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The Effect of Metabotropic Glutamate 5 Receptors Stimulation on Delayed Non-Match to Odor Performance in Long-Evans Male Rats

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Abstract

Patients suffering from Major Depressive Disorder (MDD) have cognitive impairments that modern antidepressants are unable to treat. There is a large body of research linking glutamate receptors to both cognitive functions and MDD. The present study looks at one type of glutamate receptors, the metabotropic glutamate 5 (mGlu 5) receptor, and the effects of its stimulation on a cognitive task on male Long-Evans rats. The following two hypotheses were used: 1) Memory performance in the delayed non-match to odor task will be progressively impaired as the time between the information and retention trial increases, and 2) administering a mGlu5 positive allosteric modulator such as CDPPB will improve performance on the delayed non-match to odor task. First, a time delayed non-match to odor task (DNMTO) was tested to see if it could function as a test of short-term memory. Results indicated that an increase in inter-trial intervals significantly lowered accuracy in the task. This DNMTO task thus showed a more ethologically relevant working memory task than the commonly used visual-based tasks as rats have poor vision. Then the rats were dosed with 3-Cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB), a positive allosteric modulator for mGlu 5 receptors, to test for an improvement on the task. No significant differences were found between control or any dose (1, 3, or 10 mg/kg IP) in either accuracy, latency, distance or speed. However, large effect sizes were found which implies that there was not enough power due the low sample sizes caused by attrition. This study was part of a larger research project delving into different glutamate receptors and their impact on both cognitive function and MDD.

Keywords: mGlu 5, major depressive disorder, non-matched to sample

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Major Depressive Disorder

Major depressive disorder (MDD) is a mood disorder that requires at least five of the following symptoms: 1) depressed mood, 2) loss in interest and/or pleasure, 3) increase or decrease in appetite, 4) insomnia or hypersomnia, 5) psychomotor agitation or retardation, 6) fatigue, 7) feelings of worthlessness or guilt, 8) decrease in the ability to think or concentrate, 9) recurrent thoughts of death (American Psychiatric Association, 2013). MDD effects over 300 million people globally (World Health Organization, 2018), and had an estimated economic burden of $210.5 billion for the United States in 2010 (Greenberg et al., 2015). Estimates of this number suggest that the economic burden has been increasing by a rate of 21.5% in from 2005 to 2010, when accounting for inflation (Greenberg et al., 2015).

Impaired Cognitive Function in MDD

Cognitive impairments are present 94% of the time during depressive episodes and are present 44% of the time when not in a depressive episode (Conradi et al., 2011). A large number of studies have demonstrated an impairment in cognitive function in patients with MDD. Over 20% of people with MDD suffer from cognitive impairments of at least two standard deviations in one or more of the following categories: memory, psychomotor speed, reaction time, complex attention and cognitive flexibility (Gualtieri & Morgan, 2008). This is supported by a meta-analysis that found that 20 to 30 percent of patients with MDD have impairments of at least one standard deviation in executive function, in which they included concept formation, abstraction, set shifting, set maintenance, planning, self-monitoring, and divided attention (McIntyre et al., 2013). The same meta-analysis also found that there is a consistent cognitive impairment across

several other functions, including but not limited to working memory, attention, verbal memory, and spatial memory (McIntyre et al., 2013). Unmedicated patients with depression maintain cognitive deficiencies even after other clinical symptoms are gone (Herrera-Guzmán et al., 2010). An older study found that about 15% of patients scored one or two standard deviations below normal on tasks of verbal fluency, visuo-motor tracking, visuo-spatial tracking, and verbal learning, with it increasing to 50% of patients on visual attention and task switching (Veiel, 1997). Patients with MDD also performed significantly worse on a process dissociation task (MacQueen et al., 2003). In addition, elderly patients with MDD both perform worse on cognitive measures (working memory, information-speed, attention, and episodic memory) and improve at a much lower magnitude than elderly controls even after treatment (Nebes et al., 2003). Furthermore, yet another metanalysis on cognitive deficits demonstrated that people with no depression symptoms performed better on working memory measures than those with mild or worse symptoms (Lee et al., 2012). Patients with MDD performed significantly worse on tests of working memory, including the digit span and visuo-spatial span tasks (Fossati et al., 1999). Another meta-analysis found that severity of cognitive deficiencies in MDD in tasks such as the Trail Making Test, the Stroop Task, and other memory, visuo-motor, visuo-spatial and verbal fluency tasks, were similar to individuals with moderately severe traumatic brain injuries (Veiel, 1997).

Many of the tasks that patients with MDD have impairments in are tasks that require the prefrontal cortex (PFC). The PFC is the anterior portion of the frontal lobe of the cerebral cortex. It makes up one-third of the cerebral cortex in humans (Siddiqui et al., 2008). The main functions of the PFC include executive function, memory, intelligence, language, visual search and gaze control. Working memory has long been associated with the prefrontal cortex (PFC) in humans

(Manoach et al., 1997). Working memory is defined as the temporary holding of information so that it can be manipulated (Baddeley, 1992). This memory is then either encoded into long-term memory (LTM) or lost. Working memory involves several brain regions including the PFC, the parietal cortex, and the basal ganglia. The PFC is critical to retain information during working memory tasks, particularly as the difficulty of the task is increased (Eriksson et al., 2015). Previous studies have attempted to map out how subregions of the PFC process working memory. In humans, the left ventral PFC has been shown to be more involved in verbal working memory, while the right dorsal PFC is more involved in spatial working memory (Eriksson et al., 2015). In rats, the analogous region to the PFC is the medial prefrontal cortex (mPFC), part of the anterior cingulate cortex (Paxinos & Watson, 2013).

Patients with MDD do not only have the cognitive impairments previously mentioned, but also have physical changes in the areas of the brain important for those tasks. A meta-analysis has found that MDD is associated with a reduction in gray matter in the prefrontal cortex, particularly in the dorsolateral and dorsomedial regions (Bora et al., 2012). In addition, both neuronal size and glial cell density are reduced in the prefrontal cortex for patients with MDD (Cotter, 2002). Patients with MDD have been found to have volume reductions in the gray matter regions of the dorsolateral and dorsomedial prefrontal cortex, with larger reductions in patients with multiple episodes (Bora et al., 2012). Patients with MDD also have reductions of about 20% of glial cells in the prefrontal cortex (Öngür, Drevets, & Price, 1998). Therefore, MDD has been reliably shown to cause physical changes in the PFC that can also be measured through cognitive tasks.

These data are important from the perspective that the magnitude of cognitive impairment may predict the degree of functional recovery among MDD patients (Jaeger et al., 2006), and

patients with higher levels of impaired cognitive function may be less likely to respond to antidepressant treatment (Dunkin et al., 2000). Even with the negative impact of this disease on cognitive abilities, most treatments for MDD focus on the emotional impairments and not on the cognitive impairment, despite the fact that cognitive deficiencies are one of the requirements for a MDD diagnosis (American Psychiatric Association, 2013). These cognitive dysfunctions affect the patient's ability to function in society, school, their job, or any other daily scenario. These impairments are often accompanied by measurable differences in the brain. Unfortunately, modern treatments are not efficient at alleviating these cognitive deficiencies.

Most modern antidepressants fall into either the selective serotonin reuptake inhibitor (SSRI) or serotonin-norepinephrine reuptake inhibitors (SNRI) categories. These drugs, however, suffer from high resistance rates as many patients fail to respond to them. In addition, they have a long-delayed onset before alleviating the symptoms. The antidepressants vortioxetine, duloxetine, paroxetine, citalopram, phenelzine, nortriptyline, and sertraline did not have significant effects on cognitive control or executive functioning however, there was a small improvement on psychomotor speed and delayed recall. Vortioxetine improved cognition in executive functioning, including attention, learning and memory, processing speed, and working memory (Mahableshwarkar et al., 2015; McIntyre et al., 2014). Another study looked at cognitive deficiencies in working memory, attention, and episodic memory in elderly patients and found that these impairments still persisted even after being treated with either an SSRI (parozetine) or a tricylic antidepressant (nortriptyline) (Nebes et al., 2003). Over half of patients who have suffered from MDD reported being significantly disabled, or worse, in neurocognitive tasks after 6 months of treatment after being hospitalized for psychiatric (Jaeger et al., 2006).

Thus, overall current treatments are not fulfilling the need to treat the cognitive impairments that are afflicting MDD patients.

MDD and Glutamate Neurotransmission

For a long time, research in MDD focused solely on serotonin (5-hydroxytryptamine or 5-HT) neurotransmission with some inclusion of norepinephrine. More recently however, MDD has also been tied to glutamate neurotransmission. Glutamate is the most abundant excitatory neurotransmitter in the central nervous system. It is the ionized form of glutamic acid, another amino acid. It is not only used as a neurotransmitter, but for other cellular metabolic functions as well. Glutamate is found throughout the brain, in both neurons and glial cells. Most glutamate is intracellular and located at nerve terminals. Too much extracellular glutamate will kill cells by overly activating the glutamate receptors. Astrocytes will pull in extra glutamate from the extracellular space and turn it into glutamine, which neurons can then turn into glutamate (Danbolt, 2001).

Glutamate receptors can be divided into two main types: ionotropic receptors and metabotropic receptors. The ionotropic glutamate receptors are ligand-gated ion channels and include the NMDA, AMPA and kainate receptors. When glutamate binds to these, it opens the channel and allows ions to flow in. Thus, ionotropic glutamate receptors are critical mechanisms for fast excitatory neurotransmission. NMDA receptors, a type of ionotropic glutamate receptor, function as “coincidence detectors” by requiring both glutamate and glycine to bind to their respective sites to open, followed by a cellular membrane depolarization to clear a Mg^{2+} ion that blocks the channel (Halliwell et al., 1989).

On the other hand, metabotropic glutamate (mGlu) receptors are G-protein coupled receptors that react to the neurotransmitter glutamate. When glutamate binds to the receptor, it

sends the G-protein to activate second messengers depending on the type of metabotropic receptor. There are eight subtypes of mGlu receptors divided into three groups, including group one, which contains mGlu 1 receptors and mGlu 5 receptors. Unlike the Group II and Group III receptors which are primarily inhibitory, Group I mGlu receptors have been found to facilitate cellular activity (Cartmell & Schoepp, 2002).

Metabotropic Glutamate 5 Receptors

There are two mGlu 5 receptors splice variants: mGlu 5a receptors and mGlu 5b receptors, both of which are coupled to phospholipase C by $G_{q/11}$. They are activated selectively by (S)-3,5-dihydroxyphenylglycine (DHPG), which activates phospholipase Cβ1. This releases intracellular Ca^{2+} stores, which in turn activates protein kinase C, also known as PKC (Niswender & Conn, 2010). PKC is necessary for phosphorylation of glutamate transporters and aids in higher glutamate uptake (Lortet et al., 1999). Therefore, activation of mGlu 5 receptors leads to an increase in glutamate neurotransmission. See Figure 1 for a diagram of this process.

MGlu 5 receptors can physically interact with the ionotropic N-methyl-D-aspartate (NMDA) glutamate receptor through the Shank and Homer proteins (Cartmell & Schoepp, 2002; Perroy et al., 2008; Pilc et al., 2013), which is important for many functions including brain development and cognitive function. One way depolarization can happen is with mGlu5 receptor activation, which is why mGlu 5 receptors can also biochemically potentiate NMDA receptors (Mannaioni et al., 2001). In addition, mGlu5 receptors and NMDA receptors are mutually interacting (Cartmell & Schoepp, 2002).

