EFFECT OF AN ANXIOLYTIC AGENT ON SPATIAL LEARNING STRATEGY PREFERENCE IN PREPUBERTAL MALE RATS

AN ABSTRACT
SUBMITTED ON THE FIFTEENTH DAY OF APRIL 2014
TO THE DEPARTMENT OF NEUROSCIENCE
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
OF THE GRADUATE SCHOOL
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FOR THE DEGREE OF
MASTERS OF SCIENCE
BY

Thomas Lobell

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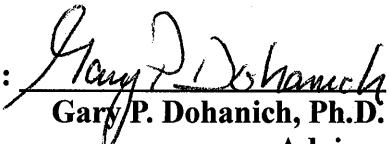

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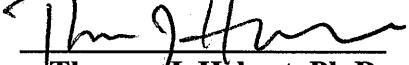

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ABSTRACT

Rodents rely on spatial learning and memory to efficiently navigate a complex environment. Distinct brain areas mediate different types of spatial learning strategies. The hippocampus-dependent *place* strategy utilizes spatial cues in the environment to guide the rodent to a goal, while striatum-dependent *response* and *stimulus-response* strategies rely on proprioceptive cues or cues proximal to a goal, respectively, to guide the rodent to a goal. The spatial strategy employed to learn a task is influenced by a range of factors including biological sex, age, and anxiety. Previous reports have found that high levels of natural, or trait, anxiety tend to bias prepubertal male rats towards a *stimulus-response* learning strategy. The effect of reduced transient, or state, anxiety on spatial strategy preference in prepubertal rats is yet to be determined. In the present study, an anxiolytic agent, diazepam, was administered to prepubertal male rats to determine its effect on expression of anxiety-like behaviors on an open field test and spatial learning strategy preference on a visible platform water maze (VPWM) task. Prepubertal male rats treated with a low dose of diazepam (2.5 mg/kg) displayed reduced anxiety in comparison to prepubertal male rats treated with vehicle or a high dose of diazepam (5 mg/kg). A separate sample of prepubertal male rats treated with vehicle displayed a significant preference for a *stimulus-response* learning strategy as reported previously, while males treated with either a low or high dose of diazepam displayed no spatial learning strategy preference. Taken together, these results support previous findings that higher levels of anxiety bias prepubertal male rats towards a *stimulus-*

response learning strategy, indicating that reduction of anxiety shifts strategy preference away from a *stimulus-response* learning strategy.

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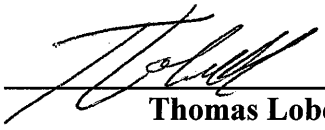

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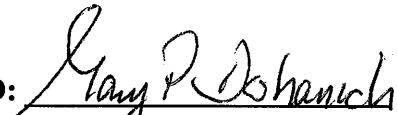
APPROVED: 

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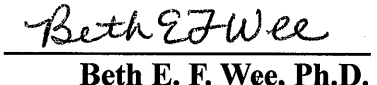

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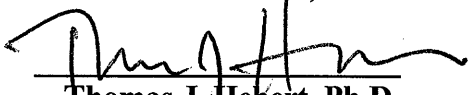

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INTRODUCTION

Both humans and rodents rely on spatial learning and memory, the cognitive ability to store and manipulate relationships between distinct objects in space, in order to navigate efficiently through a complex environment. Spatial ability allows humans to learn the layout of their home, remember the locations of objects around their home, and navigate to and from work. In rodents, these same cognitive abilities are necessary to efficiently locate food, mates, and burrows, a process that is essential to the survival of the individual.

There is a large body of literature utilizing rodent models to elucidate the neural mechanisms underlying, and factors that mediate and modulate, spatial learning and memory. Importantly, spatial memory performance in rats can be influenced by a wide variety of factors, including biological sex, age, early rearing conditions, environmental enrichment, hormone profile, and anxiety (Pompili, Tomaz, Arnone, Tavares & Gasbarri, 2010; Belviranli, Atalik, Okudan & Gokbel, 2012; Kosten, Kim & Lee, 2012; Simpson & Kelly, 2012; Sampedro-Piquero, Zancada-Menendez, Begega, Rubio & Arias, 2013). This wide range of factors influencing spatial learning and memory suggests a diverse and interactive neural basis for spatial memory that involves both cellular- and systems-level processes and interactions (McDonald & White, 1994).

Importantly, distinct brain areas mediate different types of spatial strategies. A

hippocampus-based *place* strategy requires the use of relationships between salient visual cues in the environment to guide the subject towards a specific location in space (Grissom et al., 2012; Grissom, Hawley, Hodges, Fawcett-Patel & Dohanich, 2013). A *place* strategy is a flexible strategy that involves learning the location of visual cues in an environment and the relationships between these cues, allowing for the creation of a "cognitive map" of the environment. This flexibility makes a *place* strategy effective when solving a task in which the demands of the task are changing frequently (Hawley, Grissom & Dohanich, 2011). For example, during a water maze task that requires rats to navigate to a submerged platform from multiple start points, a *place* strategy is required because the demands of the task change for each trial, depending on where the rat enters the maze (Ramos & Vaquero, 2000).

Alternatively, striatum-based *response* and *stimulus-response* strategies rely on proprioceptive cues or proximal cues, respectively, to guide the subject towards a specific location (McDonald & White, 1994; Packard & McGaugh, 1996; Grissom et al., 2012). When intra-maze and extra-maze cues are ignored, and a habitual motor response, such as turning in one direction each trial is learned, a *response* strategy is being implemented. This strategy can be used to solve one version of a T-maze mask in which rats are trained to turn right on each trial in order to receive a food reward (Chang & Gold, 2003). When extra-maze cues are ignored and intra-maze cues or cues proximal to the location of a goal are used to navigate to the goal, a *stimulus-response* strategy is being employed. This strategy is effective when learning a water maze task in which rats learn to navigate to an escape platform that is either visible or marked by a cue proximal to the escape platform (Hawley et al., 2011; Grissom, et al., 2012). Both response and stimulus-

response strategies rely on relatively simple, habitual responses, and are thus not well suited to tasks in which the demands of the task are changing frequently (Hawley et al., 2011).

The amygdala is a third structure that modulates learning strategy. The amygdala is responsible for emotion-dependent learning and memory, and is necessary for acquisition of tasks that rely on emotional states as motivation for learning. Specifically, an intact amygdalar system is necessary to acquire conditioned place preference and inhibitory avoidance tasks, in which a rewarding stimulus or an aversive stimulus is administered in one distinct environment, causing a subsequent preference for or avoidance of that environment, respectively (McDonald & White, 1993; White & McDonald, 1993). The amygdala also plays an important role in the expression of anxiety. Activation of the amygdala, particularly the centromedial nucleus, results in the expression of both autonomic and behavioral responses associated with heightened anxiety states (Tye et al., 2011).

Separate brain structures often process information simultaneously during acquisition of a task, with one structure determining the type of strategy that is used to learn the task (Packard, 1999; White & McDonald, 2002; Grissom et al., 2013). Studies examining strategy preferences often utilize dual-solution tasks. These protocols involve training on a task that can be learned using either a place strategy or a response or stimulus-response strategy, followed by a probe trial that reveals which strategy was used to learn and complete the task (Packard, 1999; Chang & Gold, 2003; Elliott & Packard, 2008; Hawley et al., 2011; Grissom, et al., 2012; Grissom et al., 2013). Interestingly, the strategy that is employed when completing a task that can be successfully learned using

either a place, response, or stimulus-response strategy depends on a variety of factors including age, biological sex, hormone profile, early rearing conditions, and anxiety (Daniel & Lee, 2004; Korol, Malin, Borden, Busby & Couper-Leo, 2004; Elliot & Packard, 2008; Grissom et al., 2012; Hawley, Grissom, Barratt, Conrad & Dohanich, 2012).

Hormonal influences are especially significant, as they underlie sex differences in spatial learning strategy preference. Varying hormonal conditions have been shown to differentially affect strategy preferences, particularly in adult rodents. Adult male rodents, for instance, are generally biased towards a place strategy during early training trials and a response strategy later in training, regardless of circulating hormone levels (Chang & Gold, 2003; Packard & McGaugh, 1996; Hawley et al., 2012). Adult female rodents, however, show different strategy preferences depending on circulating ovarian hormone levels, as evidenced by strategy preference changes across the estrous cycle and between ovariectomized rats receiving estradiol replacement when compared to no hormone replacement. Adult females tend to prefer a place learning strategy when circulating estradiol levels are high, while adult females tend to prefer a response learning strategy when estradiol levels are low, a result that has been replicated in both rats and mice (Packard & McGaugh, 1996; Korol et al., 2004; Daniel & Lee, 2004; Tunur, Dohanich & Schrader, 2010).

