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Dissociating Allopregnanolone Mnemonic Effects from Sedation

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Abstract.

Allopregnanolone (Allop) is a neurosteroid metabolite of progesterone. Allop modulates cognition, specifically learning and memory, but these effects are frequently confounded by its anxiolytic and sedative properties. We attempted to dissociate the anxiolytic effects of Allop from its mnemonic effects by employing a pharmacological challenge with d- amphetamine. Because previous research suggests that the effects of Allop may vary with the cognitive domain being tested, we assessed both spatial and non-spatial memory. Spatial memory was tested in a Morris Water Maze, and non-spatial object memory was tested on a novel discrimination task. Allop, alone or in combination with *d*-amphetamine did not have any significant effects on spatial memory. Neither Allop nor amphetamine alone affected memory of a novel object relative to controls, but the combination of the two produced a dissociation and enhanced performance. The results suggest that, depending on the type of memory being tested, the sedative effects of Allop can be dissociated from mnemonic effects by co-administering a sub-threshold dose of d-amphetamine.

Dissociating Allopregnanolone Mnemonic Effects from Sedation

The psychotropic effects of neurosteroids have generated tremendous interest among the scientific and medical community in recent years (Schüle, Eser, Baghai et al., 2011). Neurosteroids are neuroactive metabolites of steroid hormones, which are converted from peripheral hormone levels and are synthesized *de novo* in the brain in both men and women (Zheng, 2009). Fluctuations in neurosteroids have been implicated in cognitive decline in several neurodegenerative diseases as well as age-related memory changes (Naylor, Kilts, Hulette et al., 2010; Noorbakhsh, Ellestad, Maingat et al., 2011; Paris, Walf & Frye, 2011; Wang, Liu, Irwin et al., 2008). Additionally, neurosteroids may also play an important role in anxiety and mood disorders such as depression and post traumatic stress disorder (Bernardi, Pluchino, Begliuomini et al., 2004; Schüle, Eser, Baghai et al., 2011; Pinna & Rassmussen, 2011). It has been suggested that some neurosteroids may provide useful treatments for a broad range of mood and neurodegenerative disorders including age-related cognitive decline (Bernardi et al., 2004; Pinna & Rassmussen, 2011).

In particular, the neuroactive metabolite of progesterone, Allopregnanolone (Allop) possesses potent anxiolytic, sedative, and antiepileptic effects. The psychotropic effects of Allop are mediated by a mechanism similar to the action of benzodiazepines, i.e. binding to the $GABA_A$ receptor (Bitran, Shiekh, & McLeod, 1995; Brot, Akwa, Purdy et al., 1997). Like other positive allosteric modulators of $GABA_A$ such as alcohol, barbiturates, and benzodiazepines (Arolfo & Brioni, 1991; Brioni, Arolfo, Jerusalinsky et al., 1991), Allop has acute sedative effects, although its effects on memory are less clear. In the past two decades, researchers have published a variety of findings regarding the effects of Allop or progesterone on learning and memory. A number of studies have indicated that Allop and progesterone may be detrimental to

memory and cognition (Cuttler, Graf, Pawluski et al., 2011; Johansson, Birzniece, Lindblad et al., 2002; Kask, Bäckström, Nilsson et al., 2008; Sun, Luine, Zhou et al., 2010; van Wingen et al., 2007), while other studies indicate that Allop may improve certain forms of memory (Fry & Walf, 2008; Frye, Duffy, & Walf, 2007; Frye & Lacey, 2000; Lewis, Orr, & Frick, 2008; Walf et al., 2006).

Evidence for memory impairing effects. Many studies have reported that Allop and progesterone impair performance on tests of learning and memory in both animals (Johansson, Birzniece, Lindblad et al., 2002) and humans (Kask, Bäckström, Nilsson et al., 2008; van Wingen, van Broekhoven, Verkes et al., 2007) in a dose-dependent manner. Allop inhibited learning of a Morris Water Maze (MWM) in mice (Johansson et al., 2002), a test of spatial memory, and acute administration of progesterone has also been found to impair spatial working memory but not recognition memory in an object recognition task among both male and female rats (Sun, Luine, Zhou et al., 2010). Acute administration of allopregnanolone also impaired episodic memory performance in healthy middle-aged women, without affecting working memory (Kask et al., 2008). However, this study, which used a two-back memory test, found a ceiling effect on working memory performance, so it is possible that a more sensitive test could reveal Allop-induced impairments on working memory. Furthermore, some studies have reported increases in endogenous levels of Allop to be correlated with impaired memory performance and subjective reports of cognitive difficulty (Cuttler, Graf, Pawluski et al., 2011). Increased Allop serum levels have been found to be greatest in women during the third trimester of pregnancy (Genazzani, Petraglia, Bernardi et al., 1998), corresponding to the finding of significant declines in explicit memory in pregnant women from the second to the third trimester (Keenan, Yaldoo, Stress et al., 1998).

Evidence for memory enhancing effects. In contrast to aforementioned findings, several studies have reported improvements in memory, following immediate post-training administration of allopregnanolone and progesterone. This suggests that Allop may enhance memory consolidation processes. Bodensteiner, Stone, & Ghiraldi (2008) found post-training administration of progesterone to benefit spatial memory of young but not aged mice. However, Lewis et al. (2008) reported contradictory findings of beneficial effects on spatial memory in ovarectomized older mice, but no effects on ovarectomized middle aged mice following post-training injection. Lewis et al. (2008) also found a beneficial effect from certain doses of progesterone injected post-training, on object memory consolidation in both middle aged and aged female mice. Other studies also have reported enhancement in object recognition, water maze performance, and Y-maze tasks following administration of progesterone as well as Allop immediately following training (Frye, Duffy, & Walf, 2007; Fry & Walf, 2008). Additionally, Allop may enhance learning in conditioned passive avoidance tasks (Frye & Lacey, 2000; Walf et al., 2006).

