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The Effect of Substance P on Ovariectomy-Induced Memory Deficits in Rats

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**THE EFFECT OF SUBSTANCE P ON OVARIECTOMY-INDUCED MEMORY
DEFICITS IN RATS**

**Thesis submitted to
The Graduate College of
Marshall University**

**In partial fulfillment of the
Requirements for the degree of
Master of Arts
Psychology**

by

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ABSTRACT

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Jamie L. Haga

The present experiment was designed to test whether pretreatment with substance P would affect ovariectomy-induced memory deficits in rats for retention in the Morris water maze. Adult female Sprague-Dawley rats were divided to two groups: (1) control (received saline) and (2) experimental group (received substance P). All rats underwent an ovariectomy, as this has been shown to significantly impair spatial reference learning and memory (Monteiro, Matté, Bavaresco, Netto, & Wyse, 2005). Approximately 8 months after surgery, all rats were trained in the Morris water maze in order to evaluate both reference and working memory. Results showed that substance P did not significantly affect performance in any segment of the experiment, when compared with controls. The results of the present experiment do not support the hypothesis that pretreatment with substance P improves spatial and working memory of ovariectomized rats.

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The Effect of Substance P on Ovariectomy-Induced Memory Deficits in Rats

Though studies of the sex steroid estrogen and the neurotransmitter substance P have contributed to a greater understanding of mechanisms of reproductive behavior and pain perception, respectively, numerous research findings have revealed that separately, both are also able to improve performance in behavioral tasks of learning and memory (Miller et al., 1999; Packard & Teather, 1997; Singh, Meyer, Millard, & Simpkins, 1994; Hasenöhrl, Frisch, Nikolaus, & Huston, 1994). Estrogen has been shown to increase spatial and working memory performance in young and old ovariectomized mice (Miller et al., 1999). Posttraining administration of estradiol has been shown to enhance memory in ovariectomized rats in a time-dependent manner (Packard & Teather, 1997) and estrogen replacement enhances performance on a two-way active avoidance task in adult ovariectomized rats (Singh et al., 1994). Chronic administration of substance P in dosages of 50 and 250 µg/kg has been shown to improve maze performance of old rats (Hasenöhrl et al., 1994). Furthermore, in a study by Tomaz and Nogueira (1997), rats injected with substance P and tested 24 hours or 21 days after the original learning showed better retention performance than a control group (Tomaz & Nogueira, 1997). Whether the memory enhancing effects of substance P are dependent upon the presence of estrogen has received little investigation.

Substance P is a peptide belonging to the tachykinin family, which is a group of small neurotransmitters that are involved in a multitude of physiological processes due to their widespread distribution, centrally and peripherally. Substance P is referred to as a tachykinin when describing the nonmammalian peptide, but is designated as a neurokinin when referring to mammalian peptides. Tachykinins function as pain transmitters in the periphery, while centrally, they act as neurotransmitters and neuromodulators in the brain and spinal cord (Strand, 1999). Substance P can be extracted from the brain of all vertebrate species, including man (Pernow, 1983). The effects of substance P are mediated by the neurokinin-1 (NK1) receptor. A decreased concentration of neurokinins, including substance P, accompanies brain aging in rats, as well as in humans (Huston & Hasenöhrl, 1994). Substance P promotes memory in normal animals and can counter age related performance deficits (Huston & Hasenöhrl, 1994).

The release of substance P may be regulated by ovarian steroid hormones. Substance P and ovarian steroid receptors that have been identified within the same hypothalamic cells suggest that gonadal steroids may regulate the synthesis of substance P in guinea pigs (Hasenöhrl

et al, 1994). In female guinea pigs, estradiol treatment increases substance P levels in the hypothalamus and midbrain, while treatment with estradiol and progesterone decreases substance P to baseline levels found in ovariectomized controls (Bethea, Hess, Widmann, & Henningfeld, 1995). Furthermore, substance P-immunoreactive axon terminals were observed to innervate areas of the medial preoptic area of the female rat brain and to form synaptic connections at these sites with neurons which contain estrogen receptors in their nucleus (Kallo, Fekete, Coen, & Liposits, 1998). The medial preoptic area contains high concentrations of estrogen and is associated with the expression of maternal behaviors in rats (Numan, 1994). Using dual-label immunocytochemical procedures, results from a study by Kallo et al. (1998) revealed that substance P immunoreactive axon terminals play both inhibitory and excitatory roles in the innervation of estrogen sensitive neurons in the medial preoptic gray of the female rat. Kallo et al. (1998) suggest that results of their study indicate that estrogen-receptive preoptic neurons may be regulated by substance P-containing neuronal pathways via synaptic mechanisms. Similarly, substance P-immunoreactive punctate structures suggestive of boutons were found in close association with the processes of some estrogen-receptor-immunoreactive neurons (Turcotte & Blaustein, 1997). Substance P is morphologically positioned to modulate the effects of neurotransmitters with which it is colocalized (Strand, 1999). Therefore, estrogen and substance P containing structures in the rat brain that are in close proximity to one another may imply shared regulatory capabilities.

