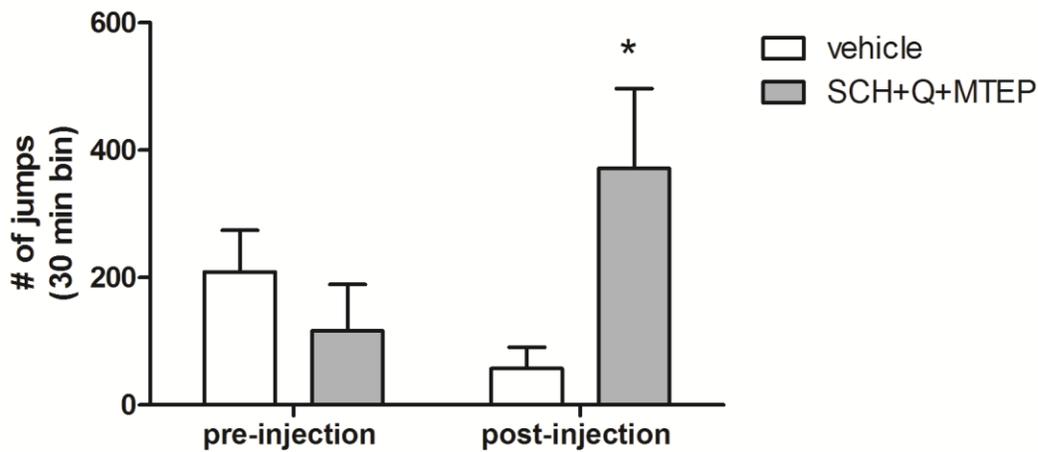


Figure 3-8. Acute administration of a dopamine D2 receptor agonist, an adenosine A2a receptor antagonist, and a glutamate mGluR5 receptor negative allosteric modulator in saline. Rates of repetitive behavior in the vehicle- and drug-injected groups were similar before injections, but diverged post-injection. The triple drug cocktail made up of the adenosine A2a receptor antagonist (SCH58261), the dopamine D2 receptor agonist (quinpirole), and the glutamate mGluR5 receptor NAM (MTEP), significantly increased repetitive behavior.

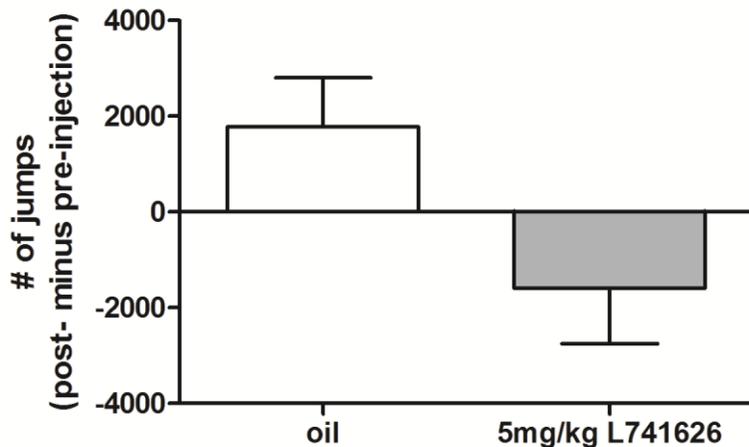


Figure 3-9. Acute administration of a dopamine D2 receptor antagonist in oil. A single dose of the dopamine D2 receptor antagonist, L-741,626, suspended in peanut oil had no significant effect on repetitive behavior.