Factors affecting observation of memory effects. These seemingly contradictory findings among different studies regarding the mnemonic effects of Allop may be the result of several factors. Most notably, the effects on memory vary depending on both administration timing and endogenous levels of Allop. Changes in endogenous levels of Allop fluctuate as a factor of age, pregnancy, stress, and phase in the estrous or menstrual cycle (Genazzani, Petraglia, Bernardi et al, 1998). These changes may reflect changes in sensitivity to Allop and account for such differences in performance among aged versus young subjects, and fertile versus postmenopausal and ovarectomized subjects. For example, Lewis et al. (2008) found enhancing effects after 24 hours in middle aged and aged mice, while effects were not observed

in the middle-aged mice after 48 hours. In addition, the effects on memory vary depending on whether progesterone is administered alone or in combination with estrogen. Fertile women in the luteal phase of the estrous cycle have also been found to have significantly higher levels of circulating Allop than women in the follicular phase (Genazzani, Petraglia, Bernardi et al, 1998); however, women in the luteal phase performed better on a sustained attention task and test of implicit memory than women during the follicular phase. This finding may be the result of concurrent changes in estrogen levels (Maki, Rich, & Shayna Rosenbaum, 2002). Estrogen may enhance the conversion of progesterone to Allop, therby modulating its effects (Vongher & Frye, 1999).

Brain regions implicated in the action of Allop. Allop may exert its effects in a variety of brain regions, particularly in the amygdala and the prefrontal cortex (Engin & Treit, 2007; Van Wingen et al., 2007). Both of these regions are rich in GABA receptors by which Allop is thought to exert its effects. Infusion into the amygdala and prefrontal cortex reliably produce anxiolytic behavioral responses in animals, enhancing exploration (Engin & Treit, 2007). Allop exhibits a similar anxiolytic profile to benzodiazepines, increasing open arm entries in the elevated plus maze, and head-dips in the Boissier test (Modol, Darbra, & Pallares, 2011). Allop also produces similar behavioral effects to benzodiazepines in the mirrored chamber test (Reddy & Kulkarni, 1997). In women, progesterone administration decreased responses in the amygdala and fusiform gyrus during memory encoding of faces; during retrieval, brain activity was likewise decreased in the fusiform gyrus and prefrontal cortex (van Wingen et al., 2007). Interestingly, van Wingen et al. did not find changes in hippocampal responses. Other studies have failed to find behavioral effects of Allop in the hippocampus (Engin & Treit, 2007). For example, hippocampal administrations of Allop failed to produce an anxiolytic response in the

elevated-plus-maze or shock-probe test (Engin & Treit, 2007), in contrast to other GABA agonists such as benzodiazepines. Allop may impair memory through an indirect mechanism, i.e. disrupt the feedback between the limbic brain structures that are responsible for memory formation and retrieval. These brain regions ordinarily provide important feedback to the hippocampus to facilitate the selective encoding of emotionally salient information.

Sedative versus mnemonic effects. Further research is necessary to clarify the inconsistent findings regarding the mnemonic effects of Allop and progesterone. Cognitive sedation might be a contributing factor to the results of studies that have reported an impairing effect of Allop on memory and learning performance. For example, intravenous infusion of Allop has been found to increase subjective ratings of sedation and decrease saccadic eye velocity (van Broekhoven, Backstrom, & Verkes, 2006). Although Allop acts on $GABA_A$ receptors, it does not produce consistent behavioral effects when infused into the hippocampus (Engin & Treit, 2007) and may not affect memory in the same manner as other GABA agonists such as benzodiazepine and alcohol, which do produce behavioral effects when infused in the hippocampus. Therefore, Allop's effects on sedation and/or motivation may detrimentally affect task performance in such studies, which might mask the observation of improvements in memory and learning induced by Allop that have been documented in studies utilizing a post-training administration technique (Lewis et al., 2008).

Although sedation can cause impairments in memory performance (Veselis et al., 2001), the relative amount to which the sedative effects of Allop contribute to Allop-induced changes in memory performance not been demonstrated, and it is not clear whether forms of memory may be differentially impacted by the sedating properties of the neurosteroid. It is possible to dissociate the mnemonic and sedative behavioral effects of other $GABA_A$ receptor agonists (i.e.

benzodiazepines) using both electrophysiological techniques and pharmacological techniques (Curran et al., 1998; Mitzner & Griffiths, 2007). Accordingly, the present study will attempt to dissociate the anxiolytic effects of Allop from its effects on spatial and non-spatial memory. This will be accomplished by co-administration of a central nervous system stimulant (CNS), a paradigm that has been successful in differentiating anxiolytic versus memory effects in benzodiazepines (Mitzner & Griffiths, 2007). The CNS stimulant *d*-amphetamine will be used in attempt to reverse Allop-associated sedation. By counteracting the sedative effects of Allop, we hope to observe the effects of Allop on memory in isolation. *d*-amphetamine promotes arousal by increasing the availability of dopamine within the CNS. (Boutrel & Koob, 2004). This arousal does not necessarily result in behavioral changes when applied at sub-threshold doses (Rabin, Hunt, & Lee, 1987)., which is a useful strategy to minimize any potential confounding behavioral effects resulting from d-amphetamine alone.