As for many neuropeptides, substance P can alter gonadal function via a direct influence on pituitary luteinizing hormone secretion (Dudas & Merchenthaler, 2006) and/or by regulation of the hypothalamic gonadotropin releasing hormone (GnRH) system. Subcutaneously administered substance P inhibits GnRH-induced luteinizing hormone release via NK1 receptors (Dudas & Merchenthaler, 2006) and microinjection of the peptide into the medial preoptic area reduces luteinizing hormone and follicle stimulating hormone levels in the plasma. A direct interaction between substance P and GnRH systems, based on electron microscopic observations in rats, may take place at the level of GnRH perikarya and dendrites in the medial preoptic area and infundibular stalk/medial eminence regions in humans and be the morphological substrate for the substance P-controlled regulation of gonadal functions in humans (Dudas & Merchenthaler, 2006).

The memory conserving actions of substance P may be partially mediated by sex steroids. The preprotachykinin A (PPT-A) gene encodes the precursor of several tachykinin neuropeptides, including substance P. Sex steroids mediate the expression of the preprotachykinin gene in the anterior pituitary. Males possess more preprotachykinin messenger ribonucleic acid than females, but substance P levels in the hypothalamus, anterior pituitary, and plasma are higher in females (Strand, 1999). Specific sequences of the tachykinin are involved since N-terminal substance P 1-7 enhances memory whereas the C-terminal hepta- and hexapeptide sequences improve reinforcement (Strand, 1999).

Changes in the neuroendocrine system due to the loss of ovarian function at menopause have an important biological role in the control of reproductive and non reproductive functions, and regulate mood, memory, cognition, behavior, immune function, the locomotor system, and cardiovascular functions (Rehman & Masson, 2005). Estrogen deficiency during menopause is often associated with memory dysfunction (Frick, Fernandez, & Bulinski, 2002) and has been implicated as a risk factor for cognitive impairment in elderly women (Bagger et al., 2005). Estrogen replacement therapy improved cognitive test performance in women with Alzheimer's disease compared to men with the disease (Miller et al., 1999) and improved both verbal and spatial memory in non-demented menopausal women (Frick et al., 2002).

A mechanism by which estrogen may enhance cognitive function is by modulating the production and release of acetylcholine in the basal forebrain (Shughrue, Scrimo, & Merchenthaler, 2000; Gibbs, 1994; McEwen, Alves, Bullock, & Weiland, 1997). This is a system that projects to the cerebral cortex and hippocampus and is thought to play a central role in learning and memory (Shughrue et al., 2000). Cholinergic neurotransmission modulates memory and learning (Fischer, Chen, Gage, & Björklund, 1992). It has been demonstrated that long-term, but not short-term loss of ovarian function produces deficits in choline acetyltransferase in the medial septum and nucleus basalis magnocellularis (Gibbs, 1998). The loss of choline acetyltransferase may partially explain the memory deficits believed to accompany estrogen deficiency.

Explanations for the deficits in memory associated with menopause, as well as certain neurological degenerative diseases, such as Alzheimer's and Parkinson's disease, have previously been explored (Blanchet et al., 1999; Miller et al., 1999). Multiple neurotransmitter systems are believed to be involved in the cognitive decline of Alzheimer's disease patients, hallmarked by