However, quite different strategy preferences have been reported in prepubertal rats, when gonadal hormone levels are low and thus minimally influential on spatial learning strategy preference. Specifically, prepubertal male rats preferred a stimulus-response strategy over a place strategy on dual-solution tasks, while prepubertal females

displayed no preference for either strategy (Grissom et al., 2012; Grissom et al., 2013). This observed sex difference may be at least partly attributed to differences in the rate of brain development between female and male rats early in life. Namely, female rats reach puberty approximately one week before male rats, and the hippocampal system develops a week earlier in female rats than in male rats. At 28 days of age, for instance, female rats have a greater number of apical dendritic segments, longer apical segments, and greater apical dendritic spine density when compared to age-matched male rats, suggesting a more complex apical dendritic arbor in females (Grissom & Dohanich, 2013). This developmental difference, which may be caused in part by the effect of early estradiol exposure on the cholinergic system (Hammond & Gibbs, 2011), may explain the increased tendency of prepubertal female rats to use a hippocampus-mediated place learning strategy on dual-solution tasks in comparison to age-matched prepubertal male rats (Grissom, et al., 2012; Grissom et al., 2013).

There is evidence that differences in strategy preferences may result from differential activation of the hippocampus and striatum. For instance, on a cross maze task that could be learned using either a place strategy or a response strategy, post-training activation of the hippocampus via glutamate infusion biased rats towards a place strategy, while post-training activation of the striatum via glutamate infusion biased rats towards a response strategy (Packard, 1999). Additionally, in a study by Chang and Gold (2003), the release of acetylcholine (ACh) was monitored throughout extensive training on a plus-maze task that could be learned using either a place strategy or response strategy. Strategy preference over the course of training replicated previous findings that adult male rats preferred a place strategy early in training but a response strategy after

extensive training (Packard & McGaugh, 1996; Hawley et al., 2012). Notably, this shift in strategy preference mirrored activation of the hippocampus and the striatum. Early in training, when a place strategy predominated, ACh release was elevated in the hippocampus, while it remained low in the striatum. Later in training, when a response strategy was preferred, ACh release was elevated in the striatum. Interestingly, hippocampal ACh release remained elevated throughout training, indicating that striatal activation can dominate hippocampal activation when both brain areas are simultaneously active (Chang & Gold, 2003).

Furthermore, the ratio of muscarinic ACh binding in the hippocampus compared to the striatum similarly reflects strategy preference in prepubertal rats, even when training is not extensive (Grissom et al., 2013). Specifically, both male and female prepubertal rats that preferred a striatum-dependent stimulus-response learning strategy on a dual-solution visible platform water maze task also exhibited a lower ratio of muscarinic ACh binding in the hippocampus compared to the dorsolateral striatum, suggesting that the increased cholinergic activation of the striatum is associated with the stimulus-response learning strategy preference. Correspondingly, both male and female prepubertal rats that preferred a hippocampus-dependent place learning strategy exhibited a higher ratio of binding in the hippocampus compared to the dorsolateral striatum, indicating that the increased cholinergic activation of the hippocampus is associated with the place learning strategy preference (Grissom et al., 2013). Overall sex differences in strategy preference mirror these findings, as prepubertal female rats, which are more likely to prefer a hippocampus-dependent place learning strategy, exhibit a higher ratio of muscarinic ACh binding in the hippocampus compared to the dorsolateral striatum

(Grissom et al., 2013). Similarly, prepubertal male rats, which are more likely to adopt a striatum-dependent stimulus-response learning strategy, exhibit a lower ratio of muscarinic ACh binding in the hippocampus compared to the dorsolateral striatum (Grissom et al., 2013).

Anxiety states also affect strategy preference in both adult and prepubertal rats. Anxiety can be divided into two types, each of which describes a different temporal context. State anxiety is anxiety that is experienced in a certain context at a certain point in time. For example, when a rat is placed into a novel context such as an open field apparatus, observed anxiety-like behaviors are a result of state anxiety (Goes, Antunes & Teixeira-Silva, 2009). Conversely, trait anxiety describes a context-independent, inherent, and enduring trait of an individual that is stable over time (Goes et al., 2009). Accordingly, when tested on an elevated plus maze, a rat with high trait anxiety will show consistently high levels of anxiety-like behaviors over the course of repeated trials, while a rat with low levels of trait anxiety will show a reduction in anxiety-like behaviors over the course of repeated trials (Andreatini & Bacellar, 2000). Though trait and state anxiety are differentiated in the literature, they are inherently related, as individuals with high levels of trait anxiety will tend to exhibit high levels of state anxiety at any given point in time. Previous studies have demonstrated that higher levels of inherent or trait anxiety tend to bias adult male rats towards a stimulus-response strategy on a visible platform water maze task (Hawley et al., 2011). In an earlier study, transient or state anxiety was manipulated by either peripheral or intra-amygdala injection of an anxiogenic drug; in both cases, increased state anxiety biased adult male rats towards

response and stimulus-response strategies on a dual-solution plus-maze task (Elliot & Packard, 2008).

Similarly, prepubertal male rats tend to prefer a stimulus-response strategy under heightened anxiety states, while prepubertal female rats show no strategy preference regardless of anxiety level (Grissom et al., 2012). This sex difference could again be the result of developmental differences. Contrary to the hippocampus, the amygdala develops earlier in male rats than in female rats due to early androgen exposure in developing males (Siddiqui & Shah, 1997). Specifically, prepubertal male rats have approximately 80% more excitatory synapses and exhibit significantly greater mEPSC frequency in certain amygdalar nuclei when compared to prepubertal female rats (Cooke & Woolley, 2005). These developmental and morphological sex differences in the amygdala could result in heightened trait and state anxiety in prepubertal males compared to prepubertal females, which may explain the observed preference for response and stimulus-response strategies seen in males and the lack of preference for either strategy seen in females.

It is possible that anxiety states exert an effect on strategy preference in dual-solution tasks through amygdala-mediated modulation of hippocampal and striatal activation. This theory is supported by findings that the hippocampus and amygdala share non-reciprocal neural connections that allow for competition between the two structures (Chang & Gold, 2003). For example, ACh release in the hippocampus is negatively correlated with performance on amygdala-dependent tasks such as conditioned place preference (McIntyre, Pal, Marriott & Gold, 2002; McIntyre, Marriott & Gold, 2003). Further, lesions of the fornix have been shown to improve acquisition of an

amygdala-dependent conditioned place preference task (White & McDonald, 1993). Both of these findings suggest that the hippocampus competes with the amygdala for control of cognitive processes, resulting in impaired acquisition of amygdala-dependent tasks. Because of the competitive nature of the relationship between the amygdala and the hippocampus, it is conceivable that under heightened anxiety states, competition between these two structures for control over learning and memory may decrease the ability of the hippocampus to guide spatial learning. As a result, rats under heightened states of anxiety may thus be more apt to adopt a striatum-mediated stimulus-response learning strategy, as reported in previous studies (Hawley et al., 2011; Grissom, et al., 2012).

Previous studies examining the effect of anxiety on strategy preference in prepubertal rats have used measures of trait anxiety to assign subjects to groups (Grissom et al., 2012). Assigning subjects to groups based on intrinsic tendencies towards anxious behaviors may also allow for the introduction of group differences that may confound results. For instance, adult rats that exhibit high levels of trait anxiety are also impaired in hippocampus-dependent learning (Hawley et al., 2011). If the same is true for prepubertal rats, the impairment in hippocampus-based place learning, rather than anxiety itself, may contribute to the observed striatum-mediated stimulus-response strategy preference. Furthermore, rats that exhibit high levels of trait anxiety have been shown to have different gene expression in the amygdala compared to rats that exhibit low levels of trait anxiety (Diaz-Moran et al., 2013). Intrinsic group differences in gene expression could influence the amygdala's modulatory role on learning and memory, which could also obscure the effect of state anxiety on spatial learning strategy preference. The effect

of varying levels of state anxiety on strategy preference within groups of rats with both high and low trait anxiety is yet to be determined.