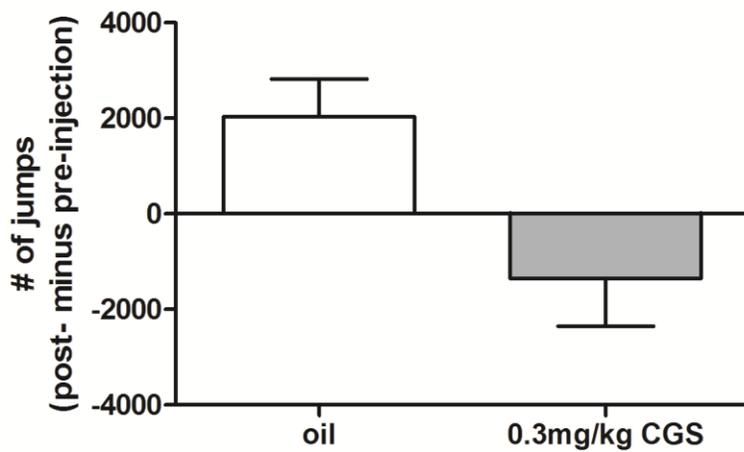


Figure 3-10. Acute administration of an adenosine A2a receptor agonist in oil. A single dose of the adenosine A2a receptor agonist, CGS21680, suspended in peanut oil had no significant effect on repetitive behavior.

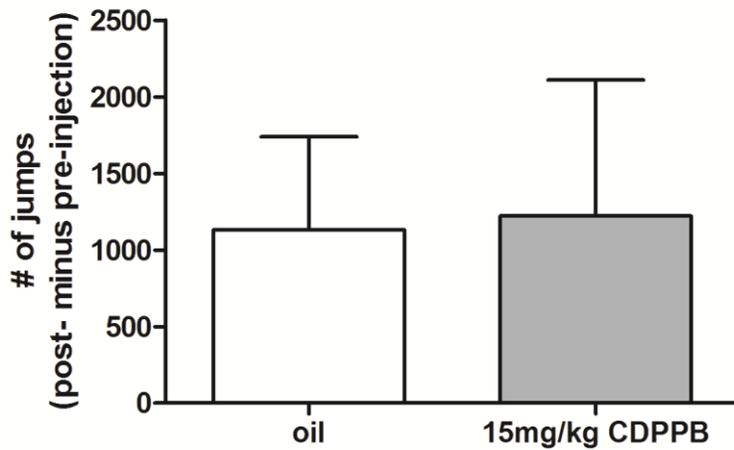


Figure 3-11. Acute administration of a glutamate mGluR5 positive allosteric modulator in oil. A single dose of the glutamate mGluR5 receptor PAM, CDPPB, suspended in peanut oil had no significant effect on repetitive behavior.

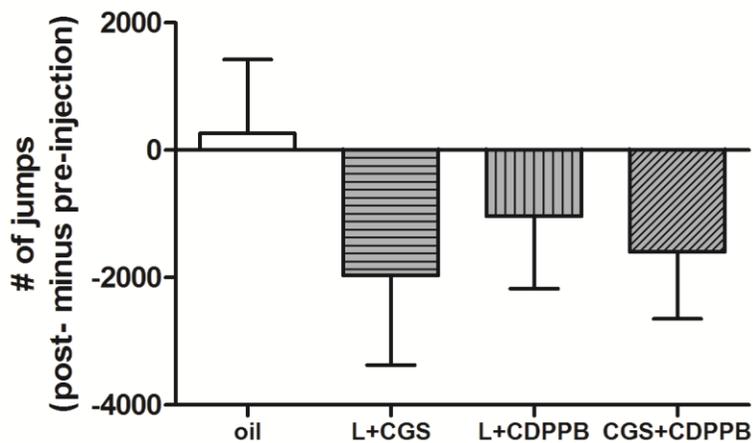


Figure 3-12. Acute administration of the double drug combinations in oil. Combinations of the double drug cocktails suspended in peanut oil had no significant effects on repetitive behavior.