cholinergic neuronal dysfunction and loss (Simpkins et al., 1997). A decline in acetylcholine and its synthetic enzyme, choline acetyltransferase, is a prominent biochemical feature of Alzheimer's disease (Miller et al., 1999). It has been determined that choline acetyltransferase activity at 28 weeks postovariectomy is reduced by 56% in Sprague-Dawley rats (Singh et al., 1994). In ovariectomized rats, estrogen treatment significantly increases the expression of choline acetyltransferase in the basal forebrain region (Miller et al., 1999). Substance P and other tachykinins coexist with acetylcholine in neurons of the myenteric plexus (Strand, 1999). Research on the neurobiological mechanisms of learning and memory has repeatedly shown that drugs with memory-promoting effects share as a common characteristic the ability to increase the activity of basal forebrain cholinergic systems (Hasenöhrl et al., 2000). Though acetylcholine has been demonstrated to be linked to memory and learning, results from some studies have not observed any age-related changes in cholinergic neuron morphology (Veng, Granholm, & Rose, 2003). The interactions between estrogen, substance P, and acetylcholine that influence memory and learning remain unclear.

In the present study, animals were ovariectomized to simulate the estrogen loss experienced by post menopausal women and because it has been shown to significantly impair spatial reference learning and memory (Monteiro, Matté, Bavaresco, Netto, & Wyse, 2005). Spatial memory is the part of memory responsible for recording information about one's environment and its spatial orientation (http://en.wikipedia.org/wiki/Spatial_memory, 2006). By removing the source of gonadal estrogen, ovariectomy eliminates the confounding variable of endogenous estrogen on the actions of exogenous substance P. However, it has also been shown that long-term (1.5-6 months) ovariectomy improves spatial memory in aged rats (Bimonte-Nelson et al., 2003; Monteiro et al., 2005). These inconsistencies highlight the need to further evaluate the role of estrogen on memory and learning.

As in humans, aging in rodents is typically accompanied by impairments in spatial memory. Spatial reference memory in rats, commonly tested in a hidden-platform version of the Morris water maze, deteriorates with age in both males and females (Frick et al., 2002). In the present experiment, a version of the original Morris water maze was utilized because this method has previously been used as a discriminatory assay of spatial memory (Morris, 1984) and requires intact spatial memory abilities and is particularly sensitive to the effects of aging (Wenk, 1998; Veng et al., 2003). Spatial reference memory of ovariectomized rats that receive

substance P should be better than those that receive saline.

Methods

Subjects

The rats tested in the present study were 16 female Sprague-Dawley rats obtained from Hilltop Labs Inc. (Scottsdale, PA) at approximately 5 weeks of age. The animals were bilaterally ovariectomized before arrival and weighed 440 +/- 5 g at the beginning of the experiments. They were housed in plastic cages with 4 rats per cage in a temperature controlled room (21 +/- 1° C) and maintained on a 14:10 h light-dark cycle (lights on at 8:00 am). Each cage housed 2 rats from the control group and 2 from the experimental group. All rats had *ad libitum* access to food and water.

Drugs and Injection Procedure

Substance P (substance P; molecular weight 1347.80) was dissolved in physiological saline containing 0.01 M acetic acid (pH 4.6), frozen in stock solutions of 1 mg/ml and diluted shortly before use. Dilutions of substance P were kept cold (at about 4° C) and used on the same day they were made. Sterile saline was stored at room temperature (22° C) throughout the experiment. Intraperitoneal injections were administered every other day (Days 3, 5, 7, 9, and 11) and 30 minutes prior to performance in any water maze trial of that day. No injections were given on any other day of the experiment (Days 1, 2, 4, 6, 8, 10, 12, 13, 14, 15, or 16). The control group rats received i.p. injections of saline and the experimental group rats received i.p. injections of substance P (50 µg/kg in a volume of 1 ml/kg). The dose of substance P was selected based on previous experiments assessing the role of substance P in learning and memory processes (Tomaz & Huston, 1986; Huston, Hasenöhrl, Boix, Gerhardt, & Schwarting, 1993; Hasenöhrl et al., 1994). Control group rats received the same volume of saline for each i.p. injection.

Water Maze Procedure

To determine the effects of long-term estrogen deprivation, training trials began 8 months after ovariectomy. All rats performed test trials over a 16-day period in the Morris water maze. The maze consisted of a white circular tank (1.83 m in diameter and 0.58 m in height) filled with water (25° C) to a depth of 20 cm and was surrounded by a variety of extramaze cues (e.g., video recorder, a poster, ceiling pipes, and a door), which remained unchanged throughout the water maze experiment. The water was made opaque with non-toxic white paint to prevent the rat

from seeing through the water. The water was drained daily. A white PVC tube (4.5 inches diameter) escape platform was submerged 2 cm below the surface. To allow the tube to remain upright underwater, it was attached using hot glue to a marble floor tile (1 ft length x 1 ft height). Four small holes, spaced approximately 2 inches apart, were drilled through the lower side of the tube to allow water to drain in and out. The tube was closed at the top with a white plastic lid, which had a 1 cm depression around the inner perimeter of the tube providing an area the rats could grip and pull themselves onto the platform.