Benzodiazepines are a class of chemicals that are known to have a wide range of therapeutic uses including sedation, seizure suppression, muscle relaxation, and anxiety suppression (Rudolph & Knoflach, 2011). A common benzodiazepine, diazepam, is known to act on the $GABA_A$ receptor complex to induce its well-documented anxiolytic effects (Atack, 2011; Hoeller et al., 2013). Because diazepam has been shown to reduce anxiety-like behaviors on a number of behavioral tasks commonly used to assess anxiety in rodents, including the elevated plus maze task and the open field task (Schmitt & Hiemke, 1998; Vekovischeva, Haapalinna, Sarviharju, Honkanen & Korpi, 1999), diazepam was used to manipulate state anxiety in the current study. Diazepam, however, also has the potential to induce sedative and locomotor side effects at high doses (Schmitt & Hiemke, 1998), which has the potential to confound results of behavioral testing. For instance, sedative effects of diazepam could increase latency to escape for diazepam-treated rats, falsely suggesting decreased cognitive performance. To avoid these possible side effects, relatively low doses of diazepam was used in the current study and locomotor activity was examined to ensure that no group differences in locomotor function exist.

The current study aimed to extend the hypothesis that heightened anxiety biases prepubertal male rats towards a stimulus-response learning strategy on a dual-solution visible platform water maze task. We predicted that administration of an anxiolytic drug to manipulate state anxiety, rather than relying on inherent anxiety states to differentiate between groups, would reduce the effect of trait anxiety on strategy preference.

Specifically, we hypothesized that vehicle-treated prepubertal male rats would display higher levels of anxiety on an open field task when compared to diazepam-treated prepubertal male rats. Correspondingly, we hypothesized that vehicle-treated prepubertal male rats would display an anxiety-induced preference for a stimulus-response spatial learning strategy, as previously reported (Grissom, et al., 2012), while diazepam-treated prepubertal male rats would show no preference for either a place or stimulus-response spatial learning strategy on a dual-solution water maze task.

METHODS

Anxiolytic Agent

The anxiolytic agent used in the current experiment was diazepam (Sigma-Aldrich Co., St. Louis, MO). Diazepam was administered in a vehicle composed of 5% poly(ethylene glycol) (Sigma-Aldrich), 5% Tween-80 (Sigma-Aldrich), and 0.9% NaCl (EMD Chemicals Inc., Gibbstown, NJ) in deionized, distilled H_2O. Diazepam was administered via intraperitoneal (i.p.) injection at low (2.5 mg/kg) and high (5 mg/kg) doses delivered in injection volumes of 2 ml/kg. Injection volume was doubled in contrast to most studies in the literature to minimize variation in dosing as a result of small injection volumes. Diazepam doses were chosen to induce an anxiolytic effect while avoiding sedative and locomotor side effects, as reported in previous studies (McNamara, Davis, & Skelton, 1996; Nakamura-Palacios & Roelke, 1997; Schroeder, Humbert, Desor, & Nehlig, 1997).

Animals

All experimental procedures were approved by the Tulane University Institutional Animal Care and Use Committee in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* (1996). All rats were obtained from Harlan Laboratories (Indianapolis, IN) and housed in Tulane University vivarial facilities under a 12:12 hour light/dark cycle (lights on at 07:00 hours). Food and water were provided *ad libitum*. Subjects were obtained by breeding 14 adult female Long-Evans rats

with 6 adult male Long-Evans rats in Tulane University vivarial facilities. Prior to mating, all breeder rats were pair-housed with a same-sex cage mate. To maximize the chances of reproductive success, adult females were mated until they were observed to have received two to three ejaculations from at least two different males. Beginning 19 days after mating, pregnant females were singly housed and provided with paper nesting material. Within 24 hours after birth, litters were culled to a maximum of 12 pups, in order to minimize the effect of maternal attention on subsequent behavioral measures. All litters were reared with their mothers for the first 21 days of life. During this period, mothers and offspring were transferred to clean cages every 5 days. On post-natal day (PND) 21, offspring were weaned from mothers and housed in groups of three to seven by litter and sex. From 13 viable litters, 74 prepubertal male and 63 prepubertal female offspring were obtained. The prepubertal male offspring were used as experimental subjects, while female offspring were transferred to another experiment.

Of the 74 prepubertal male rats, 31 subjects were assigned to anxiety testing only, and 43 subjects were assigned to water maze testing only. Previous studies have used the same groups of rats and tested anxiety at PND 26 and strategy preference on PND 28 (Grissom et al., 2012). To test the same groups of rats on both anxiety and water maze tasks in the current experiment, each subject would have to receive vehicle or diazepam treatment twice, once before each task, which could introduce possible drug tolerance effects. Furthermore, exposure to anxiety testing prior to water maze testing could affect behavioral response to water maze testing. For these reasons, different groups of rats were used for each task.

Open Field Test

A total of 31 prepubertal male rats were tested on the open field task. Depending on the size of the litter, between 0 and 4 prepubertal males from each litter were randomly assigned to undergo open field testing only, in order to determine the effect of drug conditions on measures of anxiety. To minimize litter effects, males tested on the open field test were assigned to vehicle, 2.5 mg/kg diazepam, or 5.0 mg/kg diazepam groups in a pseudo-randomized fashion, so that each litter contributed no more than 2 prepubertal males to any treatment group.

On PND 28, male littermates assigned to anxiety testing received an i.p. injection of vehicle, 2.5 mg/kg diazepam, or 5.0 mg/kg diazepam, depending on group assignment. Anxiety testing began 55 minutes after diazepam administration to ensure that the interval between drug injection and anxiety testing was identical to the interval between drug injection and the probe trial on the strategy preference task (see below). Rats were placed into the Northeast corner of a black Plexiglas arena (90 × 90 × 45 cm) and allowed to explore for 5 minutes. All trials were recorded by a camera mounted directly above the center of the open field. During subsequent video scoring, the image of the open field was divided into 16 congruent squares in a 4 × 4 grid, and the number of entries into each square during the 5-minute trial was recorded. An entry was defined as a rat moving into a new square with all four paws. Videos were scored by experimenters blind to drug treatment. Lower anxiety was indicated by higher number of entries into the center 4 squares and greater tendency to enter one of the center 4 squares at least once during the 5-minute trial. Overall activity was measured by the total number of squares entered during the 5-minute trial.

Visible Platform Water Maze Task

The effect of drug treatment on spatial learning strategy preference was determined using a visible platform water maze (VPWM) task procedure to test a separate sample of 43 prepubertal males (McDonald & White, 1994; Grissom et al., 2012; Grissom et al., 2013). Depending on the number of male offspring in each litter, between 2 and 4 offspring from each litter were randomly assigned to undergo water maze testing only, in order to determine the effect of diazepam treatment on spatial learning strategy preference. To minimize litter effects, prepubertal males tested on the VPWM task were assigned to vehicle, 2.5 mg/kg diazepam, or 5.0 mg/kg diazepam groups in a pseudo-randomized fashion, so that each litter contributed no more than 2 prepubertal males to any treatment group.

VPWM testing was conducted according to previously described procedures (McDonald & White, 1994; Grissom et al., 2012; Grissom et al., 2013). A white, circular, metal pool 180 cm in diameter and 60 cm in depth was placed in the center of a room containing both two- and three-dimensional extramaze cues of various shapes, sizes, and colors. The pool was filled to a depth of 30 cm with water made opaque by non-toxic white paint (Crayola, Easton, PA). Water was maintained at approximately 25°C throughout testing to reduce its aversive quality.

On PND 27, prepubertal males assigned to VPWM testing were handled and weighed, followed by a habituation swim for one minute during which no platform or spatial cues were present. On PND 28, subjects received i.p. injections of vehicle, 2.5 mg/kg diazepam, or 5.0 mg/kg diazepam, depending on group assignment. After a 15-minute delay, behavioral testing of males on the VPWM task began.

A circular black platform protruding 3 cm above the surface of the water was placed in the Southwest quadrant of the pool. Eight training trials were conducted with rats entering the pool from the four cardinal directions in a pseudo-randomized order, such that each rat entered from each of the cardinal directions twice while the platform remained in a fixed location in the Southwest quadrant (Fig. 2A). During each trial, rats were allowed 30 seconds to locate the platform. If a rat failed to locate the platform in 30 seconds, the experimenter guided the rat to the platform. Rats remained on the platform for 10 seconds before being removed from the maze, hand-dried with a towel, and placed in a holding cage heated by overhead lamps for an inter-trial interval of 5 minutes. Learning was indicated by decreasing latency to locate the platform and path length to reach the platform as training progressed. Equal acquisition of the task was indicated by similar latency to locate the platform and path length to reach the platform between groups on training trial 8.