MGlu 5 receptor activation and its release of Ca^{2+} activates eukaryotic elongation factor-2 kinase (eEF2K). eEF2k activates the activity-regulated cytoskeleton-associated protein (Arc, also known as Arg3.1). The activation of Arc in turn, is necessary to regulate long-term

potentiation (LTP) and long-term depression (LTD). Both LTP and LTD are long-lasting synaptic changes caused by the activity of a given synapse. While LTP strengthens the activity and makes it easier for the neuron to activate, LTD makes it more difficult. Arc is involved with both LTD and LTP, as well as dendritic plasticity, although LTP tends to happen first followed by long term LTD. With mGlu 5 receptors, after the initial LTP response, LTD is the primary response to their activation (Li et al., 2015; Park et al., 2008). These synaptic changes lead to lasting differences in how the brain processes information. LTP and LTD are both necessary for learning and memory and synaptic plasticity (Luscher & Malenka, 2012). More specifically, LTP increases dendritic spine head diameters, while LTD decreases them (LaCrosse et al., 2015). This altered neuronal plasticity has been linked to MDD and other brain disorders.

Overall, this demonstrates that mGlu 5 receptors are an important part of cognition and memory. Their activation causes a chain of sub-cellular actions, culminating in long-lasting changes to the brain's neuronal activity, far beyond the initial activation of the receptor. The mutual interaction between mGlu 5 receptors and NMDA receptors is another important function of mGlu 5. Furthermore, there is a widespread distribution of mGlu 5 receptors throughout the brain.

MGlu 5 Receptor Distribution

Mglu5 receptors are located all through the rat brain in the postsynaptic dendritic areas of neurons and astrocytes (Niswender & Conn, 2010). The lateral septum had a very strong expression of mGlu 5 receptors and the triangular nucleus has strong labeling (Romano et al., 1995; Shigemoto et al., 1993). In the brainstem, the superior superficale, dorsal cortex, and spinal nucleus all have high levels of mGlu 5 receptor expression (Luján et al., 1996; Romano et al., 1995). The basal ganglia is a region with intense mGlu 5 receptor labeling, although this

labeling is focused in the nucleus accumbens and caudate/putamen (Romano et al., 1995; Shigemoto et al., 1993), with only a light level of mGlu 5 receptors in the substantia nigra pars reticulata (Hubert et al., 2001).

The olfactory regions have high concentrations of mGlu 5 receptors. The anterior olfactory nucleus and olfactory tubercle both demonstrate high levels of mGlu 5 receptors (Romano et al., 1995; Shigemoto et al., 1993). The olfactory bub itself has the highest levels of mGlu 5 receptors in the accessory olfactory bulb (Shigemoto et al., 1993) with strong expression also found in the external plexiform layer (Romano et al., 1995, 1995). The medial dorsal nucleus of the thalamus, responsible for relaying information from the olfactory bulb has a moderate expression of mGlu 5 receptors.

mGlu 5 Receptor Expression in Cognition-Relevant Brain Regions

The hippocampal formation has intense mGlu 5 receptor expression throughout all subfields (Luján et al., 1996). The *Cornu Ammonis* 1 (CA1) region of the hippocampus, which is one of the hippocampus' primary output regions, consistently has the highest markers of mGlu 5 receptors throughout several studies (Luján et al., 1996; Romano et al., 1995; Shigemoto et al., 1993). Within the CA1 region, the stratum oriens had the highest concentration of mGlu 5 receptor expression, followed by the stratum radiatum and then the stratum lacunosum-moleculare (López-Bendito et al., 2002; Luján et al., 1996; Romano et al., 1995). The stratum pyramidale has the weakest labeling (Luján et al., 1996; Romano et al., 1995). The CA3 region also has intense labeling although it is weaker than the CA1 region (López-Bendito et al., 2002; Luján et al., 1996; Romano et al., 1995). The labeling follows the same distribution as the CA1 albeit weaker. The straum oriens has the strongest labeling, followed by the statum radium and stratum lancunosum-moleculare; the stratum pramidale has the weakest labeling (Hubert et al.,

2001; López-Bendito et al., 2002; Luján et al., 1996). The dentate gyrus has a strong expression of mGlu 5 receptors except in the molecular layer (Luján et al., 1996; Romano et al., 1995; Shigemoto et al., 1993). Within the hippocampus, mGlu 5 receptors are generally distributed extrasynaptically on pyramidal neurons (which are the main output cells in areas such as the hippocampus and frontal cortex) with stronger expression on dendritic spines and the occasional cell body (Luján et al., 1996). In addition, there is evidence suggesting that mGlu 5 receptors within the hippocampus are expressed on astrocytes (Bradley & Challiss, 2012; Biber et al., 2001).

MGlu5 receptors are distributed throughout the rat's neocortex, with the highest concentration in the middle layers, closely followed by the superficial layers. (Romano et al., 1995). Here, mGlu 5 receptors exclusively have dendritic expression, appearing only in neuropil surrounding cells bodies in both pyramidal and non-pyramidal cells (López-Bendito et al., 2002), as well as astrocytes (Biber et al., 2001). MGlu 5 receptors in the frontal cortex are localized extrasynaptically and perisynaptically near the cell membrane. (Luján et al., 1996; Négyessy et al., 1997; Shigemoto et al., 1993).

mGlu 5 Receptors and MDD

Interestingly, there have been some studies demonstrating a promising link between mGlu 5 receptor abnormalities and MDD, particularly in both the PFC and hippocampus. Postmortem and PET studies have found that unmedicated patients with MDD have lower mGlu5 binding in areas including the prefrontal cortex and hippocampus as well as lower levels of mGlu5 protein binding in the prefrontal cortex (Deschwanden et al., 2011). These observations lend further support to the hypothesis that reduced glutamate neurotransmission is related to MDD pathophysiology. Unfortunately, while there have been a large number of studies

demonstrating mGlu 5 NAMS as a possible antidepressant, these compounds have yet to perform well on clinical trials (Barnes et al., 2018). However, other have been other glutamatergic drugs that have been linked to MDD, such as ketamine.

One flaw of current antidepressants is how they usually take several weeks or even months before effectively reducing MDD symptoms. The discovery of ketamine, a noncompetitive NMDA receptor antagonist, as an antidepressant demonstrated that there could be fast acting antidepressants, as ketamine clinically improves depressant within hours(Berman et al., 2000; Krystal et al., 2013). While traditional antidepressants function by interacting with monoamine, serotonin, or a combination of serotonin and norepinephrine receptors, ketamine differs by interacting with NMDA receptors. Ketamine binds the NMDA receptors and then upregulates AMPA receptor expression. When rodents are given an AMPA antagonist, ketamine's antidepressants are blocked, demonstrating that the AMPA receptor mechanism is relevant to ketamine's results(Maeng et al., 2008). In addition, S 47445, a positive allosteric modulator of AMPA receptors, has been shown to produce antidepressant like effects in mice and rats(Mendez-David et al., 2017). Furthermore, esketamine, a noncompetitive NMDA receptor antagonist similarly to ketamine, has also recently been demonstrated to be a fast acting antidepressant(Zheng et al., 2020). Similarly, to NMDA receptors, mGlu 5 receptor activation reduces AMPA expression. Therefore, there have been similar ideas that reducing mGlu 5 receptor activation could similarly by relevant as an MDD treatment.

Glutamatergic Drugs and Cognition

Pharmacology studies have demonstrated a link between mGlu 5 receptors and cognitive function. These studies tend to look at several key drugs. These tend to be either positive allosteric modulators (PAMs), or negative allosteric modulators (NAMs). Allosteric modulators

are drugs that function by binding to a receptor site that is different than the receptor that the endogenous agonist binds to (known as the orthosteric site), and modify the activity of the endogenous agonist for that receptor. PAMs facilitate the receptor's activity, while NAMs inhibit it. The most commonly used mGlu5 receptor NAM is 2-methyl-6-(phenylethynyl)-pyridine (MPEP), while commonly used PAMs for the mGlu 5 receptor include 3-Cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB), and VU0409551.

There have been studies that mGlu 5 receptor PAMs protect against microglia-induced neuroinflammation (Spampinato et al., 2018). However, high doses of mGlu 5 receptor PAMs are neurotoxic in rats (Parmentier-Batteur et al., 2014). CDPPB administration improves performance in the novel object recognition task in rats (Uslaner et al., 2009). However, in an attentional set-shifting task (a measure of executive function), rats dosed with CDPPB did not differ from control (Darrah et al., 2008). In addition, daily doses of 30 mg/kg CDPPB prior to T-maze training caused rats to perform better than vehicle or those given 10 mg/kg (Fowler et al., 2013).

Doses of 10 mg/kg MPEP caused impairments in working memory in rats in spontaneous alternation task, a putative test of spatial working memory (Homayoun et al., 2004). MPEP has also been found to decrease performance in the 5-choice serial reaction time task, a test of sustained attention that requires use of the PFC (Totah et al., 2013), not by decreasing accuracy, but by decreasing response speed as well as the increasing the rate of premature responses (Semenova & Markou, 2007). MPEP has also been shown to change place cell firing in the hippocampus in novel locations, impacting the long-term stability of the place field (Zhang & Manahan-Vaughan, 2014). A previous study that ran two experiments on MPEP's effects on a delayed match to position task showed that 1) MPEP increased latency at 30 mg/kg, and not at 3

or 10 mg/kg, with no difference in choice accuracy and B) MPEP had a small but significant decrease in accuracy at 100 mg/kg but not at 30 mg/kg, and only at the longest interval (Ballard et al., 2005). In addition, the mGlu 5 receptor NAM basimglurant (RO4917523, RG7090) has made it to clinical trials in humans for depression, after studies have shown that it is promising in rats. A dose of 1.5 mg/d provided a significant difference compared to placebo in patient-rated Montgomery-Åsberg Depression Rating Scale (which focuses on MDD severity), but not when physician rated (Quiroz et al., 2016).

The modulation of NMDA receptor activation by mGlu 5 receptors noted previously may also be a possible treatment of cognitive deficiencies. Evidence supporting this idea can be found in several studies that look at administrations of both mGlu 5 and NMDA drugs. For example, CDPPB administration can alleviate cognitive impairments associated with NMDA receptor inhibition caused by MK-801, a noncompetitive NMDA antagonist (Cleva & Olive, 2011). MK-801 inhibition causes an impairment on novel object recognition and applying a dose of 3 mg/kg CDPPB was able to reverse the impairment, while 10 mg/kg CDPPB did not significantly differ (Uslaner et al., 2009).

There have been a multitude of previous studies demonstrating that reducing glutamatergic neurotransmission negatively affects cognitive function. MK-801 also decreased performance in a the five-choice serial reaction task and the delayed matching to position task. Furthermore, phencyclidine (PCP), another noncompetitive NMDA antagonist, impaired accuracy in the 5CSRT and the delayed matching to position task (Smith et al., 2011). PCP did not increase latency to solve the Morris Water maze, however, it did decrease performance on the reversal memory task in male rats but not females (Andersen & Pouzet, 2004). D-Serine, which binds to an NMDA co-agonist site, was able to improve the impaired performance caused by

the PCP (Anderson et al., 2003). D-Serine also improves impairments caused by MK-801 (Karasawa et al., 2008). In addition, ketamine, also a noncompetitive NMDA antagonist, decreased performance, but not latency in the 5CSRT and decreased performance in the delayed matching to position task (Smith et al., 2011). In the odor span task, 3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonicacid (CPP) a NMDA antagonist, and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), an AMPA antagonist, both impaired performance (Davies et al., 2013).