Orientation Trials

Orientation trials were performed on Days 1 and 2 by placing a rat in the pool without the platform and allowing it to swim for 120 sec. To avoid stressing the animal, the rat was placed into the water gently with the hind-quarters lower, so the head did not go under water. One 120 sec trial was performed per day, which allowed the rats to habituate to the experience of swimming in the pool. All 16 rats were able to successfully swim for 120 sec.

Training Trials

On Day 3, i.p. injections and training trials in the water maze began. A training trial consisted of each rat being placed in one of four quadrants (north, south, east, or west) along the perimeter of the tank and recording the time the rat took to reach the escape platform. The platform was located in the middle of one quadrant, equidistant from the center and edge of the pool. Each rat was released into the water with the head pointing toward the pool-side, to minimize visual bias. If the rat did not reach the escape platform within 120 sec, it was hand guided to the platform and allowed to remain there for 15 sec. If the rat did find the platform, it was allowed to remain there for 15 sec. Four trials were performed by each rat per day. The quadrant in which the rat was released and the hidden platform quadrant remained the same for all four trials of the day. Hidden platform location remained the same for each rat for all 6 days of the training trials, but release quadrant was changed daily for each rat. Between trials, the rat was towed off and placed in a towel-lined cage to help maintain core body temperature. The average intertrial interval for each rat was approximately 3 min. Training was conducted for 6 consecutive days, from Days 3-8. Animals received an i.p. injection on Days 3, 5, and 7.

Probe Trial

On Day 9, the platform was removed from the pool and the animals were tested in a 120 sec spatial probe trial. The probe trial is used to test the rat's knowledge of the precise location

of the platform. An accurate direction of the swimming behavior provides evidence that the rat has learned the spatial location of the platform relative to the available external cues (Wenk, 1998). The time spent in the platform-quadrant where the platform had been located during the training was measured. Injections were administered on Day 9.

Working Memory Trials

A repeated acquisition test (Yamada et al., 1999) was conducted, to assess working memory, for 3 consecutive days (Days 10 - 12) and consisted of five trials (one session) per day. For each trial, the rat was put into the pool at one of four starting positions, the sequence of the positions being selected randomly. The starting position and platform location remained the same throughout the remaining four trials of the day. The starting position for each rat remained the same throughout all working memory trials but the location of the hidden platform was changed daily. Since the platform position changed daily, working memory was evaluated by this task. Latency to find the platform for the first trial of the day should be longer compared to that of the last if learning is taking place.

The first trial of each session was an informative sample trial in which the rat was allowed to swim to the platform in its new location and to remain there for 15 sec. If the rat did not find the platform within 120 sec, it was hand guided to the platform and allowed to remain there for 15 sec. Each rat was allowed to swim for up to 120 sec and time to find the platform was recorded. Between trials, the rat was placed in a towel-lined cage for approximately 3 minutes. Intraperitoneal injections were given on Day 11.

Relearning Trials

To determine if substance P has any long term effects, all i.p. injections were stopped after Day 11 and testing trials similar to those performed during the first training trials were done for 4 consecutive days (Days 13 - 16). Relearning trials were performed following the same procedure as for the training trials of Days 3 - 8. Four trials were performed per day. A trial consisted of each rat being placed in one of four quadrants along the perimeter of the tank and recording the time each rat took to reach the escape platform. As before, the platform was located 2 cm beneath the water level in the middle of one quadrant, equidistant from the center and edge of the pool. The hidden platform location for each rat for the relearning trials was the same as it was during the training trails of Days 3 - 8. The quadrant in which the rat was released and the hidden platform quadrant remained the same for all four trials of each day.

Hidden platform location remained the same for each rat for all 4 days of the relearning trials, but release quadrant was changed daily for each rat.

Each rat was allowed to swim for a maximum of 120 sec to find the hidden platform. If the rat did not reach the escape platform within 120 sec, it was hand guided to the platform and allowed to remain there for 15 sec. If the rat did find the platform on its own, it was also allowed to remain there for 15 sec.