After 8 training trials were completed, a probe trial was conducted to determine spatial learning strategy preference. During the probe trial, the platform was moved to the opposite (Northeast) quadrant of the pool, and rats entered the pool from the South (Fig. 2B). During the probe trial, rats that swam to the new platform location (Northeast quadrant) were categorized as *stimulus-response learners*, rats that initially swam to within 5 cm of the previously trained platform location (Southwest quadrant) were categorized as *place learners*, and rats that showed no clear strategy were categorized as *ambiguous learners* (Fig. 2C). Examples of strategies that resulted in an *ambiguous learner* classification included thigmotaxis and not swimming to either the old or new platform location. All trials were recorded by an overhead camera interfaced with

tracking software (HVS Image Ltd., United Kingdom) that recorded escape latency and path length. The swim path taken from the start point to the platform was recorded to analyze strategy preference on the probe trials.

Statistical Analyses

An analysis of variance (ANOVA) with a between-subjects effect of drug treatment was carried out to determine the effect of drug treatment on activity (total number of squares entered) and anxiety (entries into center squares) on the open field task. A χ^2 analysis was conducted to determine the effect of drug treatment on the number of animals entering one of the center squares at least once during the open field trial.

An ANOVA with a within-subjects effect of 2-trial training trial block, with each block representing the average of two training trials, and a between-subjects effect of drug treatment (vehicle, 2.5 mg/kg, or 5 mg/kg) was carried out to determine the effect of drug condition on performance during the 8 training trials on the VPWM task. An ANOVA with a between-subjects effect of drug condition was carried out to determine the effect of drug condition on latency and path length to find the platform on the final training trial (trial 8) of the VPWM task. A χ^2 analysis was conducted to determine the relationship between drug treatment and learning strategy preference as indicated by swim path during the probe trial on the VPWM task.

RESULTS

Anxiety in the Open Field Test

The benzodiazepine diazepam is a well-documented, commonly used anxiolytic drug with the potential for adverse side-effects including sedation and psychomotor disruption (Schmitt & Hiemke, 1998; Vekovischeva et al., 1999; Atack, 2011; Hoeller et al., 2013). Despite extensive use in adult rats, diazepam has not been used in experiments with prepubertal rats. To assess the effect of treatment with vehicle, 2.5 mg/kg diazepam, or 5 mg/kg diazepam on measures of activity and anxiety, 31 prepubertal male rats were tested on the open field task. After two rats were excluded from open field test analysis due to a fire alarm during behavioral testing, open field test data from 29 rats was analyzed. A one-way analysis of variance (ANOVA) conducted on the total number of entries into both center and peripheral squares in the open field revealed no significant differences in locomotor activity between the vehicle group and the two diazepam treatment groups [$F(2,26) = 0.305$, $p = 0.740$; Fig. 3], indicating that sedation and psychomotor disturbances were absent at the doses of diazepam used in this study.

A one-way ANOVA revealed a trend toward a significant effect of diazepam treatment on the number of entries into the center squares of the open field [$F(2,26) = 2.561$, $p = 0.097$], with rats treated with a low dose of diazepam entering the center

squares more frequently than rats treated with vehicle and rats treated with a high dose of diazepam (Fig. 4). Exploratory Fisher's least significant difference (LSD) post-hoc analysis revealed a significant difference in number of entries into the center squares, with prepubertal male rats treated with a low dose of diazepam entering the center squares significantly more frequently than prepubertal male rats treated with vehicle ($p < 0.05$). Similarly, a χ^2 analysis revealed that rats treated with a low dose of diazepam showed a trend to enter the center squares of the open field at least once [$\chi^2 = 3.600$, $p = 0.058$], while rats treated with vehicle [$\chi^2 = 0.000$, $p = 1.000$] and rats treated with a high dose of diazepam [$\chi^2 = 0.091$, $p = 0.763$] showed no significant tendency to enter the center squares of the open field at least once (Fig. 5). Though not quite reaching statistical significance, these results suggest that a low dose of diazepam reduced anxiety-like behaviors on the open field task.

Anxiety in the Visible Platform Water Maze Task

A total of four rats were excluded from all visible platform water maze (VPWM) task data analysis. One rat scheduled to undergo water maze testing was observed to have motor impairment as a result of diazepam administration, and was thus excluded from all VPWM data analysis. Three rats that underwent VPWM testing were excluded from all VPWM data analysis due to a fire alarm that went off during behavioral testing.

Previous studies have reported that diazepam can impair acquisition of spatial learning tasks (McNamara et al., 1996; Nakamura-Palacios & Roelke, 1997). To assess if treatment with diazepam modulates acquisition of a spatial learning task, thirty-nine prepubertal male rats were tested on the VPWM task. To assess acquisition of the VPWM task, eight training trials were divided into four blocks representing the average

of two consecutive trials, and repeated measures ANOVAs were conducted to determine the effect of trial block on latency to escape and path length. The results of a repeated measures ANOVA revealed a significant effect of two-trial block on latency to escape [$F(3,108) = 28.251$, $p < 0.001$, Fig. 6]. A total of twelve rats were excluded from analysis of path length over trial block only, due to loss of tracking by tracking software during two consecutive trials, resulting in insufficient data to perform a repeated measures ANOVA. Despite reduced group sizes, a repeated measures ANOVA revealed a significant effect of two-trial block on path length [$F(3,72) = 10.558$, $p < 0.001$, Fig. 7]. Taken together, decreased latency to escape and path length as training progressed indicates that all groups learned the task. No interactive effect of diazepam treatment by trial block on either latency to escape [$F(6,108) = 1.667$, $p = 0.136$, Fig. 6] or path length [$F(6,72) = 0.458$, $p = 0.837$, Fig. 7] was observed, indicating that diazepam treatment did not affect acquisition of the task. Therefore, as training progressed, the latency to escape and path length declined equally regardless of treatment.

Further, we analyzed latency to escape and path length on the last training trial (trail 8), in order to assess performance at the end of training. A one-way ANOVA revealed no significant effect of diazepam treatment on latency to escape [$F(2,36) = 0.394$, $p = 0.677$, Fig. 8a] on the last training trial (trial 8). A total of seven rats were excluded from analysis of path length on trial 8 only due to loss of tracking by tracking software on trial 8. A one-way ANOVA revealed no significant effect of diazepam treatment on path length [$F(2,29) = 0.004$, $p = 0.996$, Fig. 8b] on the last training trial (trial 8). Taken together, these results indicate that all treatment groups performed similarly on the final training trial.

Previous reports from our laboratory indicated an effect of trait anxiety on strategy preference in the visible platform water maze task (Hawley et al., 2011; Grissom et al., 2012). To determine the effect of treatment with the anxiolytic drug diazepam on spatial learning strategy preference, the strategy employed to locate the platform on the probe trial of the VPWM task was analyzed, with rats being categorized as *place learners*, *stimulus-response learners*, or *ambiguous learners* depending on where rats swam during the probe trial. Rats that swam to the previously trained platform location were categorized as *place* learners, rats that swam to the new platform location were categorized as *response* learners, and rats that displayed no distinct strategy preference were categorized as *ambiguous learners*, as described previously. A total of eleven rats were excluded from strategy preference data analysis only: one rat was excluded due to loss of tracking during the probe trial; two rats were excluded due to incorrect placement at the start of the probe trial; eight rats were excluded due to displaying an *ambiguous* learning strategy. The results of χ^2 analyses indicated that rats treated with vehicle displayed a significant preference for a stimulus-response spatial learning strategy on the probe trial in the VPWM task [$\chi^2 = 4.500$, $p < 0.05$], while rats treated with a low [$\chi^2 = 1.000$, $p = 0.317$] or high [$\chi^2 = 0.818$, $p = 0.366$] dose of diazepam displayed no strategy preference on the probe trial (Fig. 9). These results indicate that treatment with either a low or high dose of diazepam shifted spatial learning strategy preference away from the stimulus-response learning strategy bias observed in rats treated with vehicle.

