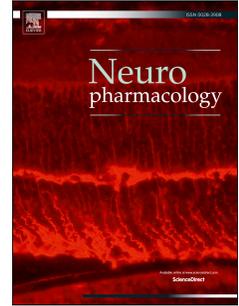


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**The Ventral Hippocampal Muscarinic Cholinergic System Plays a Key Role
in Sexual Dimorphisms of Spatial Working Memory in Rats**

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Abstract

Sex differences in cognitive processing and function have been documented in human and animal studies. Females have been found to perform better than males on non-spatial memory tasks, while males tend to outperform females on spatial memory tasks. The neural mechanisms underlying these sexual dimorphisms are unclear. However, it is known that the cholinergic system is critically involved in memory processes, and there are notable differences between males and females in cholinergic system function and receptor expression. In particular, there are sex differences in the processing of information in the frontal cortex and hippocampus. In this study, we examined the roles of muscarinic and nicotinic acetylcholine receptors in the medial frontal cortex (MfC) and ventral hippocampus (VH) on spatial working memory in male and female rats. Local infusions of scopolamine (SCOP) and mecamylamine (MEC) (10, 20, 50 $\mu\text{g}/\text{side}$) were used to antagonize these receptors in each respective brain region during performance in the 16-arm radial arm maze. Infusions of SCOP into the VH caused a significant increase in memory errors in female rats, but had no significant effect on males, while infusions of MEC into the VH had no effect on either sex. Infusions of both SCOP (50 $\mu\text{g}/\text{side}$) and MEC (20 $\mu\text{g}/\text{side}$) into the MfC caused working memory impairments in both sexes. These results show that muscarinic acetylcholine receptors in the VH are differentially vulnerable to spatial memory impairments in females. Ventral hippocampal muscarinic acetylcholine receptors may play a key role in male-female differences in spatial memory.

1. Introduction

Studies have shown that differences exist between males and females in terms of cognitive processing. These differences are particularly prevalent during spatial versus non-spatial cognition/memory tasks. For instance, female rodents have been shown to outperform males during novel object recognition (NOR) tasks; a task of working memory that, in standard form, has no spatial or aversive components (Bettis and Jacobs, 2013; Sutcliffe et al., 2007). However, when a spatial component was introduced to the NOR task in these studies, male rodents showed marked improvement in memory function compared with females. Indeed, a superior performance of males when compared to females was also reported in the radial-arm and Morris water mazes, both tests classically used to assess spatial memory function (Gresack and Frick, 2003; Jacobs et al., 1990; LaBuda et al., 2002; Roof and Stein, 1999; Saucier et al., 2008). The evidence for human cognitive processing tells a similar story to the rodent literature. Women have been shown to perform better than men on verbal and memory tasks (Seidlitz and Diener, 1998), and to be more sensitive than men to the location of objects in a personal space (Saucier et al., 2007; Voyer et al., 2007). By contrast, men were shown to outperform women during a virtual Morris water maze (MWM) task, a simulation of the common spatial working memory task performed with rodents (Astur et al., 1998; Sandstrom et al., 1998). Although some studies report conflicting results (Bimonte et al., 2000; Healy et al., 1999), a preponderance of the evidence still suggests that women perform better during non-spatial tasks, and men perform better on spatially-dependent tasks.

The neural bases for the differences observed in spatial memory between males and females remain poorly understood. Studies looking at gonadal hormones have suggested that levels of estradiol may alter the performance of spatial-related tasks in females; higher levels

typically impairing performance (Galea et al., 1995; Pleil and Williams, 2010). The role of testosterone in these tasks is less clear (for review, see Celec et al., 2015). There is also evidence that differences in hippocampal and prefrontal cortical processing between males and females may play a significant role in the results observed during cognitive tasks. These brain regions have long been known to be involved in the processing of information related to both memory and spatial navigation (Diana et al., 2010; Kennedy and Shapiro, 2004; Maguire et al., 1998). Indeed, communication between these regions appears to be critical for processing working memory (for review, see Jin and Maren, 2015; Preston and Eichenbaum, 2013). However, it has been shown that females may rely more heavily on the frontal cortical regions to perform cognitive tasks while males seem to make greater use of the hippocampus (Gur et al., 2002). More recent studies using advanced imaging techniques in humans have confirmed that structural brain differences (including functional connections) correlate with differing behavioral patterns between males and females (Tunc et al., 2016). The mechanisms underlying these structural and behavioral differences have yet to be sufficiently explored.