It is hypothesized that if Allop is directly impairing learning and memory processes in the brain, acute administration of Allop combined with *d*-amphetamine should not cause a significant change in performance from that following Allop administration alone. If Allop-induced impairments in memory performance that have been reported are caused by the sedating effects of Allop rather than a true disruption in memory processes, than the stimulant should reverse impairments on the one or more of the tasks and performance should be closer to saline controls. On the other hand, if the sedating effects of Allop are masking a consolidation or recall enhancing effect on one or more domains of memory, then administration of *d*-amphetamine might allow such memory enhancing effects to be observed.

We chose to investigate both spatial and non-spatial memory to compare whether the mnemonic effects of Allop could be dissociated, as some memory modalities may be more

susceptible to dissociation. A control measure for anxiety was also performed to evaluate the effects of combining Allop and *d*-amphetamine relative to treatment with Allop or saline, since changes in anxiety level is known to affect memory performance.

The effects of Allop on spatial memory recall and new spatial learning were examined in a Morris Water Maze. In this task, animals are trained to find the location of an underwater platform in a pool, and then tested for spatial memory recall after receiving an assigned drug treatment. Immediately after memory testing, we assessed learning of a new platform location. To examine the potential facilitation of learning consolidation, memory of the new location was tested for three consecutive days following the initial drug administration. By measuring memory recall of a previously learned location as well as learning of a new location, we were able to concurrently examine the effects of Allop on both spatial memory recall and new spatial memory encoding. It is hypothesized that animals that receive Allop alone will perform worse than controls, however the combination of Allop and d-amphetamine will partially or fully reverse impairments.

A novel object recognition task was used to measure effects on non-spatial episodic memory. In this task, animals are exposed to a single object and tested for their memory of the object following day, after receiving an assigned drug treatment. It is hypothesized that administration of Allop in combination with d-amphetamine and Allop will improve memory of an object, relative to animals administered with Allop, d-amphetamine or saline controls

Method

Subjects

Sixteen Male Sprague-Dawley rats (aged 9 months) were obtained from the animal colony at Illinois Wesleyan University. Rats were housed in individual polycarbonate cages in the animal care facility at Illinois Wesleyan University. Food and water were available *ad libitum*. The housing room was maintained at 24˚C and the animals were kept on a 12:12 hour light-dark cycle. All experimental protocols were first approved by the Institutional Animal Care and Use Committee of Illinois Wesleyan University, in adherence to the standards established by the National Institute of Health Guide for Care and Use of Laboratory Animals. One rat was excluded from the study due to failure to learn the spatial memory task.

Surgery. Rats were anesthetized using 2% isoflurane gas, delivered continuously through a nose cone. A heating pad was used to maintain constant body temperature. A stereotaxic apparatus was installed to hold the skull for the duration of the procedure. The skull was exposed along an incision, and stainless steel 22-gauge cannulae were bilaterally implanted targeting the lateral ventricles (AP: -0.6, ML: +/- 1.6, DV: -3.4). Jeweler's screws and cranioplastic cement were used to secure the cannulae to the skull. A dummy cannulae was temporarily placed into each guide cannula to maintain clearance. The animals were allowed to recover for a minimum of 5-7 days prior to behavioral training procedures.

Drug Administration. The doses of Allop and *d*-amphetamine were determined based on findings from previous research (Rabin et al., 1987). Both drugs were obtained from Sigma Aldrich (St Louis, Missouri, USA). Allop or saline was delivered bilaterally via intracranial ventricular (i.c.v.) infusion, and *d*-amphetamine was administered through intraperitoneal

injection (i.p.) Allop was suspended in a vehicle solution of cyclodextrin 45% w/v in 0.9% saline (5µg/µl). Rats received either vehicle and saline, Allop (10 mg i.c.v), *d*-amphetamine sulfate (0.05 mg/kg i.p.), or Allop and *d*-amphetamine sulfate (10 mg i.c.v, 0.05 mg/kg i.p.).. An infusion pump was used to deliver the Allop or saline treatment through the 22-gauge implanted cannulae, which was attached by polyethylene tubing to a 10-µl syringe containing the prepared treatment solution. The infusions were delivered at a rate of 1 µl/min. The needle was left in place for an additional minute to promote diffusion.

Spatial Memory Task

Morris Water Maze Training. Rats were trained for 6 consecutive days to find the location of a submerged hidden platform in the Morris Water Maze. The water maze used was a wood and fiberglass circular basin with a white-painted interior. The tank was filled with tap water, which was made opaque using nontoxic white watercolor paint. A black water-resistant marker was used to draw a 5cm x 2cm black mark on the dorsal side of each animal, to facilitate path tracking using an overhead computer-tracking device. Extra-maze cues were maintained constant among all trials. Four trials were performed across each day of training, where each rat was allowed to start in each of the four quadrants. A pseudorandom design was used to balance the order of starting location. Each animal was allowed 60 seconds to locate a transparent circular platform placed below the surface of the water. Rats were allowed to stay on the platform for 10 seconds to facilitate spatial learning based on the position of extra-maze cues. In between trials, the subjects dried with a towel and allowed to rest for 30 seconds in a holding cage. If a rat failed to find the platform within 60 seconds, it was guided to the platform and allowed to remain there for 10 seconds.

Probe Trial Testing. Spatial memory recall was tested 24 hours after the last training procedure. On the test day, the animals were divided into four groups in which they received saline (N=4), *d*-amphetamine (N=4), Allop (N=3), or *d*-amphetamine + Allop (N=4). Saline or Allop was administered via i.c.v infusion 30 minutes prior to testing, followed by an i.p. injection of saline or *d*-amphetamine 15 minutes later. A probe trail was conducted after an addition 15 minutes had lapsed since the second infusion. Here, the platform was removed from the tank and the animal was placed in the water 180º from the preexisting location of the platform. Swim distance and the percentage of time spent in each quadrant were measured to gauge memory of the platform location. Swim speed was recorded as a measure of locomotor activity.