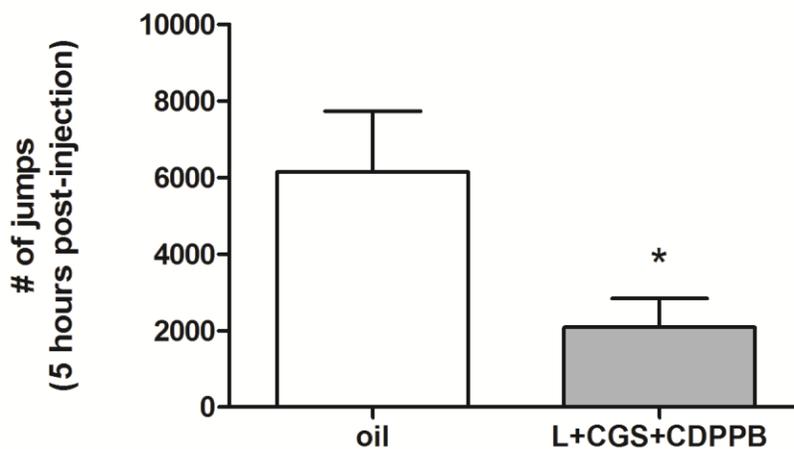


Figure 3-13. Acute administration of a dopamine D2 receptor antagonist, an adenosine A2a receptor agonist, and a glutamate mGluR5 receptor positive allosteric modulator in oil. The triple drug cocktail suspended in peanut oil significantly reduced repetitive behavior.

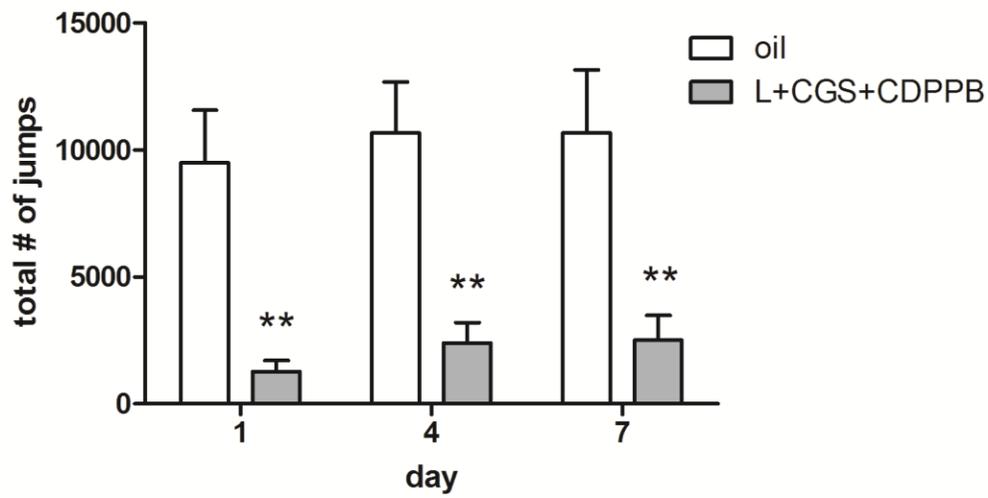


Figure 3-14. Sub-chronic administration of the triple drug cocktail in oil. Sub-chronic administration of the triple drug cocktail shows continued reduction of repetitive behavior across seven days of injections.

CHAPTER 4 DISCUSSION

Repetitive behaviors are an impairing characteristic of many neurodevelopmental, neurological, and psychiatric disorders, yet there are no medications that are specifically approved for their treatment. Reasons for this are numerous, but one of the most important factors is that we do not yet understand the neuropathologies that mediate repetitive behavior. Elucidating the dysregulated neural circuits will allow better pharmacotherapeutic strategies and reveal novel drug targets.