Inhibiting mGlu 5 receptor activity via MPEP is able to increase the effects of MK-801, a noncompetitive NMDA receptor antagonist (Homayoun et al., 2004), while activating mGlu 5 receptors via CDPPB can counteract MK-801 effects on a set-shifting task (Stefani & Moghaddam, 2010). VU0409551, an mGlu 5 receptor PAM that does not impact NMDA receptor activation has also improved cognition in the object recognition task in rats, demonstrating that the cognitive enhancements of mGlu 5 receptors are not solely caused by the modulation of the NMDA receptors (Rook et al., 2015). Together this data suggests that reduced glutamatergic neurotransmission negatively affects cognitive function, and that increasing glutamatergic neurotransmission via pharmacological means can thus improve cognitive function.

Preclinical Models of Working Memory

When comparing working memory between animal and human cognitive studies it is important to remember that there is a translational difference. What is often considered working memory in animal research would be considered short-term memory, or “long-term working memory” in humans (Carruthers, 2013), due to the longer retention times that are used in animal research. It is not yet known what exactly causes the differences between animal and human

working memory performance. The following experiments are all commonly used working memory tasks in rodents in current literature.

The Morris water maze task consists of a large pool of water that contains a submerged platform. In the working memory form of the Morris water maze, the platform is moved to a different location each day. In the first trial of the day the rat must swim around and find the platform. By the second trial (and each subsequent trial) the rat will find the platform much faster than the first trial. This task tests working spatial memory, and rodents that had received a hippocampectomy suffered serious impairments in performance (Dudchenko, 2004).

The delayed alternation task is a spatial working memory task. In a delayed alternation task, a Y or T shaped maze is used. The subject is placed on the start arm and allowed to enter an arm. The subject is then removed from the maze for a predetermined time-interval. When placed back in the field after the delay, the subject will normally choose the arm they did not chose last time. There have been a few studies that demonstrate that rats use different strategies to remember which arm they last entered, depending on which information was available to them (Dudchenko, 2004).

The odor span task is an extended non-match to sample task designed to measure working memory in rats in a manner similar to how the Digit Span task tests working memory in humans. The subject is first introduced to a scented cup (Dudchenko, 2004). The subject is removed from the field, and then a new scented cup is placed on the field along with the original cup. If the subject choses the original stimulus, which is the correct choice, it is removed again, and a third scented cup is placed in the field. This is repeated with additional cups until the subject choses an incorrect cup, thus generating a memory span measurement similar to those used in human subjects working memory tasks. Unfortunately, this task takes an extended period

of time to be trained and does not allow researchers to easily assess basic relationships between time and memory performance. This issue may be particularly important in order to verify that performance is consistent with the construct of working memory. Thus, we altered this task in order to simplify it and improve our ability to assess time-related differences in performance.

In the current study, we used a variant of a delayed nonmatch to sample task that we are calling the delayed non-match to odor task. In this task subjects are first introduced to an odor stimulus, which is paired with a food reward. After a time, delay, they are introduced to both the original odor stimuli as well as a novel one. The subject must choose the novel stimuli (no match) in order to get another food reward.

Hypothesis

We made the following hypotheses:

1) Memory performance in the delayed non-match to odor task will be progressively impaired as the time between the information and retention trial increases.
2) Administering a mGlu5 positive allosteric modulator such as CDPPB will improve performance on the delayed non-match to odor task.

Methods

Subjects

22 naïve male long Evans rats (Envigo, Indianapolis, IN), aged 7-8 weeks at arrival, were used and housed in intraventilated cages. Rats were food deprived to 90% of their free feeding weight and only fed once the day's testing is complete. They were kept on a 12-hour light/dark schedule (lights on 8am) and had *ad libitum* access to water while in their home cage. The rats were pair-housed unless there was fighting between the cage mates. Rats were given one week to acclimate to the environment before experimental procedures begin. All methods were approved

by the Montclair State University Institutional Animal Care and Use Committee prior to the start of the study and were consistent with the National Institute of Health's *Guide to the Care and Use of Laboratory Animals* (National Research Council, 2011).

Apparatus and Materials

The task was based on the design used in Davies et al. (2013). The task took place on a 91.5 cm x 91.5 cm blue plexiglass platform placed on a metal frame. The field has 3 cm tall walls. It is 91.5 cm off the floor. It is surrounded by thick black welding curtains to block any distal visual cues. An Infaimon video camera was mounted above the apparatus and used to record all training and testing within the open field. Sixteen spots, 4 on each side, (3 cm away from each other and 4 cm away from the corner) were marked by a Velcro strap. The containers with the scented spice were attached via the Velcro to prevent the animals from knocking it over. White noise was played in the room during all procedures. Panlab Smart 3.0 was used to record all video data as well as collecting data on distance traveled and speed. Kellogg's (Battle Creek, MI). Froot Loops cereal was used a food reward throughout the task.

Drugs and Chemicals

3-Cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (Tocris Bioscience, Bristol, UK), also known as CDPPB, is a positive allosteric modulator (PAM) for mGlu 5 receptors. CDPPB was suspended in a solution of 20% (w/v) cyclodextrin (Sigma Aldrich, St. Louis, MO), and injected intraperitoneally (I.P.) 30 minutes before testing as based on current literature (Uslaner et al., 2009; Vardigan et al., 2010). All doses were administered at a volume of 1 mL/kg of body weight.

Procedures

Shaping

Each rat was first individually placed on the open field and given 5 minutes to become habituated to the field before beginning shaping. Shaping was done so that the rats could reliably dig for the food reward before the training phase of the experiment. The food reward was buried so that the animal could not see or smell the reward so that during testing they would be required to use odor to identify cups. In phase 1 of shaping, a reward was placed on top of a cup with 100 grams of unscented sand. This cup was then placed in a randomized location on the field. Each rat had 5 minutes to retrieve the reward. Once the rat has finished eating the reward, it was returned to its cage and the field was vacuumed and wiped down with Vikron S (a cleaning solution). In phase 2, the reward was incrementally buried. As before, the cup was randomly placed in the field. In phase 3, the reward was fully buried to its final depth of ¼ of an inch. The rat moved on to training once it can retrieve the reward under 30 seconds for 3 consecutive days.

Delayed Non-match to Odor Training

The following odors were used: fennel seed, ginger, parsley, caraway seed, oregano, cumin, sage, anise seed, basil, mint, paprika, Jamaican all spice, and lemon. Any odors that demonstrate a consistent accuracy rate of less than chance during training was removed from the study. During the training the rats was exposed to all of the odors used in the task. The pair of scents used for each day was randomly assigned. The sand-scent mixture used in the trials consisted of 99.5 grams of sand and 0.5 grams of scent. Any spice that did not come in a fine powder was ground into a powder by mortar and pestle immediately before use. Food rewards were buried ¼ of an inch below the surface of the sand.

The procedure consists of 6 sessions, each consisting of two trials: an information trial and retention trial. In the information trial the cup containing the scented odor and buried reward was placed on the field in a random location. The rat needed to dig out the reward and eat it. The subject was then removed from the field. In retention trial, the cup was moved to a new location, and a second cup containing a different odor and a reward was placed on the field. The session was marked as correct if the subject could retrieve the reward from the cup containing the novel odor. The session was marked as incorrect if the subject dug in the wrong cup, did not retrieve the reward in 60 seconds, or jumped off the field. Once finished, the rat is then removed from the field. The field was vacuumed to remove sand and wiped down with Vikron between each trial and session to prevent odor trails. Subjects were always placed in the center of the field facing away from the curtain opening.

Rats were considered to have passed training when they correctly choose the novel odor in 5 out of the 6 sessions for 3 consecutive days. Rats who struggled to learn the task were given errorless training. The procedure was the same as in the regular training with the exception of having only the novel cup in the retention trial.

Probe Trials

In order to determine whether animals used an alternate strategy to perform this task, such as 1) marking the cups with their own scent to identify the old container, or 2) using the reward's scent to identify the new container, two separate probe trials were used. To test whether animals use the smell of the reward to decide which cup to dig in, no food reward was buried in the correct cup during the retention trial. Instead, the reward was handed to the rat once they made a correct choice. When testing for scent marking, the basic procedure was the same as the

training procedure, except that during the retention trial, two entirely new cups were used, thus making it impossible to mark the old container. All cups were wiped down with Vikron after use.

Intertrial Interval Procedure

To determine the degree to which memory performance in this task is time-dependent, experiments were conducted to investigate the effect of long intertrial intervals (ITI) on memory performance. The following ITI's were used: 30 seconds, 100 seconds, 300 seconds, and 1,000 seconds. 30 seconds was used as the control time as that was the fastest interval the researchers could reliably and consistently achieve. To prevent any order effects, each rat had their own sequence as determined by a randomized Latin square design.

Drug Trial Procedure

In order to assess the effects of mGlu5 receptor activation on performance in the delayed non-match to odor task, rats were injected with the mGlu5 receptor PAM CDPPB intraperitoneally 30 min prior to the start of the first information trial of the day. Rats received either vehicle or CDPPB at 1, 3, or 10 mg/kg. The order of doses was determined for each animal using a randomized Latin square design in order to prevent confounding due to drug history. The dose range of CDPPB was chosen on the basis of other studies that investigated the effects of this drug on memory performance (Uslaner et al., 2009). The intertrial time was set at 300 seconds based on the results of the intertrial interval testing.

Data Analysis

Alpha was set at 0.05, and sample sizes were set at 8 per group. Animals that failed to complete a drug or time curve in its entirety for any reason were entirely removed from the data set. All data analysis was done in R. Normality was first assessed via Lilliefor's test. If the data is normal, one-way repeated measures ANOVAs were to be used. The Tukey-Kramer procedure

was used for any post-hoc. If the data was non-normal, Friedman's test was used along with Wilcoxon Ranked Sign for post hoc tests. Accuracy was be the main measure of performance, although latency to dig, distance, and speed were also be measured.

Results

Training Data for the DNMTO Task

As seen in Figure 2, 21 out of 22 (95%) rats successfully completed shaping in an average of 13 days (SEM = 1.36). The only rat that did not complete shaping was removed due to aggressive behavior and not due to performance.

Rats took an average of 16 (SEM =1.75) days to successfully reach the training criteria of 3 consecutive days getting 5 out of 6 trials correct. 16 out of 21 (76%) rats were able to pass the criteria of the non-matched sample at least once (Figure 2).

Probe Trial Performance

Probe trial data is presented in Figure 3. 14 rats reached the probe trials and 11 passed (79% passed). All rats passed the reward probe trial, and the 3 that failed the marking probe were removed from the task.

The Effects of ITI on DNMTO Performance

There was an overall decrease in performance as the time between the retention and information trial increased during the ITI tests (Figure 4). A Lillifors test was used to evaluate normality and found that accuracy data was not normally distributed ($D_n = .16$, $p < 0.05$). A non-parametric Friedman Test with repeated-measures was conducted and rendered a significant effect of time ($\chi^2(8) = 16.3$, $p<0.001$; $W = 0.681$). Wilcoxon Signed-Ranks post hoc tests indicated that the 100 ($p < 0.01$, $r = 0.792$), 300 ($p < 0.001$, $r = 0.869$) and 1000 ($p < 0.001$, $r = 0.861$) second interval all had significantly worse performance than the 30 second interval. Rats

also performed significantly worse at the 1000 second interval by comparison to the 100 second interval ($p<.05$, $r=0.577$). There was no significant difference between the other times.