New Learning Task. Immediately following the probe trial test for each rat, the platform was replaced in the quadrant opposite to its original position. Each rat was trained to learn the location of the new platform across four trials according to the training procedure previously described. To evaluate the duration of Allop's effect on new learning, the training procedure for the new platform location was repeated after twenty-four hours, for three consecutive days.

Episodic Memory Task

Object Familiarization. One at a time, the subjects were placed in a square wooden arena (50 cm wide x 50 cm long x 50 cm tall) containing a single object positioned 5 cm from the wall in either the northeast or northwest corner of the box. Rats were placed on the opposite side of the arena, facing the wall. The subjects were allowed to freely explore the box and the object (a plastic yo-yo) until accumulating 30 sec of exploration time with the object, which served to control for variability in activity (Lewis et al., 2008). Total exploration time was recorded, and object exploration was timed separately by two experimenters. Object exploration time was

recorded when the animals front paws made contact with the object or when its nose was within 1 cm of the object. The box and objects were cleaned with a mild disinfecting spray in between trials, to eliminate olfactory cues.

Novel Object Recognition. Twenty-four hours after the sample phase, the subjects were each administered the treatment they had been assigned in the spatial memory test. Drug and control treatments were administered in the same procedure described above for the MWM, and the animals were tested within 15 minutes of second treatment administration. In the choice phase, two objects were placed in the same square arena, one in the northeast corner and one in the northwest corner. Each object was positioned 5 cm from the wall of the box. One of the objects was novel (a plastic horse), while the other was the same as the object used in the sample phase (a plastic yo-yo). Total exploration time and total time spent with each object was recorded by two experimenters who were blind to the drug condition of the animals. Animals were allowed up to 30 minutes to accumulate a total of 30 seconds total exploration time divided between two objects. Time spent with the novel object objects was compared to a chance performance of 15 sec, reflecting 50% of exploration time spent exploring the novel object. It is established that rats spend greater time exploring objects that are novel to them; so greater percentage of time spent with the novel object compared to the familiar object indicates greater episodic memory of the familiar object (Dere, Huston, & De Souza Silva, 2006). Exploration time was defined by nose proximity being within 1 cm of the object or front paws touching. Sitting or leaning on the object was not counted towards exploration time.

Elevated Plus Maze

In order to rule out changes in anxiety levels as a confounding factor in the memory performance of the treatment groups, an elevated plus maze test was conducted immediately

after the choice phase of the novel object recognition task. Each animal was placed in the center of a four-arm maze, containing two open arms and two closed arms. Animals were allowed 5 minutes to explore closed and open arms, and video data was recorded and coded at by experimenters who were blind to the animal's treatment condition. Measured included number of entries as well as total time spent in open arms, closed arms and center.

Results

The effects of allopregnanolone MWM performance and novel object recognition were investigated in a pharmacological dissociation design (N=15). After the animals were trained in the water maze task, a one-way ANOVA was conducted on the final-day mean swim distance to confirm a constant level of acquisition among the groups ($p>0.05$). The animals were assigned to treatment groups, and the subjects were counterbalanced to eliminate significant differences in pre-treatment group means for swim distance on the final day of training. One animal was excluded due to poor swimming ability and failure to learn the location of the platform following six days of training. There were no differences among the pre-treatment group means on final swim distance for saline + vehicle (M = 272.97, SD = 281.73) Allop (M = 240.45, SD = 87.6), d-amphetamine (M = 237.9, SD = 77.29), or Allop + d-amphetamine (M = 241.67, SD = 117.17).

Morris Water Maze. The effects of allopregnanolone on acute spatial memory recall were examined alone an in combination with *d*-amphetamine. A two-way ANOVA without repeated measures was used to examine the dependent variables: swim speed, total swim distance, percent of swim distance in target quadrant, percent of time in target quadrant, entries into target quadrant, and latency to enter the target quadrant. Statistical analysis (*d*-amphetamine x Allop) revealed no significant main effects of Allop or *d*-amphetamine for any of the

dependent variables examined (p>0.05) (see figures 1,2). We did not observe any Allop x *d*-amphetamine interactions for swim speed, percent of swim distance in target quadrant, percent of swim time in target quadrant, total swim distance, latency to enter the target quadrant (p>0.05). As such, it was not possible to dissociate the acute mnemonic and sedative effects of allopregnanolone for spatial memory, since no impairment or improvement in memory by Allop or *d*-amphetamine alone was observed in the probe trial.

The effects of allopregnanolone on new learning and memory consolidation were also examined across a four-day water maze training procedure of a new platform location following treatment administration. A three-way 2x2x4 ANOVA (Allop x *d*-amphetamine x Training Day) was conducted examining the dependent variables: total swim distance, latency to locate the platform, and latency to enter the target quadrant. In addition, percent of total swim distance spent in the target quadrant (see figure 3) and in the previously learned target quadrant were compared among the treatment groups in a three-way ANOVA. A significant main effect of Training Day was found for total swim distance, $F(1,3) = 22.34$, $p = .001$, latency to locate platform $F(1,3) = 21.98$, $p = .001$, percent swim distance in target quadrant $F(1,3) = 81.21$, $p < .001$, and percent swim distance in previously learned target quadrant $F(1,3) = 68.27$, $p < .001$. No significant main effects of Allop or *d*-amphetamine were observed for any of the dependent variables listed (p>0.05). There were no significant two-way or three-way interactions between Allop x Day, *d*-amphetamine x Day, or Allop x *d*-amphetamine x Day. A repeated-measures ANOVA was also performed for all of the measures listed above as well as for the difference scores for the behavioral measures between the 1st and 2nd day of learning. No significant main effects of treatment or interactions were observed for any of the measures (p>0.05). In summary,

we did not observe any behavioral effects of Allop, alone or in combination with *d*-amphetamine on either spatial recall or new spatial learning.