DISCUSSION

The results of the current experiment indicate that treatment with the benzodiazepine diazepam affected both anxiety and spatial learning strategy preference in prepubertal male rats. On the open field test, prepubertal male rats treated with a low dose of diazepam (2.5 mg/kg) showed reduced levels of anxiety-like behavior in the open field task when compared to rats treated with vehicle or a high dose of diazepam (5 mg/kg). On the VPWM task, all groups learned the task equivalently. Prepubertal male rats treated with vehicle displayed a significant stimulus-response strategy preference on the probe trial of the VPWM task, while prepubertal male rats treated with either a high or low dose of diazepam displayed no learning strategy preference. Taken together, these results indicate that heightened states of anxiety biased prepubertal male rats towards a stimulus-response learning strategy, while reduction of anxiety following administration of an anxiolytic drug shifted strategy preference away from a stimulus-response learning strategy.

During open field testing, rats treated with a low dose of diazepam displayed a trend towards a marked reduction in anxiety, as evidenced by a greater tendency to enter the center of the open field in diazepam-treated rats when compared to vehicle-treated controls. Interestingly, the direction of the effect of diazepam treatment on anxiety level was dose-dependent and U-shaped, with rats treated with a low dose of diazepam

showing the greatest reduction in anxiety and rats treated with either vehicle or a high dose of diazepam displaying comparable anxiety levels. Simply, this result indicates that the low dose of diazepam was the most effective anxiolytic dose in the current experiment. U-shaped trends have been previously reported in studies using diazepam and measuring anxiety (Ennaceur, Michalikova, van Rensburg & Chazot, 2010a; Ennaceur, Michalikova, van Rensburg & Chazot, 2010b; Kohtz, Paris & Frye, 2010), though the mechanisms causing this trend are not well understood. These trends may be the result of minor locomotor side effects or desensitization of $GABA_A$ receptors at the high dose of diazepam (Ennaceur et al., 2010a; Ennaceur et al., 2010b), both of which could affect behavioral measures of anxiety. Previous studies have reported that some doses of diazepam can cause locomotor impairment or sedative side effects (Nakamura-Palacios & Roelke, 1997; Schmitt & Hiemke, 1998). However, diazepam treatment did not affect locomotor activity at any dose used in the current experiment, as evidenced by similar total entries across all treatment groups on the open field test. Therefore, we can conclude that any subsequent group differences were not attributable to locomotor side effects of diazepam treatment.

Contrary to previous reports that the doses of diazepam used in this study can impair working memory and the acquisition of spatial tasks (McNamara et al., 1996; Nakamura-Palacios & Roelke, 1997), no effect of diazepam treatment on acquisition of the VPWM task was observed in this experiment, as evidenced by similar decreases in latencies and path lengths to locate the platform as training progressed, regardless of drug treatment. Furthermore, all groups displayed equivalent escape latencies and path lengths on training trial 8 of the VPWM task, indicating that all groups acquired and performed

the task equally well at the end of training. These results clearly indicate that group differences in strategy preference were not caused by group differences in the ability to acquire or perform the VPWM task.

The current experiments extend a growing literature examining the effect of anxiety on cognition. Previous studies have found that heightened states of anxiety affect cognition by biasing prepubertal male rats towards a striatum-dependent stimulus-response strategy on dual-solution tasks. A recent report by Grissom et al. (2012) relied on inherent levels of trait anxiety to differentiate between low- and high-anxiety animals, allowing for confounding variables between groups that could have influenced the reported stimulus-response strategy preference bias in high-anxiety males. By utilizing random assignment of a heterogeneous group of prepubertal male rats into drug treatment groups, the current experiment minimized the possible effects of trait anxiety on spatial learning strategy preferences.

In support of previous findings, the current results indicate that vehicle-treated prepubertal male rats show a significant preference for a stimulus-response spatial learning strategy in a dual-solution VPWM task (Grissom et al., 2012; Grissom et al., 2013). Importantly, both groups of diazepam-treated prepubertal male rats showed no significant bias towards either a striatum-dependent stimulus-response strategy or a hippocampus-dependent place strategy. In conjunction with the finding that groups of prepubertal male rats treated with diazepam displayed a trend toward significantly lower levels of anxiety than groups treated with vehicle, these results indicate that reduced levels of anxiety in diazepam-treated rats shifted strategy preferences away from the stimulus-response bias observed in vehicle-treated rats.

Interestingly, the strategy preference trends between the two diazepam treatment groups mirrors the anxiety reduction trends observed in the open field test. Specifically, prepubertal male rats treated with a low dose of diazepam showed a trend toward a significant reduction of anxiety in the open field, and twice as many rats treated with a low dose of diazepam utilized a place strategy rather than a stimulus-response strategy on the probe trial of the VPWM task. Correspondingly, prepubertal male rats treated with a high dose of diazepam displayed anxiety levels comparable to vehicle-treated rats in the open field, and nearly twice as many rats treated with a high dose of diazepam employed a stimulus-response strategy rather than a place strategy on the probe trial of the VPWM task, showing a trend toward the strategy preference displayed by vehicle-treated animals. Though the strategy preference biases observed in diazepam-treated groups were not statistically significant, there was a trend suggesting that heightened states of anxiety biased prepubertal male rats towards a stimulus-response strategy, while reduced states of anxiety biased prepubertal male rats away from a stimulus-response learning strategy, and possibly towards a place learning strategy.

A previous study from our laboratory examining sex differences in anxiety and strategy preference in prepubertal rats found that prepubertal males tend to display higher levels of anxiety in comparison to females and tend to prefer a stimulus-response learning strategy, while females display no strategy preference (Grissom et al., 2012). In the current experiment, prepubertal males treated with a low dose of diazepam displayed lower levels of anxiety and displayed no learning strategy preference, paralleling the behavioral tendencies of unmanipulated prepubertal females in the previous study. Taken together, the current findings further support the hypothesis that anxiety contributes

significantly to the previously reported sex differences in spatial learning strategy preference.

The shift in strategy preference away from a stimulus-response strategy in diazepam-treated rats may be a result of decreased amygdalar activity. Previous studies have shown that amygdalar activity can modulate activity levels between the hippocampus and striatum (Chang & Gold, 2003; McIntyre et al., 2003). Under heightened states of anxiety, control of cognition can shift from the hippocampus to the striatum, resulting in the use of habitual behaviors such as a stimulus-response learning strategy rather than the more flexible place learning strategy. Based on our results, it is conceivable that in vehicle-treated rats, high levels of amygdalar activity may induce an increase in activity in the striatum, allowing the striatum to control cognition. The increased activity in the striatum may then result in the observed stimulus-response strategy preference. Conversely, in diazepam-treated rats, reduced levels of activity in the amygdala may result in less activation of the striatum, allowing the hippocampus to control cognition. Consequently, the increased influence of the hippocampus may result in the observed shift away from a stimulus-response strategy preference. This hypothesis is in line with previous findings from our laboratory that prepubertal rats that preferred a stimulus-response spatial learning strategy also exhibited a significantly lower ratio of muscarinic acetylcholine binding in the hippocampus relative to the dorsal striatum when compared to prepubertal rats that preferred a place learning strategy (Grissom et al., 2013).

Similarly, the current findings further support the hypothesis that sex differences in brain development contribute to sex differences in spatial learning strategy preference.

In prepubertal female rats, the hippocampus develops approximately one week earlier than in prepubertal males, resulting in a more complex apical dendritic arbor characterized by increased dendritic length and spine density (Grissom & Dohanich, 2013). This developmental difference may ultimately result in the lack of preference for either a stimulus-response strategy or a place strategy observed in prepubertal female rats (Hammond & Gibbs, 2011; Grissom, et al., 2012; Grissom et al., 2013). Conversely, the amygdala develops earlier in prepubertal male rats than in female rats (Siddiqui & Shah, 1997). The earlier development of the amygdala in prepubertal male rats may accelerate its activity, as evidenced by a greater mEPSC frequency and greater numbers of excitatory synapses in the amygdala of males when compared to age-matched female rats (Cooke & Woolley, 2005). Consequently, a shift in cognitive control towards the striatum in prepubertal males may contribute to the stimulus-response strategy preference reported in the current and previous studies (Grissom, et al., 2012; Grissom et al., 2013). The reduction in amygdalar activity in diazepam-treated prepubertal males may mediate the observed shift away from a stimulus-response strategy preference. Correspondingly, in prepubertal female rats, it is conceivable that the later development of the amygdala may lead to reduced amygdalar influence over cognition, resulting in the same shift away from a stimulus-response strategy preference as is seen in diazepam-treated male rats. The parallel between a less active amygdala and a lack of strategy preference in both diazepam-treated prepubertal male rats and unmanipulated prepubertal female rats supports the hypothesis that developmental differences may contribute to sex differences in spatial learning strategy preference.