The Effects of CDPPB Administration on DNMTO Accuracy

Data on the effects of CDPPB administration on DNMTO task accuracy performance is presented in Figure 5. A Lillifors test was used to evaluate normality and found that accuracy data was normally distributed ($D_n = .13$, n.s.). A repeated measures ANOVA found that CDPPB administration (30 min IP) had no effect on DNMTO accuracy [$F(3,21) = 1.6$, n.s., $\eta^2 = 0.186$].

Figure 6 presents the data on the effects of CDPPB administration on distance travelled in the DNMTO task. A Lillifors test was used to evaluate normality and found that distance travelled data was normally distributed ($D_n = 0.16$, n.s.). A repeated measures ANOVA found that CDPPB administration (30 min IP) had no effect on DNMTO distance traveled [$F(3,21) = 1.532$, n.s., $\eta^2 = 0.180$].

Data on the effects of CDPPB administration latency to dig in the DNMTO task is presented in Figure 7. A Lillifors test was used to evaluate normality and found that latency data was normally distributed ($D_n = 0.16$, n.s.). A repeated measures ANOVA found that CDPPB administration (30 min IP) had no effect on DNMTO latency [$F(3,21) = 1.456$, n.s., $\eta^2 = 0.172$].

Figure 8 presents the data on the effects of CDPPB administration on DNMTO task speed. A Lillifors test was used to evaluate normality and found that speed data was normally distributed ($D_n = 0.16$, n.s.). A repeated measures ANOVA found that CDPPB administration (30 min IP) had no effect on speed [$F(3,21) = 1.396$, n.s., $\eta^2 = 0.166$].

Discussion

The present study revealed that increasing the time between the information and retention trial over 30 seconds significantly decreased accuracy. The accuracy also significantly decreased

when the time was increased between 100 and 1000 seconds (Figure 4). This is despite a strong floor effect of the test; since there are only two options for the rats to choose from, it should not be possible for the accuracy to go lower than a 50% chance rate in a memory task such as this. The hypothesis that memory performance in the delayed non-match to odor task would be progressively impaired as the time between the information and retention trial increases is supported by the data. There was a clear impairment once the time between the trials was increased beyond 30 seconds. Thus, these data support the idea that increasing the ITI intensifies memory load. This reduced accuracy based on increased ITI is a hallmark of working memory performance in animal behavior tasks. Although spatial and olfactory working memory consist of two independent working memory systems in rats (Bratch et al., 2016), these results are similar to previous studies with spatially based DNMS tasks (Mumby et al., 1990). This non-match to odor task is a more ethologically relevant working memory task than a visual-based task as rats have poor eyesight.

The hypothesis that administering the mGlu 5 receptor PAM CDPPB would improve performance on the delayed non-match to odor task was not supported by the data. CDPPB caused no significant difference on an odor based delayed non-match to sample task. Neither accuracy (Figure 5) nor latency (Figure 7) were significantly different. There were also no differences in the amount of distance traveled (Figure 6) or in the speed (Figure 8). The moderate effect sizes (η^2 values from 0.17 to 0.19) may mean there was not sufficient power with the low sample size of 8 rats. Although we originally planned for a larger sample size of 10 rats, the attrition rate led to a smaller sample size than was ideal. While an increase in performance was expected to be found with an increase in mGlu 5 receptor activation via CDPPB administration, the data gathered showed a trend in the opposite direction.

Previous Studies

Previous work has suggested that CDPPB, as other glutamatergic drugs, has some impact on cognitive abilities. While CDPPB has had interesting results when used to combat the cognitive impairment induced by drugs such as MK-801 (Stefani & Moghaddam, 2010). It may be that CDPPB and other PAMs are better suited to bring mGlu 5 receptor performance after it or NMDA receptors have been inhibited, whether by another drug or by disease. Prior studies also demonstrate that CDPPB increases performance on spatial memory based tasks (Cleva & Olive, 2011). CDPPB has also been found to increase novel object recognition in rats at 10 mg/kg but not at 30 mg/kg, and it likewise increases markers of neuronal plasticity (Uslaner et al., 2009). Chronic CDPPB administration in demonstrate animals staying in their home cage did not cause differences on dendritic spine density in the medial PFC, however it did in animals undergoing extinction training (LaCrosse et al., 2015). In addition, daily doses of 30 mg/kg of CDPPB prior to delayed alternation T-maze training caused rats to perform better than vehicle or those given 10 mg/kg (Fowler et al., 2013).

While CDPPB did not differ from control on this task, another type of glutamatergic drug could alter performance. VU0409551 (another mGlu 5 receptor PAM) increased novel object recognition, as well as performance, in the delayed non-matching to position task, a task similar to this study's non-matching to odor task.

In addition, if an mGlu 5 receptor antagonist or NAM were to be tested, it could decrease performance. This would mean that while a decrease in mGlu 5 impairs performance in cognitive tasks such as this nonmatch to odor task, an increase of mGlu 5 above a set point has no impact. There is a large body of research demonstrating that MPEP reliably causes cognitive impairments in spatial memory tasks including the spontaneous alternation test (Homayoun et

al., 2004) and the 5-choice serial reaction time task (Semenova & Markou, 2007). On the delayed match to position task, MPEP only altered accuracy at 100 mg/kg at the longest delay (24s), with no significant difference at 30 mg/kg (Ballard et al., 2005).

Limitations

Within the rodent brain, there are high concentrations of mGlu 5 receptors within the hippocampus and neocortex, as well as the olfactory regions. These receptors are located postsynaptically in the dendritic areas of neurons and astrocytes (Niswender & Conn, 2010). Since this was an odor based task, the high concentrations of mGlu 5 receptors in the rat's olfactory regions (Shigemoto et al., 1993) may have caused some interference as CDPPB interacted with the receptors in that brain region. Due to this, it may be that a non-odor based non-match to sample task may be more suited to test mGlu 5 receptor drugs. There was also no direct confirmation that CDPPB was binding to the PFC. A test such as autoradiography would be able to access the levels of binding had it been done. However, as previously mentioned, there have been other studies that have demonstrated significant behavioral and cognitive effects at this dose and route of administration; implying that it was a relevant dose.

Another limitation is the low sample size used in the task. A higher sample size would have more power to find an effect if there was one. In addition, a depression model was not used during the task. We wished to find the effects of mGlu 5 receptors on cognition in healthy rats before applying a depression model.

Conclusion

Overall, this study demonstrates a novel method of testing short-term memory in rodents using the delayed non-match to odor task, providing an alternative frontal-cortex dependent task for a short-term memory model. CDPPB did not improve performance by any measure in a non-

matched to odor task in rats; therefore, administering a PAM may not lead to a suitable method of treating cognitive deficiencies. Although CDPPB did not show any improvement in this task, it may still be a promising task to be used in conjunction with other drugs. Future research could apply a depression model before testing the effects of a mGlu 5 receptor drug on cognition. Future research could also use a method, such as autoradiography, to see if CDPPB is binding to the neocortex during the task. Further research should be done on possible glutamatergic treatments for those with major depression disorder.

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Diagram of mGlu 5 Receptor Activation

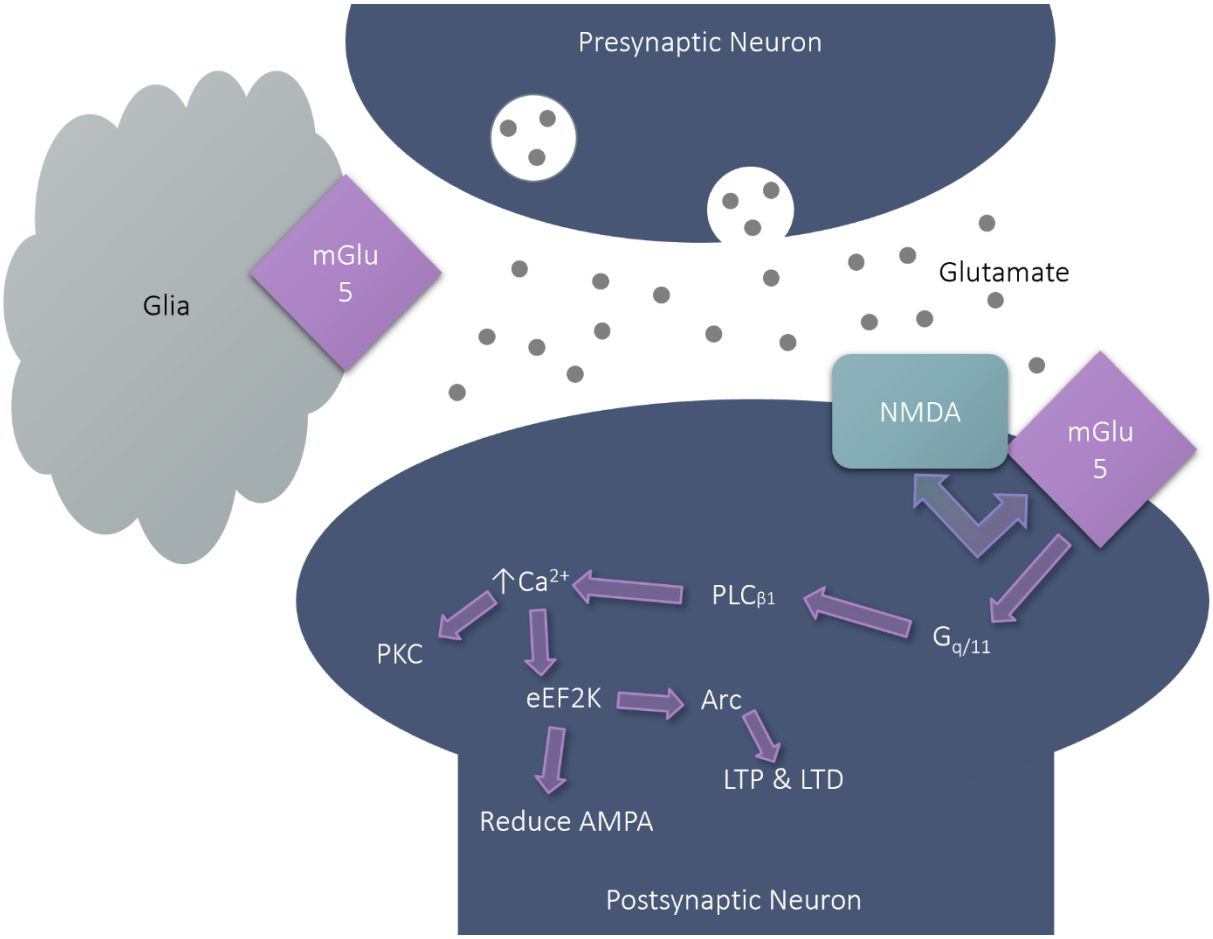

Figure 1. A diagram of mGlu 5 activation on the post synaptic neuron. The mGlu 5 receptor is located perisynaptically on the postsynaptic dendrite. Glutamate is released from the presynaptic terminal into the synaptic space. It activates the mGlu 5 receptor, which can potentiate the NMDA receptor. The mGlu 5 receptor also activates the $G_{q/11}$ protein, which activates phospolipase Cβ1 (PLCβ1), which in turn releases the internal stores of Ca^{2+}. That activates both PKC (protein kinase C) and eEF2k (eukaryotic elongation factor-2 kinase). eEF2k activates Arc, leading to both long term potentiation and depression.