Results

Statistical Analysis

Reference, spatial, and working memory data were analyzed by an analysis of variance (ANOVA). Alpha was set at 0.05 for determining statistical significance.

Training Trials

The Training data were analyzed using a 2 x 4 x 6 x 4 analysis of variance with drug and group (starting position) as between-subject factors and test day and trial as within-subject factors. The animals' abilities to find the hidden platform improved as latencies to find the hidden platform for all animals significantly decreased across days of testing, $F(5, 40) = 4.39, p < .01$ (See Figure 1). There was also evidence of learning across trials as latencies also decreased across the four trials of each day, $F(3, 24) = 15.89, p < .001$ (See Figure 2). Finally, there was a significant interaction between day and trial, $F(15, 120) = 2.05, p < .05$. Performance generally improved from trial 1 - 4 each day, but the differences were larger on the early days. Latencies decreased from a mean of 72.7 sec to 21.3 sec from Trial 1 to 4 on Day 1 and decreased from 22.3 sec to 11.2 sec from Trial 1 to 4 on Day 6. None of the effects or interactions involving the drug treatment were significant, $p > .2$ in all cases. However, a separate analysis of variance indicated that on days animals received an i.p. injection (saline or substance P), overall latency to find the platform was significantly slower compared to days with no injection (30.6 sec versus 23.4 sec), $F(1, 8) = 7.10, p < .05$.

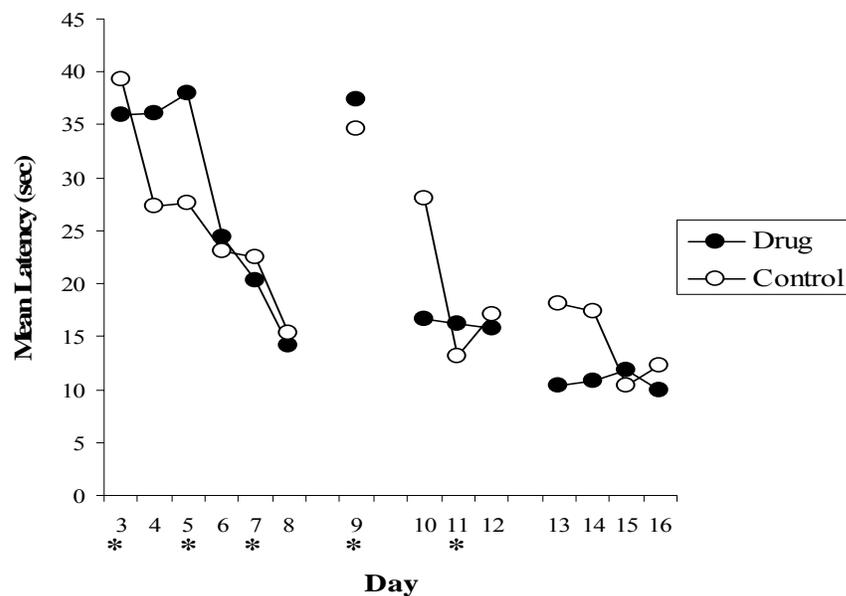


Figure 1. Mean latencies of drug versus control groups across all days. An asterisk (*) indicates an i.p. injection day (Days 3, 5, 7, 9, and 11).

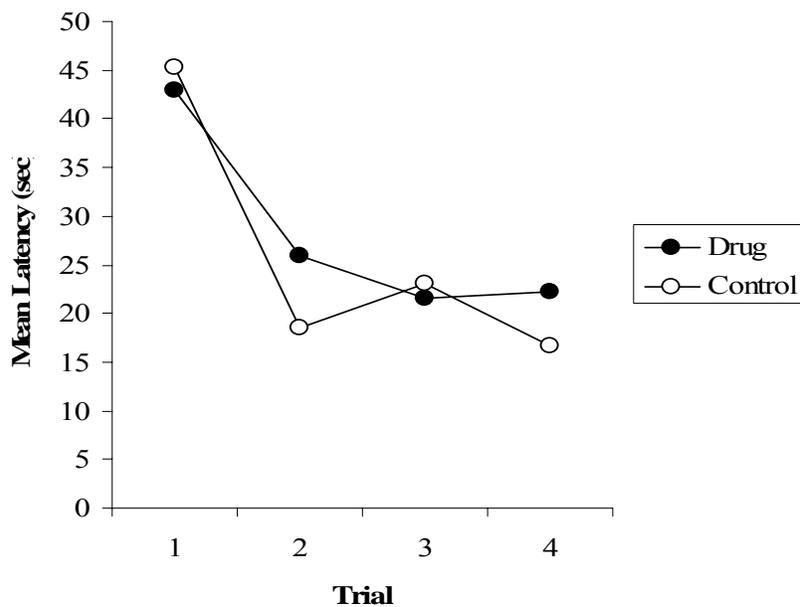


Figure 2. Mean latencies of the four trials collapsed across days of the training trials