Furthermore, the effect of anxiety on cognition in prepubertal rats is translatable to human research as well. Heightened anxiety states have been linked to a reduction in working memory, especially when the demands of the task are complex (Owens, Stevenson, Hadwin & Norgate, 2012). Anxiety also has been linked to poor academic and test performance (Keogh, Bond, French, Richards & Davis, 2004; Owens et al., 2012) and deficits in hippocampal learning in children (Mueller et al., 2009). The tendency of high-anxiety, vehicle-treated prepubertal rats to use the more rigid, striatum-mediated stimulus-response strategy rather than the more adaptable, hippocampus-mediated place strategy suggests that heightened states of anxiety may reduce cognitive flexibility. Conversely, low-anxiety, diazepam-treated prepubertal males show no strategy preference and an ability to use either learning strategy, suggesting that reduced anxiety can increase cognitive flexibility. Taken together, these findings suggest the possibility that in anxious children, impaired hippocampal function and reduced cognitive flexibility during learning and during examinations may contribute to diminished academic performance. Further study and understanding of the effect of anxiety on cognition in children will help in development of novel treatment methods for children with anxiety, which is especially important in urban neighborhoods with low socioeconomic status and high social environmental risk (Theall, Drury & Shirtcliff, 2012; Theall, Brett, Shirtcliff, Dunn & Drury, 2013).

Figure 1. Timeline of behavioral testing for rats undergoing open field testing and rats undergoing VPWM task testing. The delay between diazepam injection and anxiety testing in the open field was identical to the interval between drug injection and probe trials to assess strategy preference on the VPWM task.

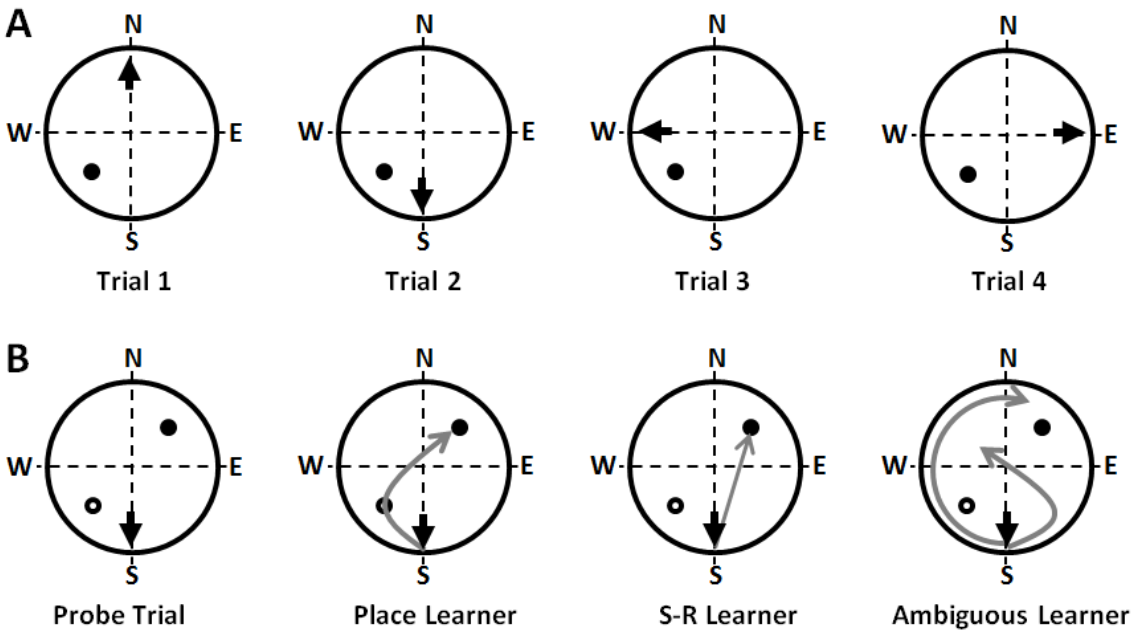

Figure 2. Visible platform water maze (VPWM) task protocol. A: During the 8 training trials of the VPWM task, rats enter the pool from each of the cardinal directions in a pseudo-randomized order while the platform remains stationary in the Southwest quadrant of the pool. Example of start points for the first 4 trials are shown with the closed circle representing the platform location and arrows indicating each trial's start point. B: After the 8 training trials, a probe trial was conducted during which the platform was relocated to the Northeast quadrant on the opposite side of the pool. The probe trial is pictured, with the open circle representing the training platform location, the closed circle representing the relocated platform, and the arrows indicating the probe trial start point. The probe trial was used to determine spatial learning strategy preference. Rats that first swam to the training platform location were categorized as *place learners*, rats that swam directly to the relocated platform were categorized as *stimulus-response* learners, and rats that displayed thigmotaxis or did not swim to either location were categorized as *ambiguous learners*. Gray arrows represent swim path.

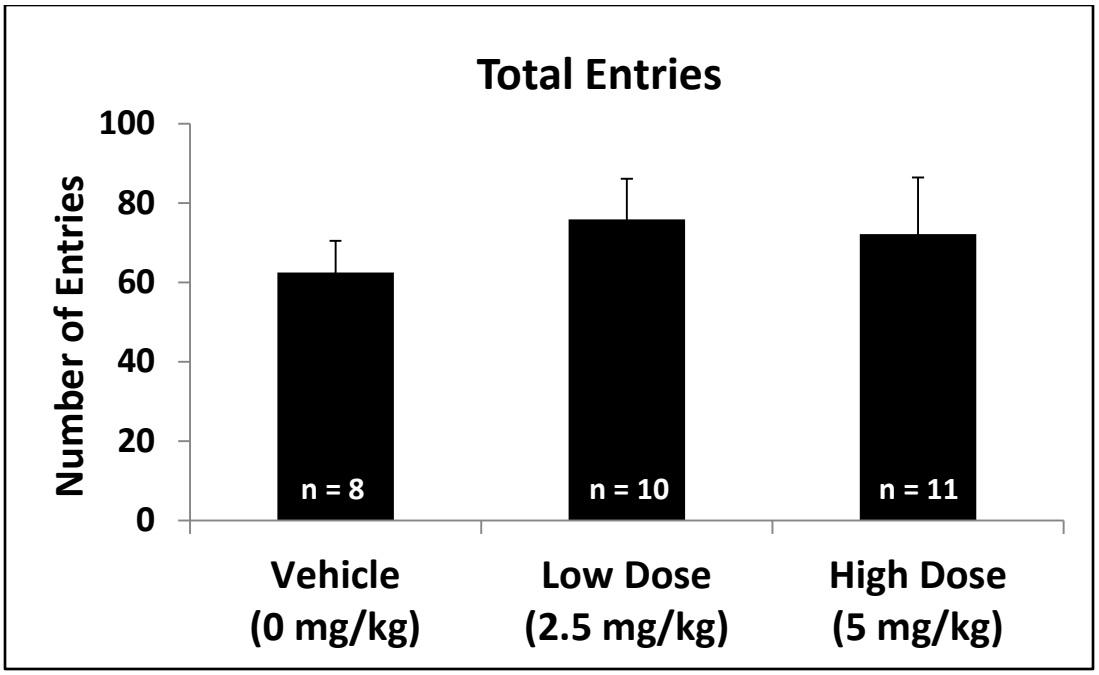

Figure 3. Total number of entries into both inner and outer squares during the 5-minute open field trial. Diazepam treatment had no significant effect on locomotor activity in the open field ($p = 0.740$). Bars represent means ± standard errors.