Novel Object Recognition. For the object recognition task, a two-way ANOVA without repeated measures was conducted to analyze percent of time spent with the novel object in the choice phase among the treatment groups. A 2x2 ANOVA (*d*-amphetamine x Allop) for percent of time spent with novel object revealed no main effects for *d*-amphetamine or allopregnanolone *(p*>0.05). However, there was a significant interaction effect for percent time spent with novel object $F(1,11) = 8.43$, $p=.014$. A one-way ANOVA followed by Tukey's HSD post hoc test was used to evaluate significant differences between groups' means for the dependent variable percent time spent with the novel object. There were no significant differences between the means of Allop, controls, and d-amphetamine alone ($p>0.05$). However, there was a significant difference between the d-amphetamine group and the Allop + *d*-amphetamine group $q[4,11] = 4.26$, $p<0.05$ (see figure 4). This implies that the combination of Allop + *d*-amphetamine resulted in significantly improved memory of an object, relative to the performance of *d*-amphetamine alone.

Elevated Plus Maze. For the elevated plus maze, a 2x2 ANOVA without repeated measures (*d*-amphetamine x Allop) was performed to examine differences the anxiolytic effects of the drug treatments. Number of entries and total time spent in closed arms, open arms, and center platform were examined. Additionally, percent time spent in closed arms, open arms, and center was computed and analyzed (see figure 5). Analysis revealed no significant main effects for Allop for number of entries in open arms, closed arms, or center, as well as total time spent in closed arms, open arms, or center. In addition, no significant main effects or interactions were observed for number of entries into closed or open arms, ($p>0.05$), although there was a

significant main effect for *d*-amphetamine for entries into the center of the maze $F(1,11) = 8.43$, $p=.048$. However, a one-way ANOVA followed by Tukey's HSD post hoc found that there were no significant differences between any of the group means.

Discussion

A number of previous studies have examined the effects of Allop on novel object recognition and episodic memory, and there are reports of impairment (Kask et al., 2007; Matthews et al., 2002) and enhancement (Frye & Walf, 2008; Lewis et al., 2008; Walf et al., 2006) across multiple domains of memory. In attempt to resolve such findings, the purpose of this study was to dissociate the sedative and mnemonic effects of Allop. While sedation can produce memory impairments, there is evidence that drug induced amnesia is a separate phenomenon that can be dissociated using pharmacological and electrophysiological techniques (Curran et al., 1998; Mitzner & Griffiths, 2006; Veselis et al., 2001). The results indicate that the sedative effects of Allop can be dissociated from mnemonic effects differentially for spatial and non-spatial memory. Our main finding is that the combination of Allop and *d*-amphetamine interacted to improve performance of a novel object recognition task. The presence of this interaction suggests Allop may benefit recognition memory when its sedative properties are negated. We observed that Allop in combination with *d*-amphetamine produced greater exploration of a novel object versus a familiar object. It is well established that rats spend greater time exploring objects that are new to them (Dere et al., 2006), so greater time spent exploring a novel object versus a familiar object indicates that the animal has a memory of the familiar object.

Object recognition memory is thought to be mediated primarily by the medial temporal lobe brain structures—in particular, the perirhinal and parahippocampal cortices (Dere et al., 2006; Winters et al., 2008). Lesions to these regions cause far greater deficits in tasks of object recognition memory than lesions to the amygdala and the hippocampus combined (Murray & Mishkin, 1998; Winters et al., 2008). Given that combination of Allop and *d*-amphetamine was found to alter object recognition in this study, it would be interesting to repeat the procedure infusing Allop directly into the perirhinal cortex.

In addition to investigating object memory, we also attempted to dissociate the sedative and amnestic effects of Allop on spatial memory. Although the majority of previous studies that examined the effects of Allop and progesterone on acute spatial memory have reported impairments (Frye & Sturgis, 1995; Johansson et al, 2002; Mayo et al, 1993), we did not observe any acute impairing or enhancing effects. In addition, we found no treatment effects on new spatial learning over the course of four consecutive days following initial training of a new platform location. All of the groups learned the task equivalently according to behavioral measures examined. Thus, it was not possible to dissociate the mnemonic effects from sedation in these tasks. Although the groups were balanced for spatial learning on the last day of testing, there was variability in the probe trial performance, among all of the groups. In particular, we observed high variability in the control group for the MWM probe trail. Consequently, we were unable to identify specific trends in performance as a function of drug treatment. Perhaps a larger sample would have revealed a more conspicuous pattern of results. It is also possible that the effects of Allop on spatial memory performance in the MWM are short lived and below impairment thresholds 30 minutes after administration when we conducted memory testing. (Johansson et al. 2002)

There are several limitations to this study. One important limitation is that we have not yet confirmed the cannula placement through which the Allop or saline was delivered. Although measurements were taken with care and pilot placements were confirmed, slight deviations in location or angle of installation could have prevented proper delivery of Allop into the lateral ventricles. This could potentially affect the cognitive and behavioral results in the tasks performed. While this information would be very useful in interpreting our results, it is beyond the scope of this project. Although we did find a significant interaction for the effects of Allop and *d*-amphetamine on object recognition memory, it is also worth mentioning that the mean baseline performance of the control animals was less than 50% for time spent with the novel object. Lower than expected performance of the control animals due to variability may limit our ability to make meaningful comparisons among our groups.