Probe Trial

Latencies to cross the target quadrant did not differ significantly among animals who received substance P versus those of the control group, $F(1, 8) = 0.29$, $p > .6$ (See Figure 1).

There also was no interaction between group and drug on latency to find the target quadrant, $F(3, 8) = 0.42, p > .7$.

Working Memory Trials

There was a significant effect of trial on latencies to find the platform, $F(4, 32) = 11.78, p < .05$. As expected, rats showed learning across trials as displayed in Figure 3. Substance P did not have a significant effect on performance since the main effect for drug treatment and all interactions involving the drug treatment were not significant, $p > .1$. Intraperitoneal injections were given on Day 11. Analysis revealed no significant difference when comparing latencies of the injection day trials (Day 11) versus latencies of non-injection day trials (Days 10 and 12), $F(3, 24) = 0.56, p > .05$.

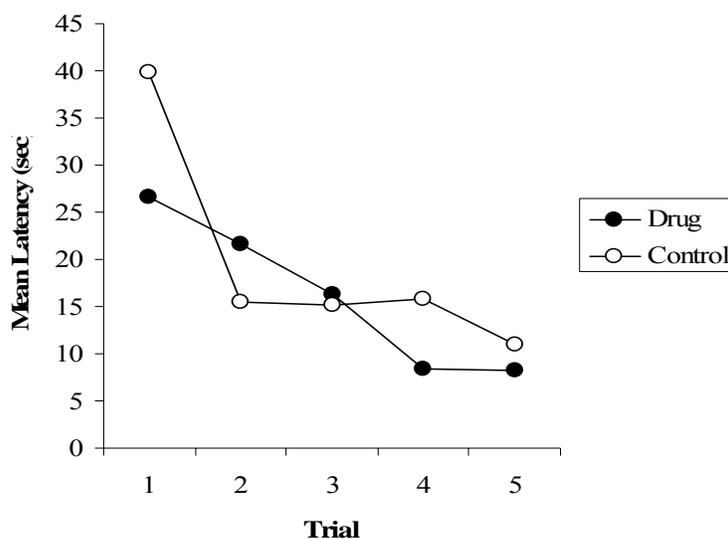


Figure 3. Mean latencies of trials collapsed across days of the working memory trials

Relearning Trials

Analysis of the relearning data for Days 13 - 16 indicated that the only significant effect was a significant improvement in performance across trials, $F(3, 24) = 6.05, p < .01$. These results are displayed in Figure 4. While no other effects or interactions were statistically significant, the main effect for drug treatment may be worthy of mention, $F(1, 8) = 3.56, p < .1$. Overall mean latency to find the hidden platform for drug subjects was 10.7 sec versus 14.5 sec for control subjects (See Figure 1).

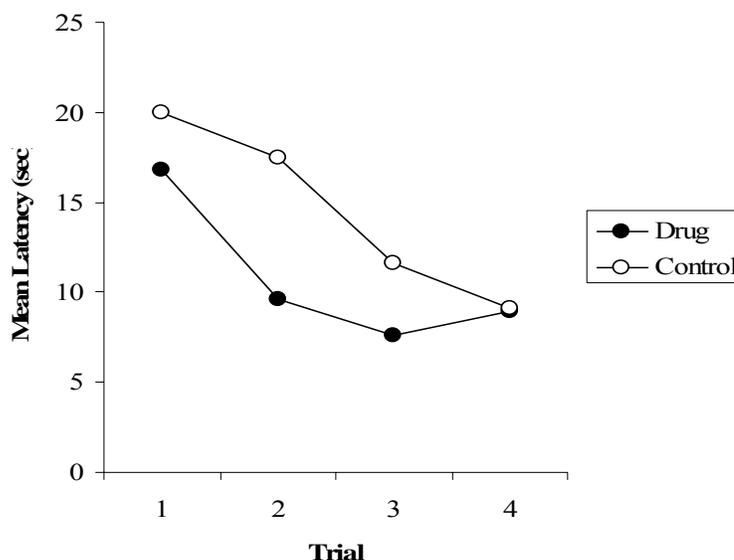


Figure 4. Mean latencies of trials of the relearning trials

Discussion

The purpose of the present study was to determine if i.p. injections of substance P could improve performance in a version of the Morris water maze by counterbalancing the memory deficits that accompany estrogen deficiency. The results of the present study did not support the hypothesis that substance P could improve memory; however, they do demonstrate that spatial learning can occur in ovariectomized rats regardless of whether they received a pretreatment of substance P or saline.