Shaping and Training Data

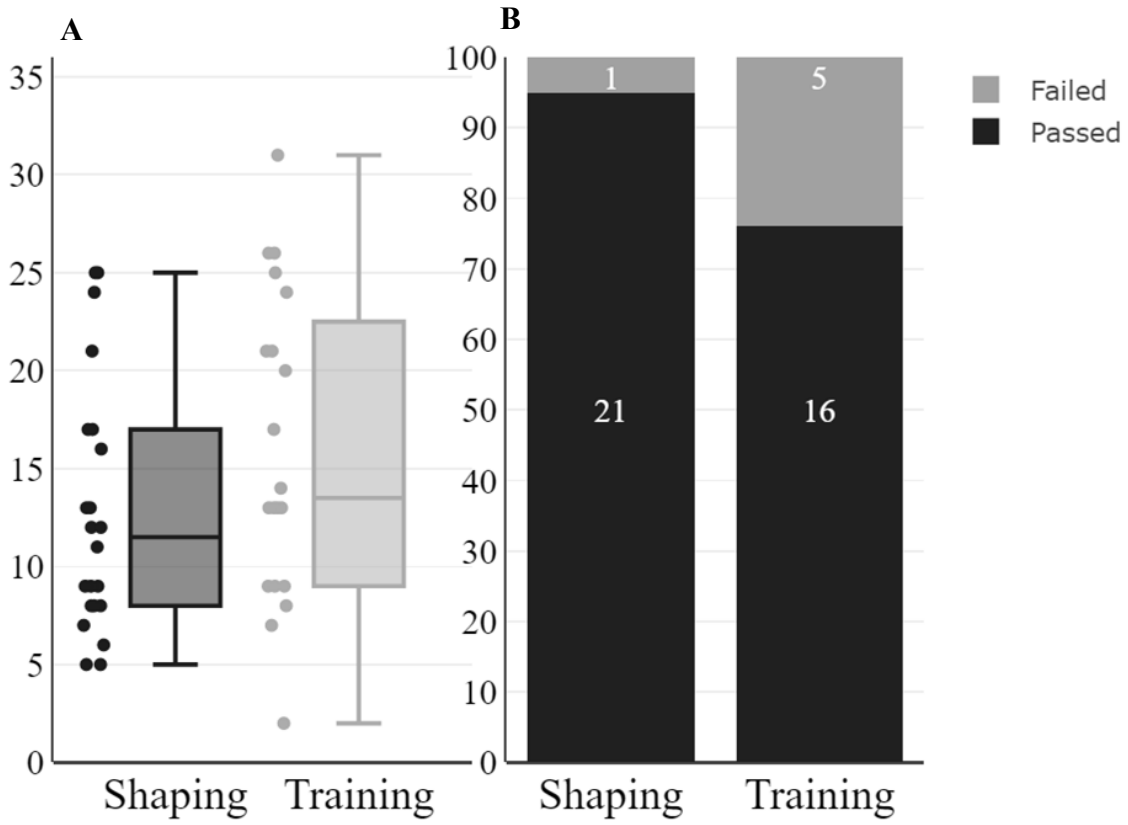

Figure 2. Training and shaping performance. (A) The number of trials each rat completed, due to either reaching criteria or being removed. (B) A bar graph demonstrating the percent of animals that completed each task or failed to learn it, along with the number of animals.

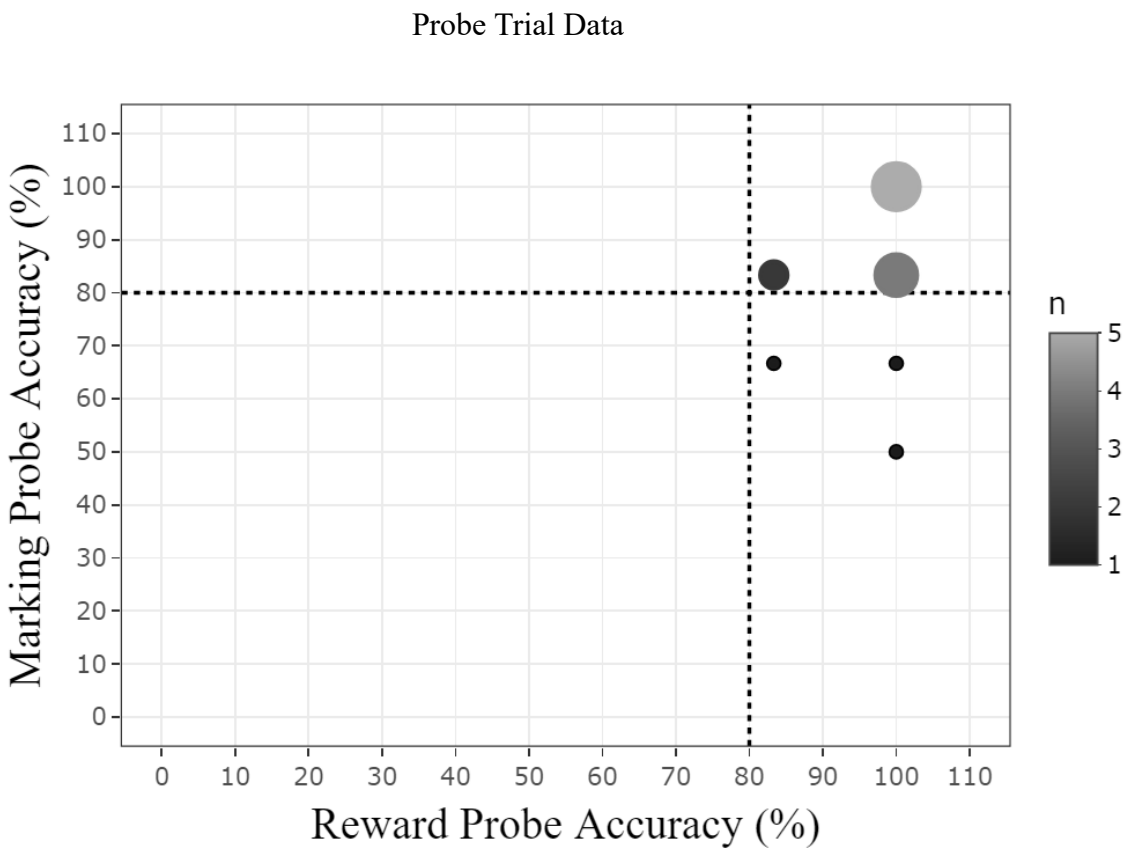

Figure 3. Performance accuracy for both odor and reward probe. The color and size of each point correspond to the number of points the same scores. The dotted lines at 80% represent the cut off points; rats that did not pass these were removed from the task. Three animals failed to pass the marking probe.

The Effects of ITI on DNMTO Performance

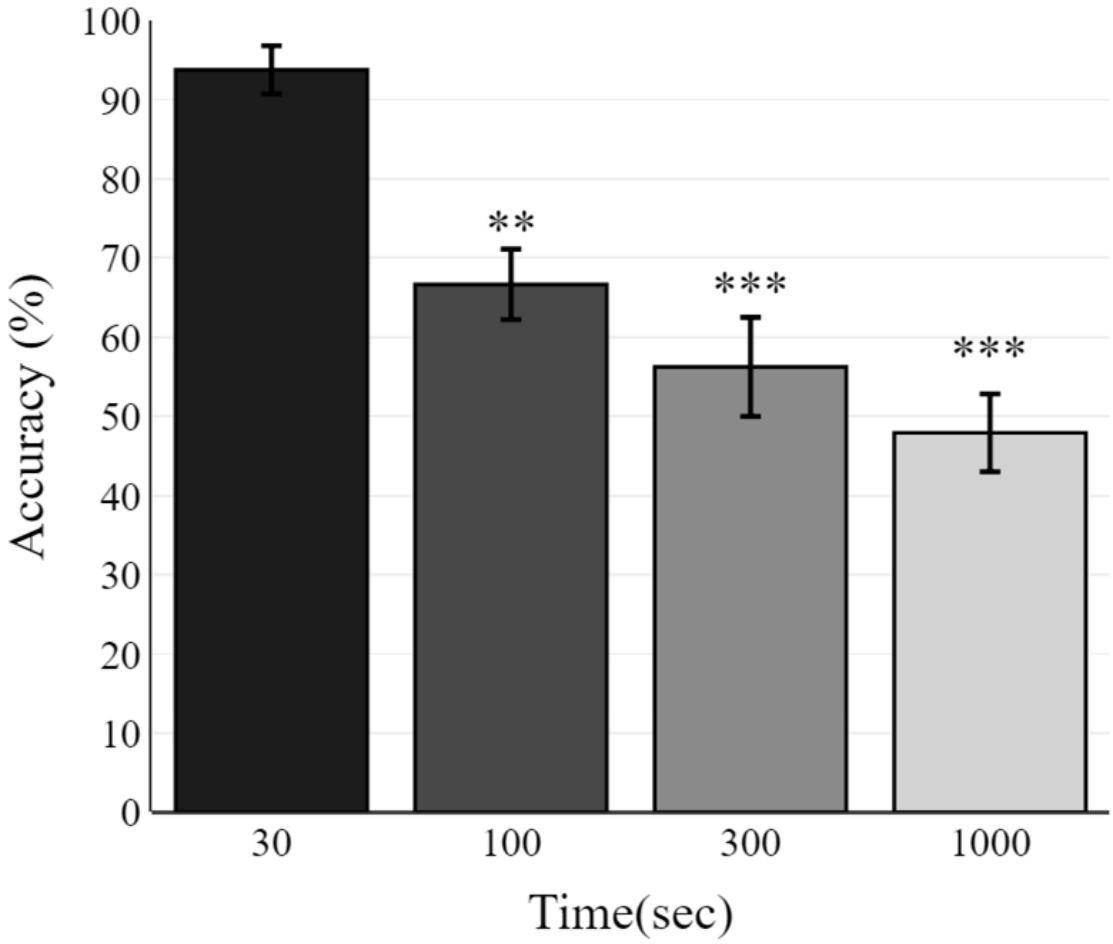

Figure 4. The effects of changing the time interval between information and retention trials on DNMTO accuracy performance. Accuracy performance was significantly impaired by increasing the time at 100 ($p<.01$, $r=0.792$), 300 ($p<.01$, $r=.869$), and 1000 ($p<.001$, $r=.861$) second intervals. Accuracy performance was also significantly impaired between the 100 and 1000 second intervals ($p<0.5$, $r=0.577$). Data are presented as mean ± SEM. *Note* **p < .01 compared to control, ***p <.001 compared to control.