Previous work using the deer mouse model has revealed specific downregulation of indirect basal ganglia pathway function (Presti et al., 2003; Presti & Lewis, 2005; Tanimura et al., 2010; Tanimura et al., 2011). We hypothesized that targeting dopamine, adenosine, and glutamate heteromeric receptors, which are only found on the indirect pathway neurons of the striatum, would significantly reduce repetitive behavior. In fact, we found that in both an aqueous and an oleaginous formulation, the triple drug cocktail targeting these receptor heteromers (a dopamine D2 receptor antagonist, an adenosine A2a receptor agonist, and a glutamate mGluR5 receptor PAM) significantly and selectively reduced repetitive behaviors. Single and double drug preparations were not effective. In addition, our sub-chronic administration protocol continued to show a significant drug effect across seven days of injections, indicating that tolerance or supersensitivity of the receptors to the drugs would not reduce drug efficacy with repeated administration. Finally, to further our understanding of the role of the indirect pathway neurons of the striatum in the expression of repetitive behavior, we tested whether a converse drug cocktail could increase the expression of repetitive behavior. We found that a combination of a dopamine D2 receptor agonist, an

adenosine A2a receptor antagonist, and a glutamate mGluR5 NAM, which we hypothesize to reduce striatal indirect pathway neuron function, did significantly increase repetitive behavior.

These experiments represent our initial attempts to find targeted pharmacotherapy for repetitive behaviors and will be the basis for future drug development. There are numerous advantages to a polypharmacy strategy targeted to heteromeric receptor complexes. Targeting particular receptor complexes on particular neurons allows selectivity of receptor activation and eliminates the necessity for high doses of singular drugs that may bind to receptors all over the central nervous system (CNS). These relatively lower doses of drugs reduce the side effect profile of each drug and may improve the likelihood of approval in the sensitive neurodevelopmental, neurological and psychiatric populations. This pharmacological strategy is also preferred over standard single target drugs because they take advantage of the normal physiological functioning of the cell. Numerous heteromeric complexes have been identified throughout the CNS and researchers are beginning to understand the differential functioning of receptor monomers and heteromers upon activation at the level of cell signaling cascade pathways (Agnati et al., 2003). Additionally, the effect of heteromeric receptor activation on cell signaling cascades can have synergistic effects such that targeting heteromeric receptor complexes can have significantly more impact on cell functioning than single drug exposure would.

Although there are many advantages to using multiple drugs to bind to receptor heteromers, designing a single molecule to bind within the binding pore of the heteromeric complex would be preferred. Finding a single molecule to preferentially

activate particular cell signaling cascades linked to the dopamine, adenosine, and glutamate receptor heteromer would significantly improve pharmacotherapeutic effect and reduce side effects.

Our finding of significant and selective reduction of repetitive behavior in deer mice with a triple drug cocktail designed to target the dopamine D2, adenosine A2a, and glutamate mGluR5 receptor is encouraging and suggests that further study of the mechanism of action is needed. We formulated the triple drug cocktail based on our understanding of each receptor's association with cell signaling cascades and their effect on cellular activation. Future studies should confirm our hypothesis that the triple drug cocktail increased activation of the indirect pathway neurons of the striatum. Additionally, the effect of the triple drug cocktail on the neuropeptide enkephalin should be explored. Antagonism of dopamine D2 receptors and agonism of glutamate mGluR5 receptors both increase enkephalin content (Steiner & Gerfen, 1998). Previous work from our lab showed significantly lower levels of enkephalin in the striata of deer mice with high rates of repetitive behavior, as compared to deer mice with low rates of repetitive behavior (Presti & Lewis, 2005). Future studies should also elucidate which cell signaling pathways and transcription factors mediate the positive pharmacotherapeutic effect. Adenosine A2a and glutamate mGluR5 cascades utilize overlapping molecules, including MAP kinase and CREB (Agnati et al., 2003). It will be important to understand which pathways are beneficial to drug response and to confirm that any single molecules that are designed to hit the receptor heteromer signal through the same cascades. These future experiments will improve our understanding of the

neurobiological mechanisms that mediate repetitive behavior reduction and will lead to the elucidation of more potential targets for novel pharmacotherapies.

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BIOGRAPHICAL SKETCH

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