Data from the Training trials revealed that the latencies to find the hidden platform for all animals significantly decreased across days of testing. This decline in latency can be attributed to two factors: (1) the rats learning to swim away from the side walls and thus increasing their likelihood of contacting the platform by chance; (2) true spatial learning about the location of the platform (Morris, 1984). During the training trials, none of the effects or interactions involving the drug treatment were significant. Interestingly though, overall latency to find the platform was significantly higher on days when injections were given than on days with no injection. This could imply that there was some mechanism, such as pain at the injection site, which impaired motor activity, thus increasing latencies.

Probe trial data revealed that latencies to cross the target quadrant did not differ significantly among animals who received substance P versus those of the control group. The

rationale for recording time taken by the rat to find a target quadrant is that the platform was removed during this test, but rats trained to find a platform in a particular quadrant should head towards it from a distance. For the working memory trials, rats showed learning across trials, however, substance P did not have a significant effect on performance. These findings did not support the notion that substance P may have long-lasting effects on maze performance. Results of the relearning trials indicated that the only significant effect was a significant improvement in performance across trials. Relearning is generally very rapid (Morris, 1984).

Injections were administered 30 minutes prior to water maze performance because this time falls within the pre- and post-trial period during which the neurokinin has been found to enhance performance in various learning tasks (Huston & Oitzl, 1989; Hasenöhrl et al., 1994). Furthermore, previous research has shown that substance P improved maze performance in aged rats when administered prior to acquisition trials in a four-choice water-filled maze, which was indicative of a mnemogenic action of the peptide, that is, a direct facilitatory effect on learning and/or memory processes (Hasenöhrl et al., 1994). Injections were administered every other day because rats injected with substance P and tested 24 hours after the original learning have shown better retention performance than control group rats (Tomaz & Nogueira, 1997). Furthermore, the i.p. route of administration was chosen because there is evidence that small amounts of systemically given substance P can penetrate the blood-brain barrier (Hasenöhrl et al., 1994). It is not known though if substance P passes the blood-brain barrier as a whole molecule and it cannot be ruled out that substance P gains access to the central nervous system via the circumventricular organs, for which the blood-brain barrier is significantly diminished (Hasenöhrl et al., 1994). Since the neurokinin was administered systemically, various peripheral effects of the peptide, such as on cardiovascular functions, nociception, and gastrointestinal motility or on plasma hormone levels could have influenced central nervous system functions (Hasenöhrl et al., 1994). A method that places substance P directly into rat brain tissue, such as a push-pull or third ventricle cannulae, may help eliminate possible peripheral effects.

Future research should be done using a larger number of rats. Small group size was a limitation of the present study. Furthermore, albino rats do not see as well as the traditionally used hooded rats. The rats' vision must be intact in order to accurately assess the value of extra maze cues in measuring tasks of spatial memory. Furthermore, data from human research suggests that cognitive functioning may be related more to psychosocial predictors such as

depression, stress, marital status, work, activity level, smoking status, overall health and obesity than estrogen loss. If these predictors play a major role in cognitive function, it is comprehensible then that estrogen loss may be a factor, but not a significant one, in the interactions that occur in order for substance P to exert memory enhancing effects. Transition through menopause may not be accompanied by a decline in working memory and perceptual speed (Meyer et al., 2003). Meyer et al. (2003) hypothesized that a decline in cognitive functioning occurs as women progress through the menopausal transition, independent of age, educational level, family income, ethnicity, and baseline self-perceived health (Meyer et al., 2003). Contrary to their hypothesis, results from their study suggest that transition through menopause is not accompanied by a decline in working memory and perceptual speed (Meyer et al., 2003). These findings defy the notion that deficits in memory and cognitive function accompany estrogen loss. Further investigation as to how hormones and substance P interact to effect memory and learning is needed.

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