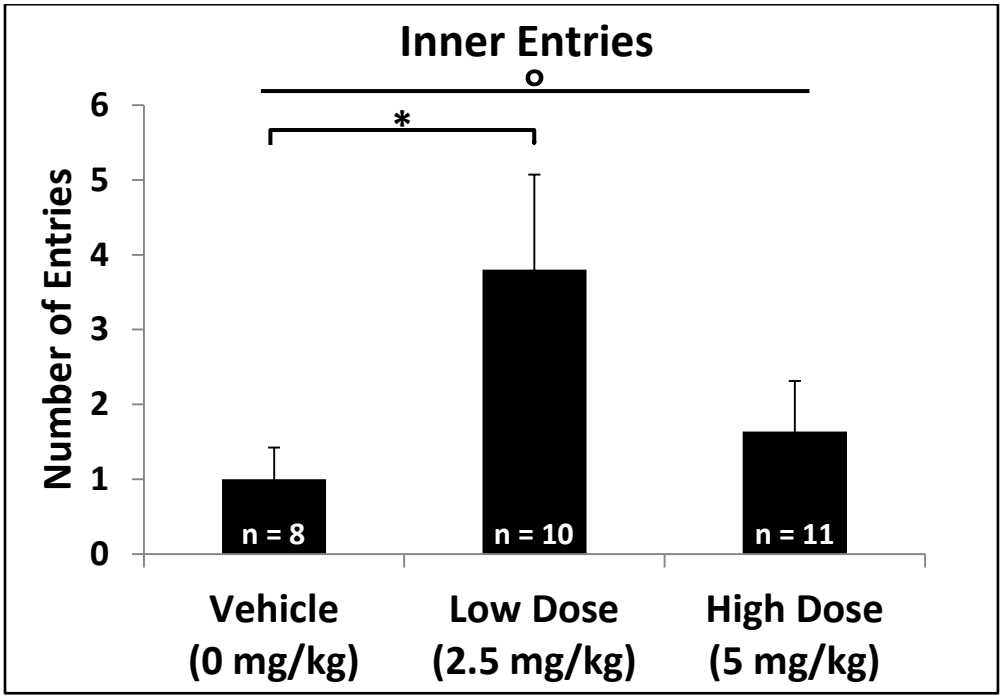

Figure 4. Number of entries into inner squares during the 5-minute open field trial. There was a trend toward a significant effect of diazepam treatment on entries into inner squares. Exploratory post-hoc analysis indicated that rats treated with a low dose of diazepam entered the inner squares significantly more frequently than rats treated with vehicle (°$p < 0.10$, *$p < 0.05$, exploratory post-hoc analysis). Bars represent means ± standard errors.

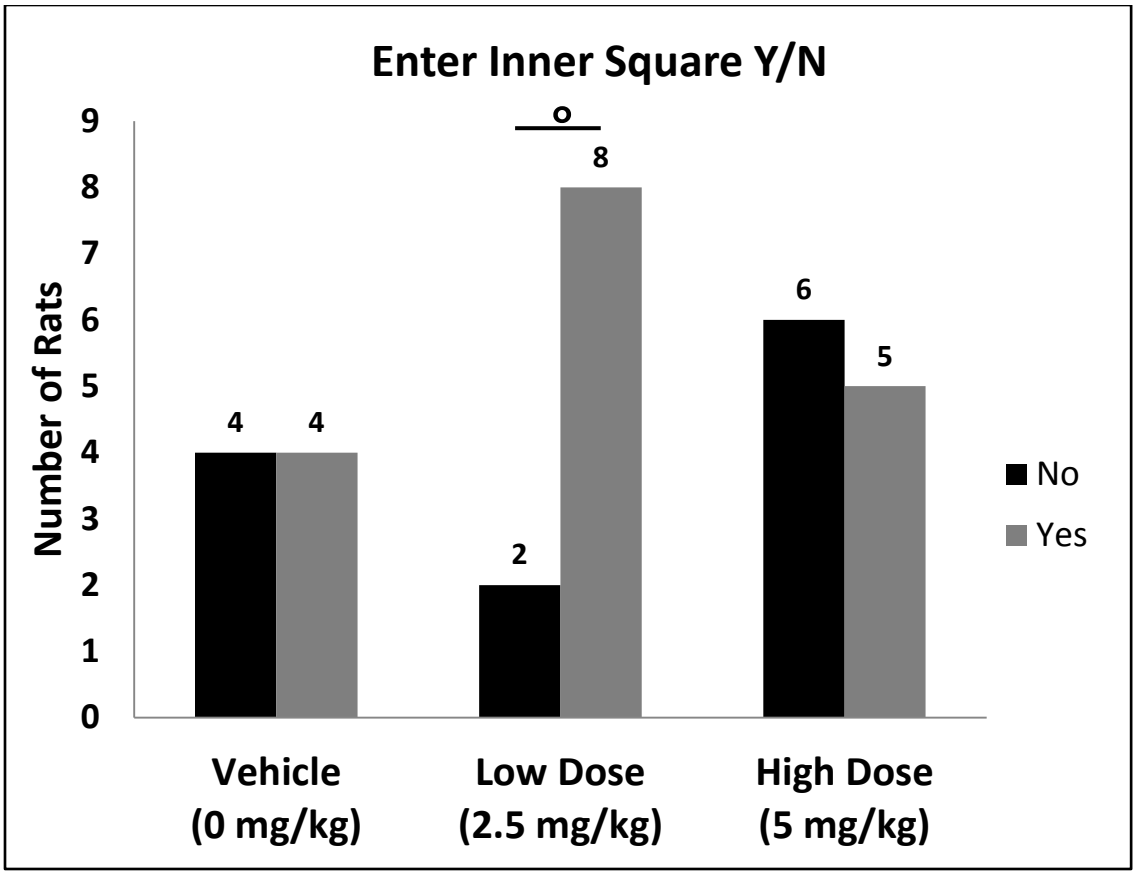

Figure 5. Number of rats entering a center square at least once during the 5-minute open field trial. Rats treated with a low dose of diazepam displayed a trend to enter a center square at least once (°$p < 0.10$). Rats treated with either vehicle or a high dose of diazepam showed no tendency to enter the center squares of the open field.

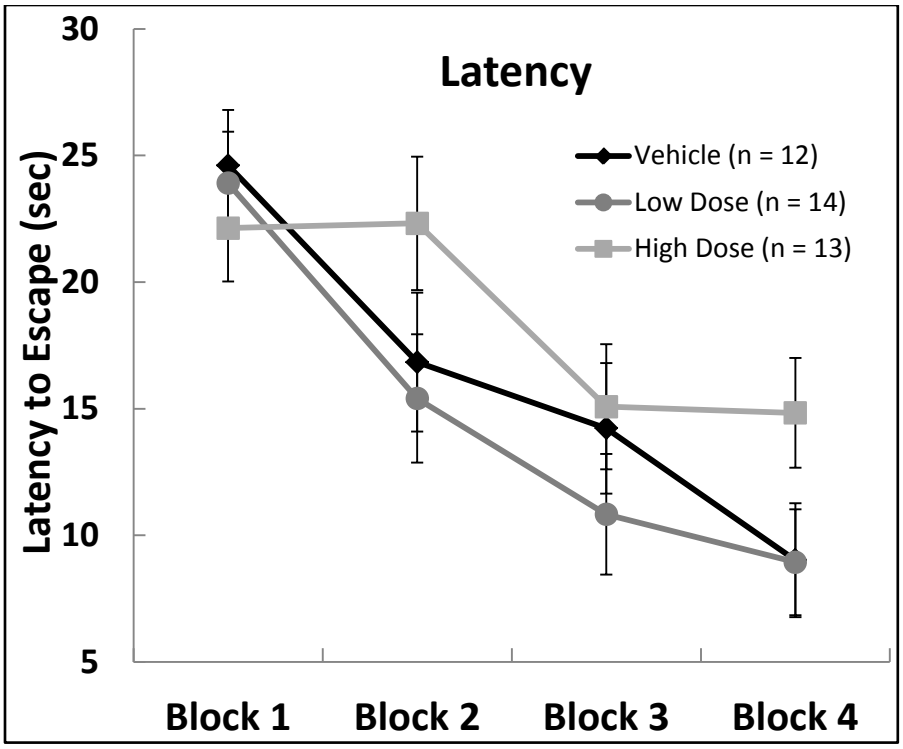

Figure 6. Latency to escape over four two-trial training blocks. All rats learned to locate the platform, as evidenced by decreasing latency to escape as training progressed ($p < 0.001$). Diazepam treatment had no effect on acquisition of the task.