Although we did not examine the effects of Allop on post-training memory consolidation in the object recognition task, there is evidence that Allop improves memory consolidation (Escudero et al, 2012) and subsequent memory performance the following day (Lewis et al., 2008). Future directions might include examining the effects of post-training administration of Allop and *d*-amphetamine on object recognition. It would also be useful to verify the effects of Allop in combination with *d*-amphetamine by examining other tests of spatial and non-spatial memory.

Despite its sedative effects, Allop has many beneficial properties including anxiolytic and antiepileptic effects that may make it an appealing pharmacological agent for treating a variety of conditions including anxiety disorders, depression, epilepsy, and bipolar disorder (Bernardi et al., 2004; Pinna & Rasmussen, 2011; Schüle et al., 2011), as a replacement or adjunct to other current drug treatments. It is important to characterize the effects of Allop on learning and

memory to establish whether it may be a useful alternative treatment to benzodiazepines in anxiety disorders and Post Traumatic Stress Disorder (PTSD). Commonly prescribed benzodiazepine treatments for generalized anxiety disorder and PTSD exert potent memory impairing effects that have been well established (Curran et al., 1998; Mitzner & Griffiths, 2007; Veselis, 2001); this was demonstrated in both electrophysiological dissociations and pharmacological dissociations similar to this current study. Thus, if Allop does not directly impair forms of memory, and its sedative effects can be reversed with a sub-threshold stimulant, it may be preferable to drug treatments that directly affect memory. In addition, changes in endogenous levels of Allop may play a role in post-menopausal memory decline (Paris et al., 2011) and in certain neurodegenerative diseases such as Alzheimer's disease (Naylor et al., 2010; Wang et al., 2008) and Multiple Sclerosis (Noorbakhsh et al., 2011). Both of these diseases are characterized by decreases in brain concentrations of Allop. Allop may also have important implications in the treatment of normal age related cognitive decline (Paris et al., 2011). Thus, it is vital to continue explorations of the role of Allop on cognitive and disease states, which may provide improved treatment options for a wide range of disorders.

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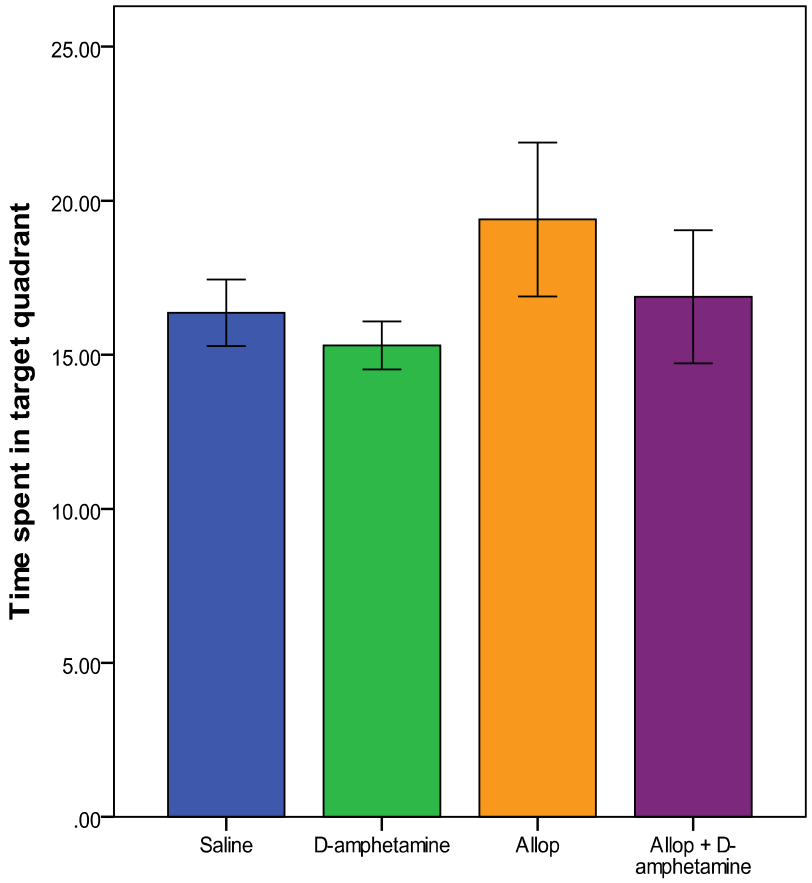

Figure 1. Effects of drug treatments on spatial memory recall. Bars represent the effects of Saline, Allopregnanolone, *d*-amphetamine, and *d*-amphetamine + Allopregnanolone on percent of time spent in target quadrant in probe trial of MWM. Error bars represent +/- 1 standard error. No significant effects of any of the treatments were observed on spatial memory recall ($p>0.05$).