The Effects of CDPPB on DNMTO Accuracy Performance

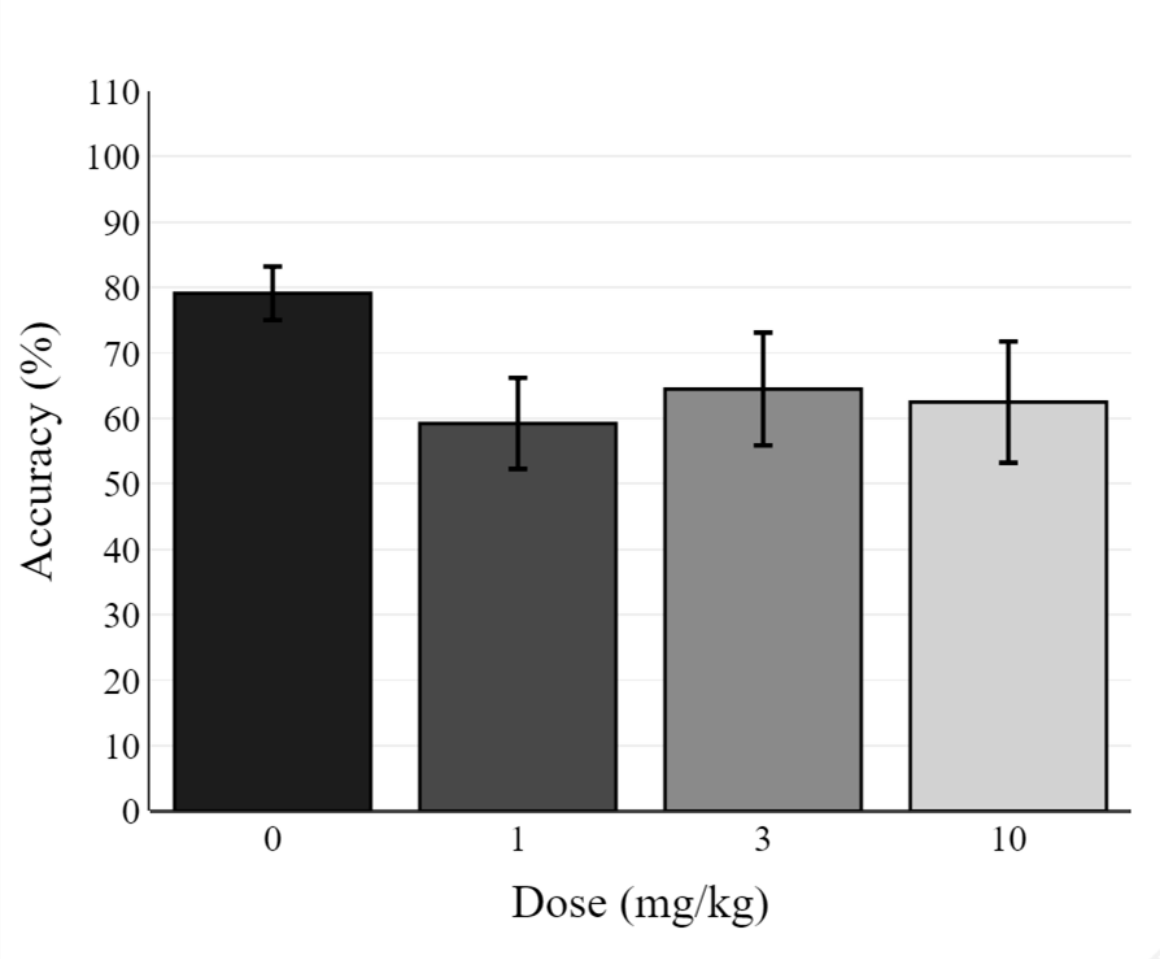

Figure 5. The effects of CDPPB administration (30 min IP) on DNMTO accuracy performance. Accuracy performance was not altered by CDPPB administration at 1, 3, or 10 mg/kg compared to vehicle [$F(3,21)=1.603$, $p= 0.219$, $\eta^2 = 0.186$]. Data are presented as mean ± SEM.

The Effects of CDPPB on DNMTO Distance Travelled

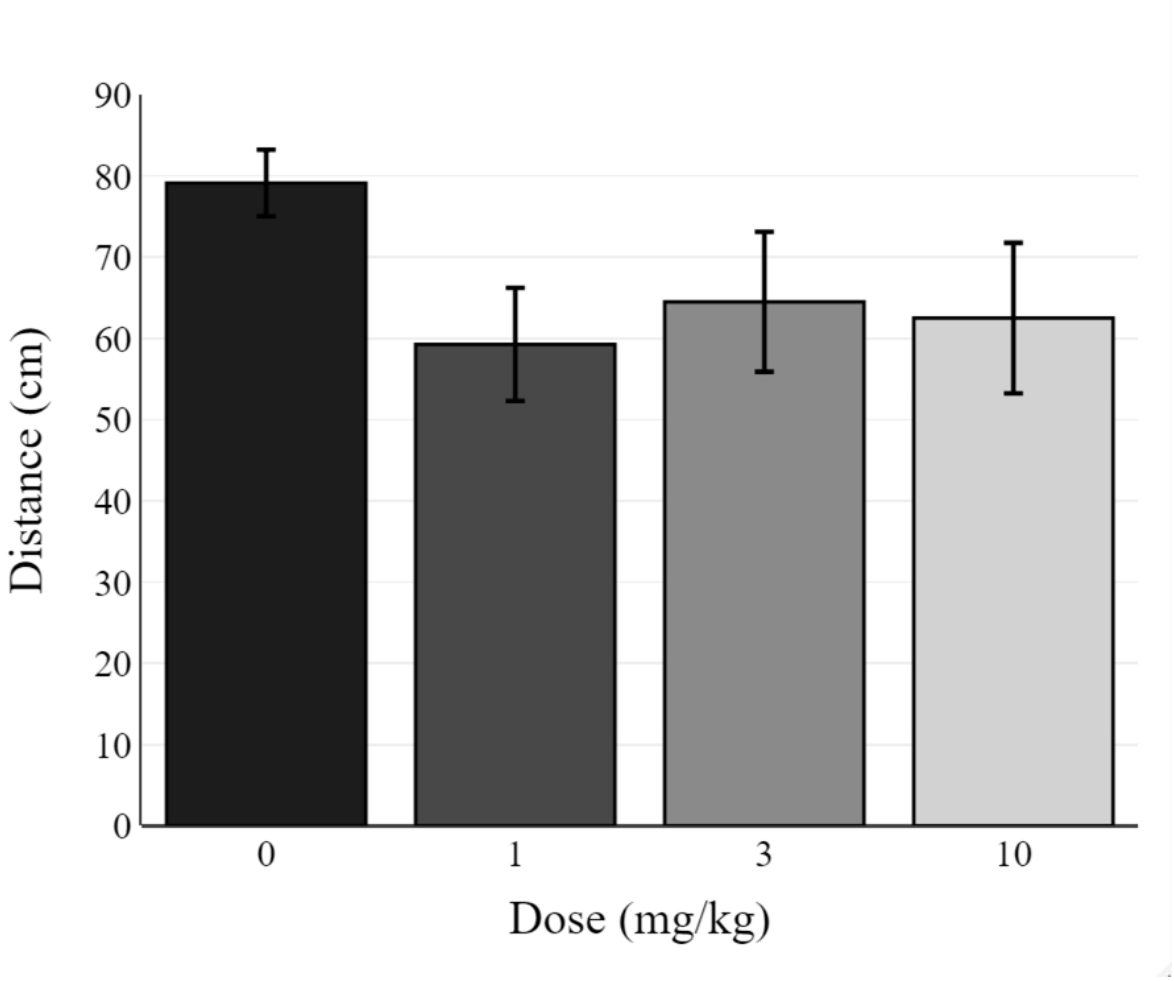

Figure 6. The effects of CDPPB administration (30 min IP) on DNMTO distance travelled. Distance travelled was not altered by CDPPB administration at 1, 3, or 10 mg/kg compared to vehicle [$F(3,21)=1.532$, $p= 0.236$, $\eta^2 = 0.180$]. Data are presented as mean ± SEM.

The Effects of CDPPB on DNMTO Latency to Dig

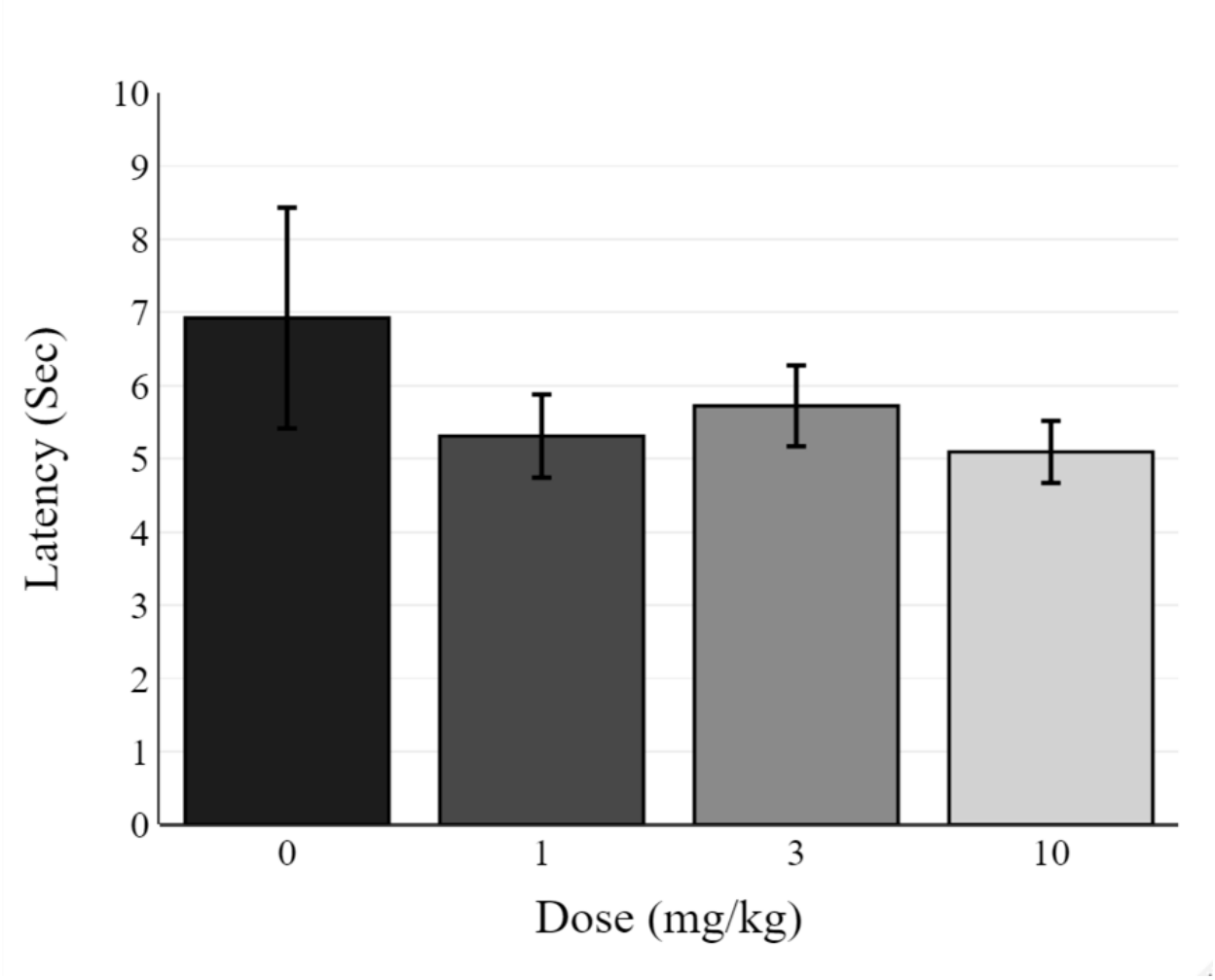

Figure 7. The effects of CDPPB administration (30 min IP) on DNMTO latency to dig. Latency to dig was not altered by CDPPB administration at 1, 3, or 10 mg/kg compared to vehicle [$F(3,21)=1.456$, $p= 0.255$, $\eta^2 = 0.172$]. Data are presented as mean ± SEM.