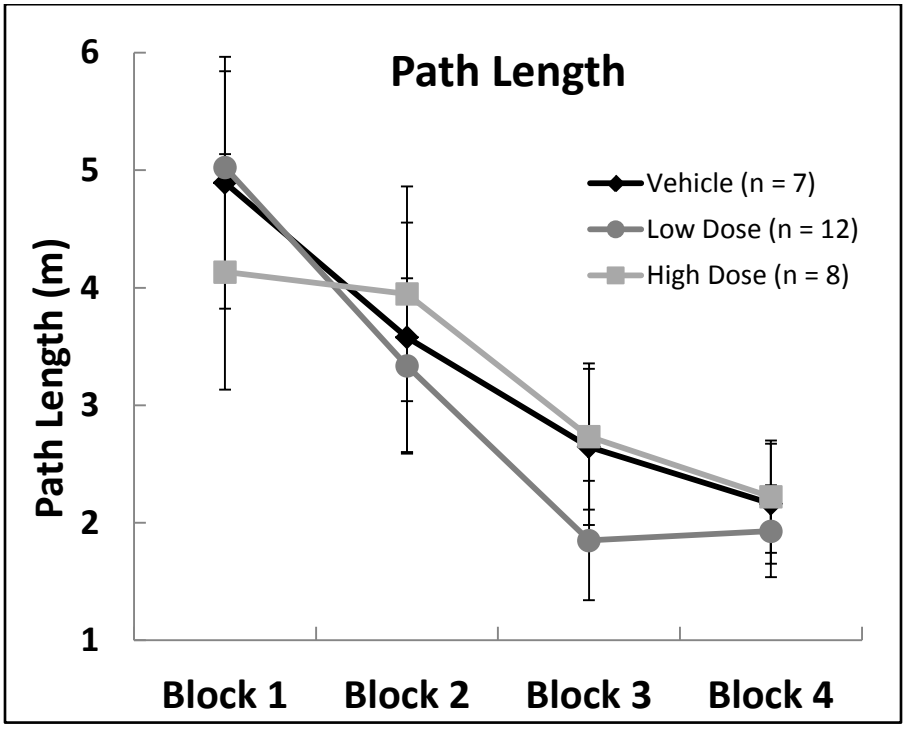

Figure 7. Path length over four two-trial training blocks. All rats learned to locate the platform, as evidenced by decreasing path lengths as training progressed ($p < 0.001$). Diazepam treatment had no effect on acquisition of the task.

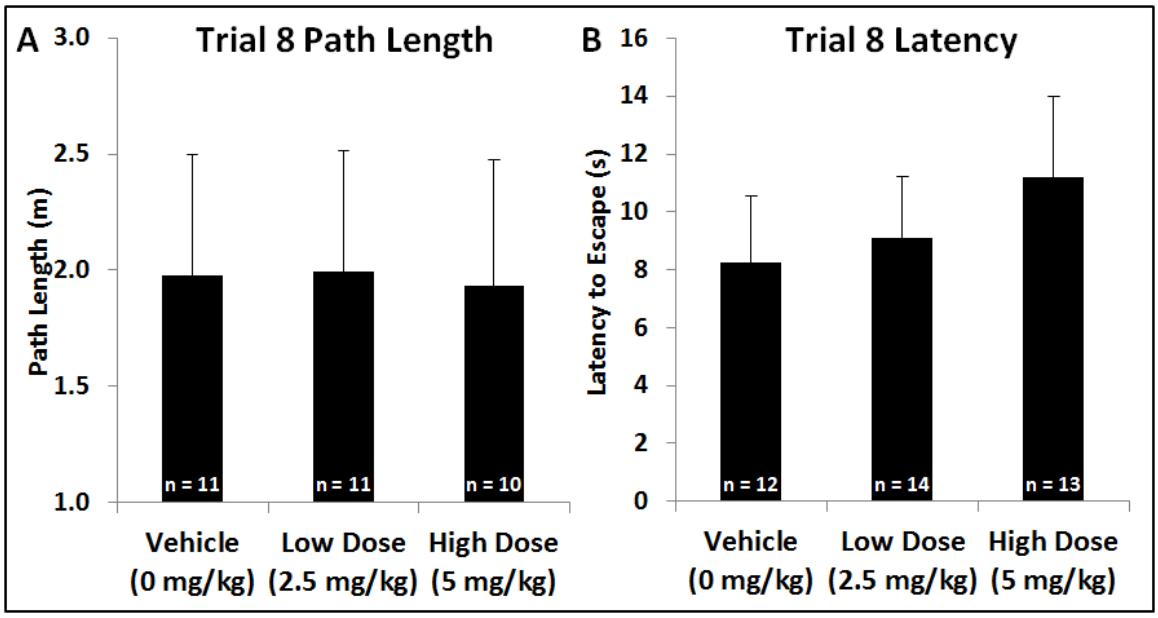

Figure 8. Performance on the final training trial of the visible platform water maze task. Diazepam treatment had no effect on either path length (A) or latency to escape (B) on the training trial 8. This indicates that by the end of training, all treatment groups were performing equally on the VPWM task.

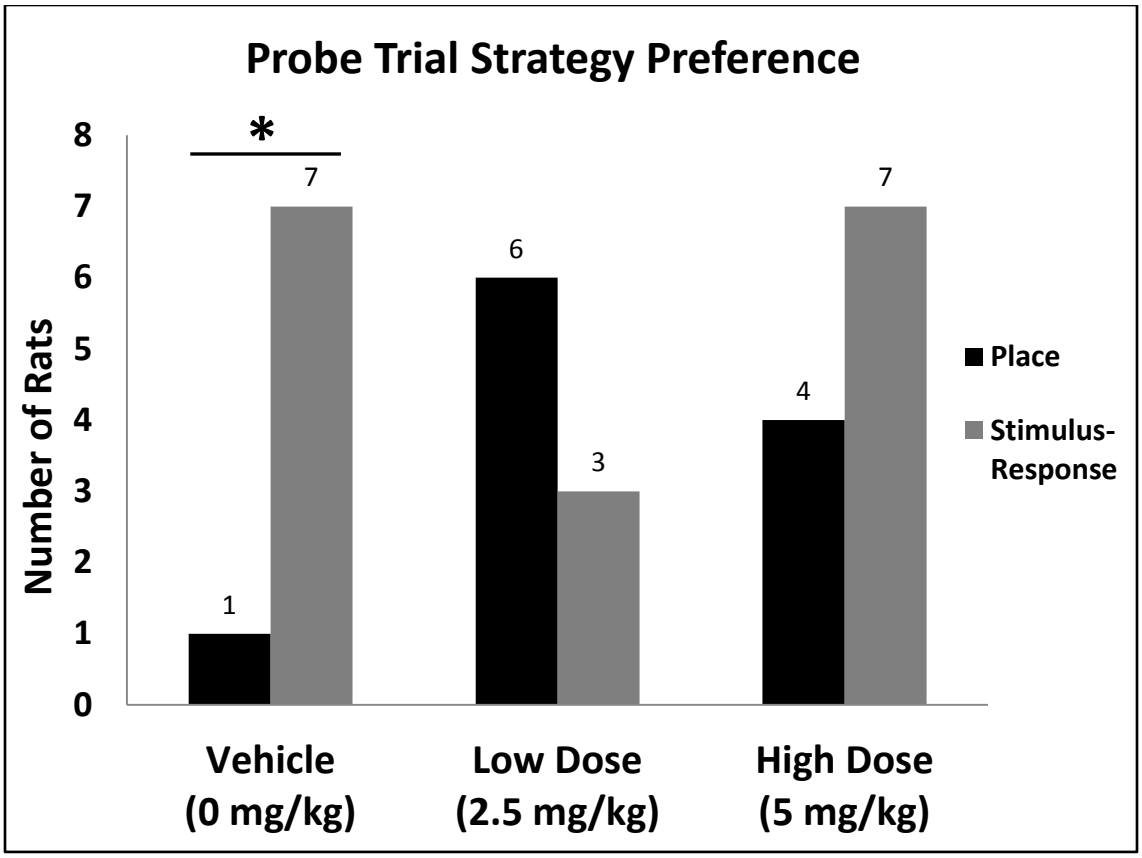

Figure 9. Spatial learning strategy preference on the probe trial. Rats treated with vehicle displayed a significant preference for a stimulus-response learning strategy (*$p < 0.05$). Rats treated with either a low dose or a high dose of diazepam exhibited no preference for either a place or a stimulus-response learning strategy.

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BIOGRAPHY

Thomas Lobell was born in Baltimore, MD on November 11, 1991. He attended high school at Catonsville High School Baltimore, MD before attending Tulane University in New Orleans, LA. At Tulane, he obtained a Bachelor's of Science in Neuroscience in the Spring of 2013, before entering the 4+1 program to pursue a Masters Degree in Neuroscience. Upon completion of the 4+1 program, Tom plans on taking a year off to travel the world before returning to academia to pursue a Ph.D. or an M.D.