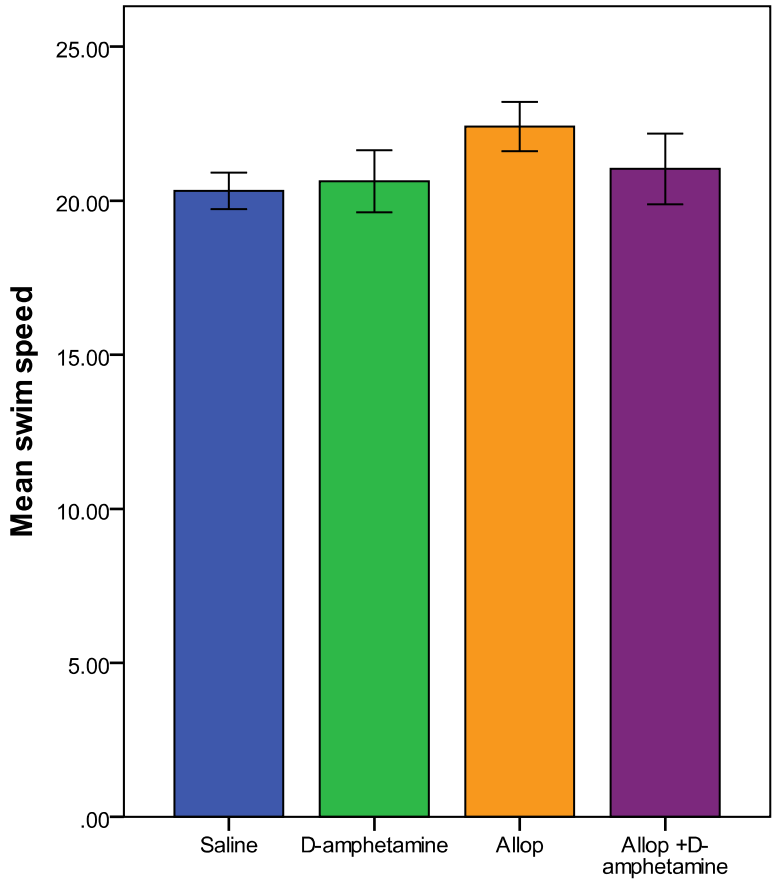

Figure 2. Effects of drug treatments on motor functions in the MWM. Bars represent the effects of Saline, Allopregnanolone, *d*-amphetamine, and *d*-amphetamine + Allopregnanolone on swim speed in target quadrant in probe trial of MWM. Error bars represent +/- 1 standard error.

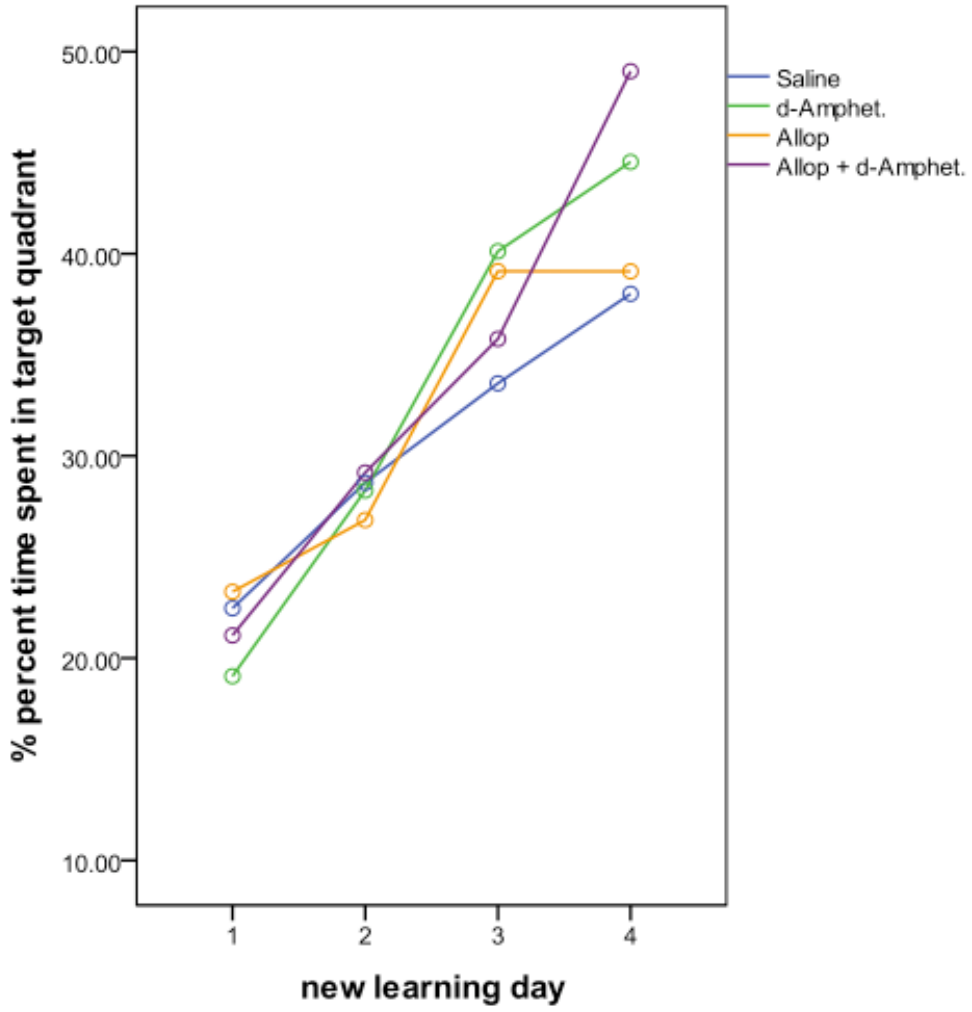

Figure 3. Effects of drug treatments on spatial new learning in male rats. Separate lines indicate drug treatment. X-axis represents the day of testing following the initial training of a new platform location in the MWM under the influence of allopregnanolone, *d*-amphetamine, saline, or *d*-amphetamine + Allopregnanolone. Y-axis represents percent of swim time spent in target quadrant. Positive slope indicates learning has occurred.