The Effects of CDPPB on DNMTO Speed

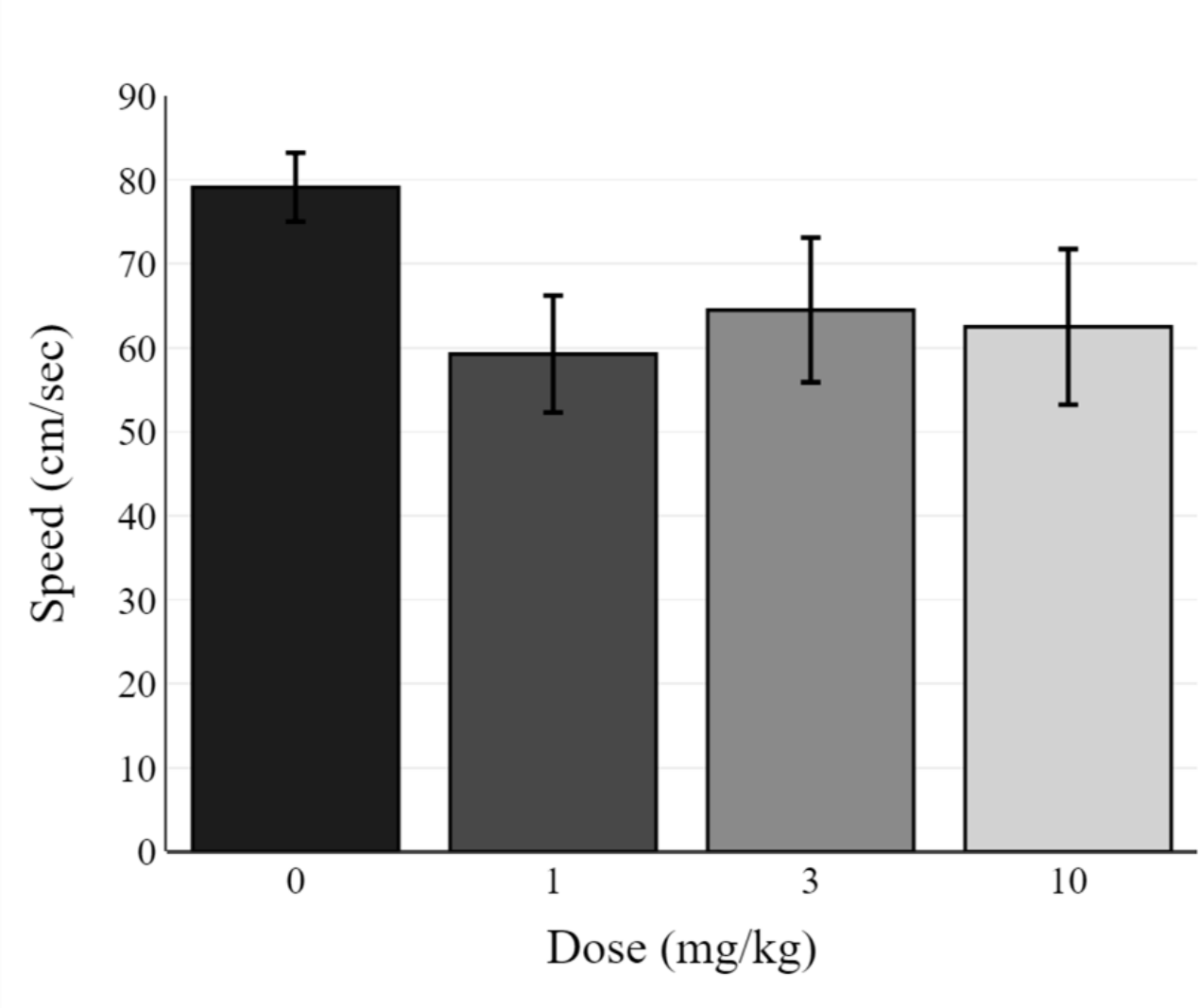

Figure 8. The effects of CDPPB administration (30 min IP) on DNMTO speed. Speed was not altered by CDPPB administration at 1, 3, or 10 mg/kg compared to [$F(3,21)=1.396$, $p= 0.272$, $\eta^2 = 0.166$]. Data are presented as mean ± SEM.

Appendix A: R Code

Drug Trial Code

```
library(tidyverse)
library(nortest)
library(sjstats)
#Data Import
drug.trial.data <- read_csv(file.choose())
#----------------------------------------------------------------
#Pre-setup
#----------------------------------------------------------------
 drug.trial.data$Rat <- factor(drug.trial.data$Rat)
 drug.trial.data$Dose <- factor(drug.trial.data$Dose)

 vehicle <- drug.trial.data %>% filter(Dose == '0')
 dose.1<- drug.trial.data %>% filter(Dose == '1')
 dose.3 <- drug.trial.data %>% filter(Dose == '3')
 dose.10 <- drug.trial.data %>% filter(Dose == '4')
################################################################################
#Accuracy Data
################################################################################
 accuracy.summary <- drug.trial.data %>% group_by(Dose) %>% summarize(
 count = n(),
 mean = mean(Accuracy),
```

```
 sd = sd(Accuracy),
 SEM = (sd(Accuracy)/sqrt(count))
 )
#------------------------------------------------------------------
#Normality Test: Lillifors (Accuracy)
#------------------------------------------------------------------
 vehicle.residuals <- tbl_df(vehicle$Accuracy - mean(vehicle$Accuracy))
 dose.1.residuals <- tbl_df(dose.1$Accuracy - mean(dose.1$Accuracy))
 dose.3.residuals <- tbl_df(dose.3$Accuracy - mean(dose.3$Accuracy))
 dose.10.residuals <- tbl_df(dose.10$Accuracy - mean(dose.10$Accuracy))
 acc.residuals<- bind_rows (vehicle.residuals, dose.1.residuals, dose.3.residuals,
dose.10.residuals)

 lillie.test(acc.residuals$value)
#------------------------------------------------------------------
#one-way repeated measures ANOVA (Accuracy)
#------------------------------------------------------------------
 aov.accuracy <- aov(Accuracy ~ Dose + Error(Rat/Dose), data=drug.trial.data)
 sum.aov.accuracy <- summary(aov.accuracy)
 sum.aov.accuracy
#------------------------------------------------------------------
#eta squared (Accuracy)
#------------------------------------------------------------------
```

```
 temp.dose <-sum.aov.accuracy[["Error: Rat:Dose"]][[1]][["Sum Sq"]][[1]]
 temp.error <- sum.aov.accuracy[["Error: Rat:Dose"]][[1]][["Sum Sq"]][[2]]
 eta.sq.accuracy <- temp.dose / (temp.dose + temp.error)
 eta.sq.accuracy
################################################################################
#Latency Data
################################################################################
 latency.summary <- drug.trial.data %>% group_by(Dose) %>% summarize(
 count = n(),
 mean = mean(Latency),
 sd = sd(Latency),
 SEM = (sd(Latency)/sqrt(count))
 )
#------------------------------------------------------------------
#Normality Test: Lillifors (Latency)
#------------------------------------------------------------------
 vehicle.residuals <- tbl_df(vehicle$Latency - mean(vehicle$Latency))
 dose.1.residuals <- tbl_df(dose.1$Latency - mean(dose.1$Latency))
 dose.3.residuals <- tbl_df(dose.3$Latency - mean(dose.3$Latency))
 dose.10.residuals <- tbl_df(dose.10$Latency - mean(dose.10$Latency))
 lat.residuals<- bind_rows (vehicle.residuals, dose.1.residuals, dose.3.residuals,
dose.10.residuals)
```

```
 lillie.test(lat.residuals$value)
#------------------------------------------------------------------
#one-way repeated measures ANOVA (Latency)
#------------------------------------------------------------------
 aov.latency <- aov(Latency ~ Dose + Error(Rat/Dose), data=drug.trial.data)
 sum.aov.latency <- summary(aov.latency)
 sum.aov.latency
#------------------------------------------------------------------
#eta squared (Latency)
#-----------------------------------------------------------------
 temp.dose <-sum.aov.latency[["Error: Rat:Dose"]][[1]][["Sum Sq"]][[1]]
 temp.error <- sum.aov.latency[["Error: Rat:Dose"]][[1]][["Sum Sq"]][[2]]
 eta.sq.latency <- temp.dose / (temp.dose + temp.error)
 eta.sq.latency
#############################################################################
#Distance Data
#############################################################################
 distance.summary <- drug.trial.data %>% group_by(Dose) %>% summarize(
 count = n(),
 mean = mean(Distance),
 sd = sd(Distance),
 SEM = (sd(Distance)/sqrt(count))
 )
```

```
#-----------------------------------------------------------------
#Normality Test: Lillifors (Distance)
#-----------------------------------------------------------------
 vehicle.residuals <- tbl_df(vehicle$Distance - mean(vehicle$Distance))
 dose.1.residuals <- tbl_df(dose.1$Distance - mean(dose.1$Distance))
 dose.3.residuals <- tbl_df(dose.3$Distance - mean(dose.3$Distance))
 dose.10.residuals <- tbl_df(dose.10$Distance - mean(dose.10$Distance))
 dist.residuals<- bind_rows (vehicle.residuals, dose.1.residuals, dose.3.residuals,
dose.10.residuals)

 lillie.test(dist.residuals$value)
#-----------------------------------------------------------------
#one-way repeated measures ANOVA (Distance)
#-----------------------------------------------------------------
 aov.distance <- aov(Distance ~ Dose + Error(Rat/Dose), data=drug.trial.data)
 sum.aov.distance <- summary(aov.distance)
 sum.aov.distance
#-----------------------------------------------------------------
#eta squared (Distance)
#-----------------------------------------------------------------
 temp.dose <-sum.aov.distance[["Error: Rat:Dose"]][[1]][["Sum Sq"]][[1]]
 temp.error <- sum.aov.distance[["Error: Rat:Dose"]][[1]][["Sum Sq"]][[2]]
 eta.sq.distance<- temp.dose / (temp.dose + temp.error)
```

```
 eta.sq.distance
##############################################################################
#SPEED Data
##############################################################################
 speed.summary <- drug.trial.data %>% group_by(Dose) %>% summarize(
 count = n(),
 mean = mean(Speed),
 sd = sd(Speed),
 SEM = (sd(Speed)/sqrt(count))
 )
#------------------------------------------------------------------
#Normality Test: Lillifors (Speed)
#------------------------------------------------------------------
 vehicle.residuals <- tbl_df(vehicle$Speed - mean(vehicle$Speed))
 dose.1.residuals <- tbl_df(dose.1$Speed - mean(dose.1$Speed))
 dose.3.residuals <- tbl_df(dose.3$Speed - mean(dose.3$Speed))
 dose.10.residuals <- tbl_df(dose.10$Speed - mean(dose.10$Speed))
 speed.residuals<- bind_rows (vehicle.residuals, dose.1.residuals, dose.3.residuals,
dose.10.residuals)

 lillie.test(speed.residuals$value)
#------------------------------------------------------------------
#one-way repeated measures ANOVA (Speed)
```

```
#---------------------------------------------------------------------

 aov.speed <- aov(Speed ~ Dose + Error(Rat/Dose), data=drug.trial.data)

 summary(aov.speed)

 sum.aov.speed <- summary(aov.speed)

#---------------------------------------------------------------------

#eta squared (Speed)

#---------------------------------------------------------------------

 temp.dose <-sum.aov.speed[["Error: Rat:Dose"]][[1]][["Sum Sq"]][[1]]

 temp.error <- sum.aov.speed[["Error: Rat:Dose"]][[1]][["Sum Sq"]][[2]]

 eta.sq.speed <- temp.dose / (temp.dose + temp.error)

 eta.sq.speed
```