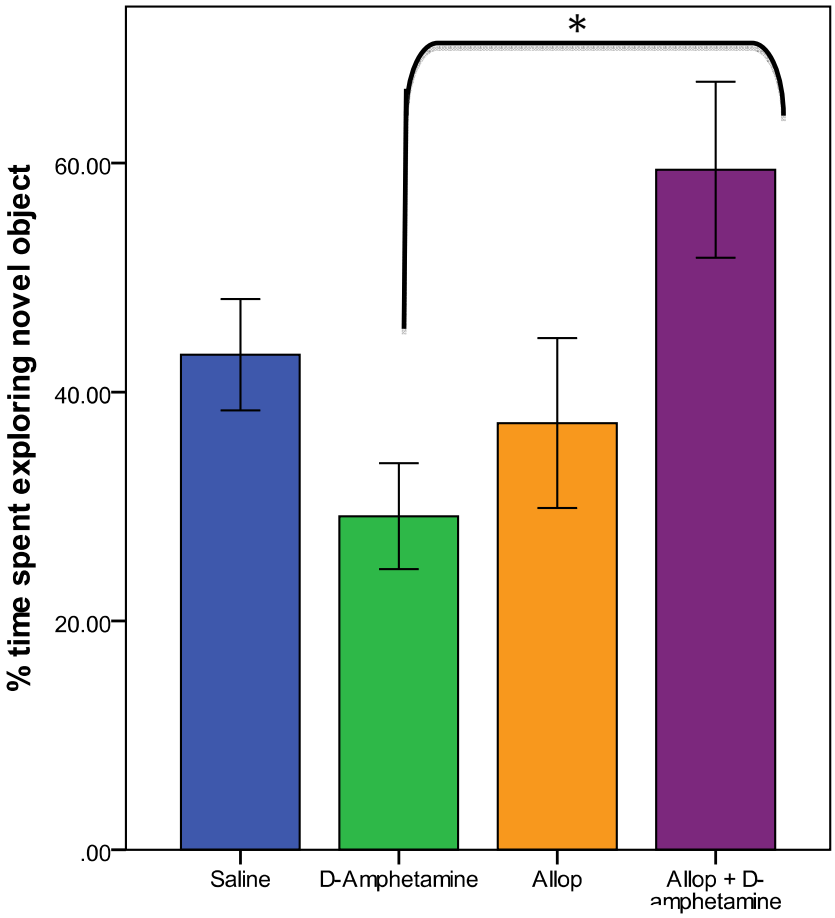

Figure 4. Effects of drug treatments on object recognition memory in male rats. Rats received allopregnanolone or saline, alone or combination with *d*-amphetamine. Bars represent mean percent of time exploring a novel object versus a familiar object (data not shown). Error bars represent +/- 1 standard error.

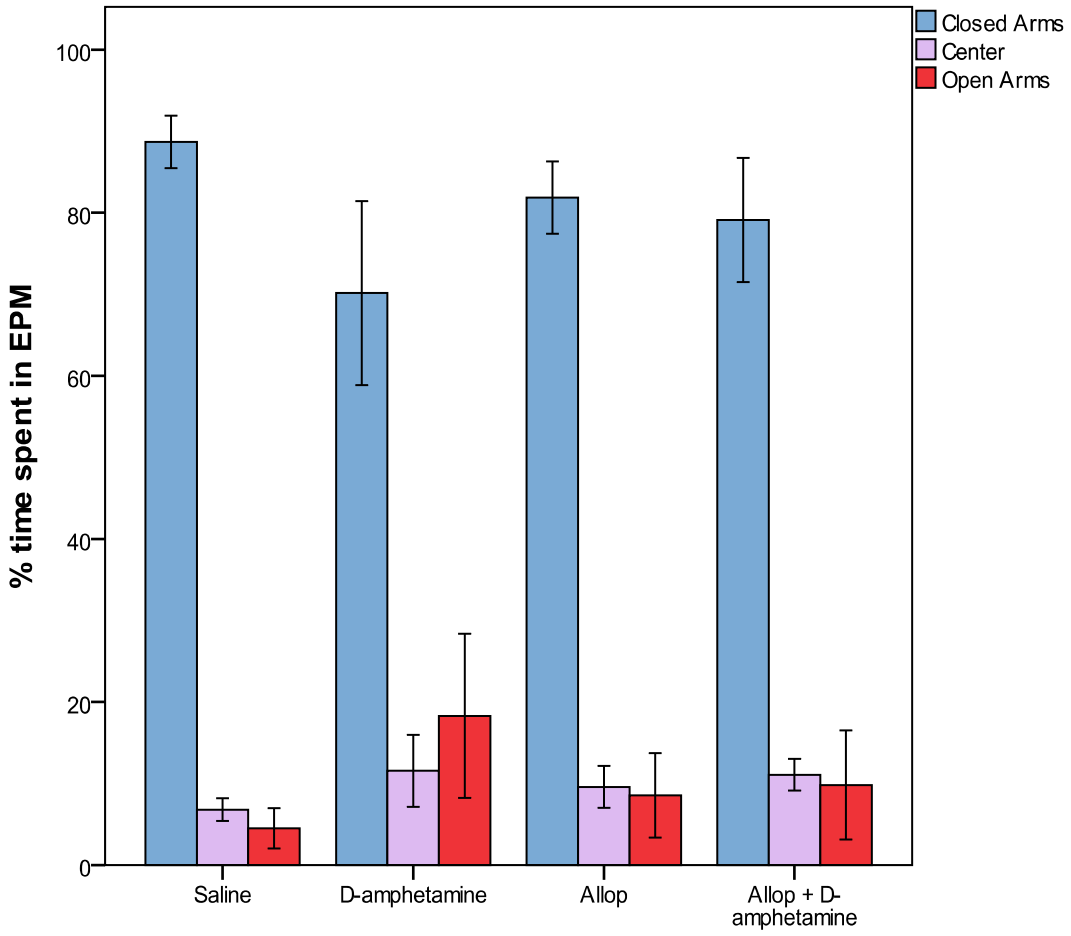

Figure 5. Effects of drug treatments on anxiety in the Elevated Plus Maze (EPM) in male rats. Bars represent percent of time in closed, center, and open arms of maze over 5 minutes of run time. Greater time spent in open arms and center indicates lower levels of anxiety. Error bars represent +/- 1 standard error.