ITI Code

```
library(tidyverse)
library(nortest)
library(rstatix)


#Data Import
iti.data <- read_csv(file.choose())
#------------------------------------------------------------------
#Pre-setup
#------------------------------------------------------------------
 iti.data$Rat <- factor(iti.data$Rat)
 iti.data$ITI <- factor(iti.data$ITI)


 time.30<- iti.data %>% filter(ITI == '30')
 time.100<- iti.data %>% filter(ITI == '100')
 time.300<- iti.data %>% filter(ITI == '300')
 time.1000<- iti.data %>% filter(ITI == '1000')
#------------------------------------------------------------------
#Summary
#------------------------------------------------------------------
 iti.summary <- iti.data %>% group_by(ITI) %>% summarize(
 count = n(),
 mean = mean(Accuracy),
```

```
 sd = sd(Accuracy),
 SEM = (sd(Accuracy)/sqrt(count))
 )
#---------------------------------------------------------------------
#Normality Test: Lillifors (Accuracy)
#---------------------------------------------------------------------
 time.30.residuals <- tbl_df(time.30$Accuracy - mean(time.30$Accuracy))
 time.100.residuals <- tbl_df(time.100$Accuracy - mean(time.100$Accuracy))
 time.300.residuals <- tbl_df(time.300$Accuracy - mean(time.300$Accuracy))
 time.1000.residuals <- tbl_df(time.1000$Accuracy - mean(time.1000$Accuracy))
 iti.residuals<- bind_rows (time.30.residuals, time.100.residuals, time.300.residuals,
time.1000.residuals)

 lillie.test(iti.residuals$value)
#---------------------------------------------------------------------
#Friedman's Test
#---------------------------------------------------------------------
 iti.fried<-friedman_test(data=iti.data, Accuracy ~ ITI | Rat)
 iti.fried
 iti.fried.eff <- friedman_effsize(data=iti.data, Accuracy ~ ITI | Rat)
 iti.fried.eff
```

```
#-----------------------------------------------------------------
#Wilcoxon Rank Sum and Signed Rank Tests
#-----------------------------------------------------------------
 iti.wilcox1 <- wilcox_test(data=iti.data, Accuracy~ITI, ref.group = "30",
          p.adjust.method = 'none', conf.level =0.95)
 iti.wilcox1
 iti.wilcox2 <- wilcox_test(data=iti.data, Accuracy~ITI, comparisons = list (c("100",
          "300"), c("300", "1000"), c('100', '1000')), p.adjust.method = 'none',
          conf.level =0.95)
 iti.wilcox2
#-----------------------------------------------------------------
#Effect sizes
#-----------------------------------------------------------------
 iti.effectsize1 <- wilcox_effsize(data=iti.data, Accuracy~ITI, ref.group= '30',
          p.adjust.method = 'none', conf.level =0.95)
 iti.effectsize1
 iti.effectsize2 <- wilcox_effsize(data=iti.data, Accuracy~ITI, comparisons =
          list(c("100", "300"), c("300", "1000"), c('100', '1000')), p.adjust.method = 'none',
          conf.level =0.95)
 iti.effectsize2
```

Appendix B: ITI Raw Data

Date	Rat	ITI (s)	Accuracy (Out of 6)	Accuracy (%)
20190306	5	30	6	100%
20190306	6	100	3	50%
20190306	8	300	4	67%
20190306	10	1000	3	50%
20190306	11	100	5	83%
20190310	5	100	4	67%
20190310	6	300	4	67%
20190310	8	1000	2	33%
20190310	10	30	6	100%
20190310	11	300	4	67%
20190313	5	300	1	17%
20190313	6	1000	4	67%
20190313	8	30	5	83%
20190313	10	100	3	50%
20190313	11	30	5	83%
20190318	5	1000	2	33%
20190318	6	30	6	100%
20190318	8	100	5	83%
20190318	10	300	4	67%
20190318	11	1000	3	50%
20190403	3	300	3	50%
20190406	3	100	4	67%
20190407	3	30	6	100%
20190408	3	1000	2	33%
20190705	17	300	4	67%
20190708	17	1000	4	67%
20190709	17	30	6	100%
20190712	17	100	4	67%
20190725	18	30	5	83%
20190729	18	300	3	50%
20190801	18	100	4	67%
20190806	18	1000	3	50%
20190306	5	30	6	100%
20190306	6	100	3	50%

Appendix C: Drug Trial Raw Data

Date	Rat	Dose (mg/kg)	Accuracy (out of 6)	Accuracy (%)	Latency (s)	Distance (cm)	Speed (cm^2/s)
20190720	3	1	2	33.33%	6.05	203.81	15.06
20190720	6	0	5	83.33%	4.61	177.56	14.08
20190720	11	3	5	83.33%	6.59	169.63	13.05
20190727	3	3	2	33.33%	5.05	200.80	17.37
20190727	5	10	3	50.00%	3.52	216.85	22.48
20190727	6	1	3	50.00%	4.06	218.68	21.13
20190727	11	10	6	100.00%	4.48	197.28	15.72
20190802	11	1	4	66.67%	5.71	265.88	19.63
20190806	3	0	5	83.33%	7.69	250.97	17.03
20190806	5	1	3	50.00%	3.64	246.69	21.87
20190810	3	10	4	66.67%	4.87	175.63	18.88
20190810	5	1	4	66.67%	4.10	197.44	24.77
20190810	11	0	5	83.33%	4.67	192.20	23.81
20190821	5	0	5	83.33%	4.65	198.88	17.25
20190821	17	10	5	83.33%	5.86	187.29	14.14
20190826	5	3	4	66.67%	6.38	194.62	15.19
20190826	6	10	1	16.67%	5.50	231.11	19.37
20190830	6	3	3	50.00%	4.92	199.46	17.77
20190830	16	0	4	66.67%	5.09	214.21	17.07
20190830	17	3	5	83.33%	8.02	219.16	12.75
20190830	18	3	4	66.67%	4.43	208.61	16.29
20190830	19	10	3	50.00%	6.16	207.43	15.91
20190906	17	0	6	100.00%	16.52	315.46	13.91
20190906	18	1	2	33.33%	4.00	229.34	19.26
20190906	19	1	5	83.33%	5.98	219.43	14.91
20190912	17	1	4	66.67%	8.54	241.26	16.56
20190912	18	10	5	83.33%	6.80	219.37	16.58
20190912	19	3	6	100.00%	7.11	221.23	16.28
20190912	22	1	4	66.67%	7.58	241.17	16.71
20190921	19	0	4	66.67%	8.75	217.44	10.55
20190921	22	10	5	83.33%	11.50	249.89	11.02
20190927	16	3	2	33.33%	3.28	183.45	24.23
20190927	18	0	4	66.67%	3.39	186.86	24.11
20191004	16	10	3	50.00%	3.56	192.87	25.80
20191017	16	1	5	83.33%	4.27	206.15	27.98

Appendix D: Probe Trial Raw Data

Rat	Marking probe Accuracy	Reward Probe Accuracy
3	100.00%	100.00%
5	100.00%	100.00%
6	100.00%	100.00%
8	100.00%	83.33%
10	100.00%	100.00%
11	100.00%	83.33%
16	100.00%	83.33%
17	100.00%	83.33%
18	83.33%	83.33%
19	100.00%	100.00%
22	83.33%	83.33%

Appendix E: Order Effects

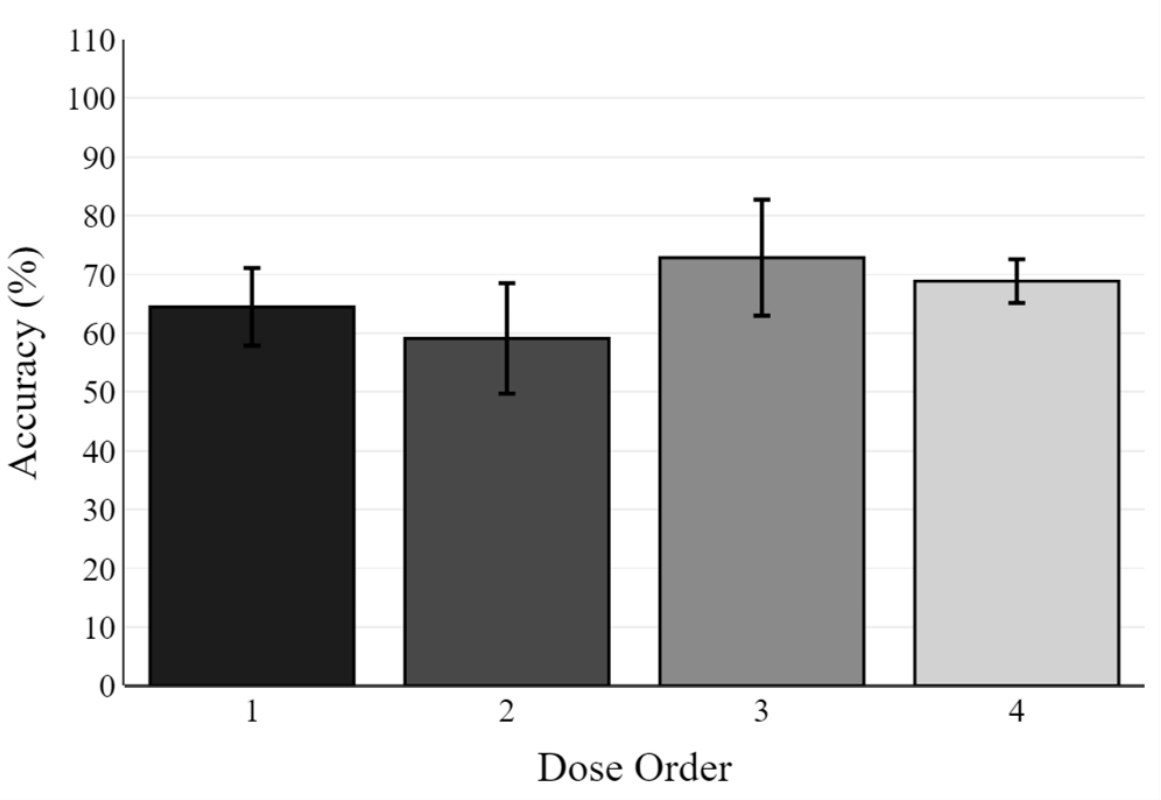

Figure 9. The effects of drug dose order on DNMTO accuracy performance. Accuracy performance was not significantly different between the order of drugs given [$F(3,21)=0.118$, $p= 0.949$, $\eta^2 = 0.0121$]. Data are presented as mean ± SEM.