The cholinergic neurotransmitter system has long been known to be critically involved in cognitive function. Early work by our lab and others showed that administration of nicotine improves performance in cognitive tasks (Buccafusco and Jackson, 1991; Levin et al., 1992; Levin and Rose, 1990). Moreover, it has repeatedly been shown that blockade of both nicotinic (nAChRs) and muscarinic (mAChRs) acetylcholine receptors with mecamylamine (MEC) and scopolamine (SCOP), respectively, results in significant impairments in working memory tasks (Cozzolino et al., 1994; Levin et al., 1989). It is known that sexual dimorphisms exist regarding cholinergic receptor expression and system function (Avisar et al., 1981; Brown and Brooksbank, 1979; Hortnagl et al., 1993; Koylu et al., 1997). How these differences may

contribute to spatial memory tasks is less well characterized. The present study was performed to demonstrate the relative roles of both nAChRs and mAChRs during a spatial memory task in male and female rats. It was hypothesized that blocking these receptors would result in differential impairment in radial-arm maze performance in males and females. Because of previous literature detailing the sex differences in cognitive processing between the frontal cortex and hippocampus, these brain areas were chosen as specific sites for determining the roles of nAChRs and mAChRs in spatial memory in males and females. The results of this study will shed light on how cholinergic function contributes to the observed differences in males and females during the performance of spatial memory tasks.

2. Materials and Methods

2.1. Subjects. Experimental procedures in this study were conducted in accordance with AAALAC guidelines, and were also approved by the Duke University Animal Care and Use Committee. Young adult male and female rats were purchased from Charles River Laboratories (Raleigh, NC, USA), and used in the study. Animals began behavioral training after one week of acclimation to the laboratory housing environment (postnatal day 60). The animals were housed in a temperature-controlled vivarium at Duke University under standard laboratory conditions and were kept on a 12:12 reverse light/dark cycle. Behavioral testing occurred during the active (dark) phase of the light cycle. The animals were housed in groups of three per cage until brain cannulation surgery. After surgery the rats were housed singly to prevent damage to the surgical crown by cage mates. All animals were given unlimited access to water while in their home cage environment. Unlimited food was also provided until behavioral training commenced. Once training began all animals were kept on a food-restricted diet for the remainder of testing

procedures, so that the animals' weights were maintained at approximately 85% of free-feeding levels. All animals progressively gained weight throughout the study.

2.2. Drugs. Scopolamine HCl was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mecamylamine HCl was obtained via the National Institute on Drug Abuse (NIDA) Drug Supply Program. Both drugs were dissolved in artificial cerebrospinal fluid (aCSF), which served as the vehicle for both compounds. The drug doses and vehicle were administered in a repeated measures counterbalanced design with every rat receiving all of the doses and vehicle administration so that dose-effects were not confounded with order of administration. Scopolamine was given first, then mecamylamine.

2.3. Cannulation Surgery. Brain cannulation surgeries were performed in a manner similar to Hall et al. (2015). Briefly, upon successful completion of training criteria for the radial-arm maze (see Section 2.4) each rat received a bilateral local infusion cannula implanted via stereotaxic surgery (David Kopf Instruments, Tujunga, CA, USA). Two specific brain areas were selected for the study: the medial frontal cortex (MfC) and ventral hippocampus (VH). Rats were anesthetized with a combination of ketamine (60 mg/kg i.p.) and dexmedetomidine (0.15 mg/kg i.p.) and placed on the stereotaxic instrument. An incision was made down the midline of the head to expose the skull. Coordinates for both brain regions were taken relative to the bregma of the skull. The MfC coordinates were based on Paxinos and Watson, 2007 (Paxinos et al., 2007), and were as follows: anteroposterior +2.70 mm, lateral ± 0.75 mm, dorsoventral -2.70 mm. Coordinates for the VH were derived from Pellegrino, 1979 (Pellegrino et al., 1979), and were: anteroposterior -3.20 mm, lateral ± 5.20 mm, dorsoventral -7.00 mm. Cannulae were secured via screw and wire structure and a mixture of carboxylate cement (Durelon™, 3M, St. Paul, MN, USA) that covered the surgical site. Surgical wounds were

treated with the topical anesthetic bupivacaine, and all animals were given ketoprofen (5 mg/kg, s.c.) for postoperative pain. Animals were given a five-day recovery period from surgery before behavioral testing with SCOP and MEC treatments began. Upon completion of behavioral testing, animals were sacrificed and brains were removed and placed in a formalin solution. To ensure proper placement of the infusion cannulae, each brain was then sliced on a cryostat. Only those animals with the correct histological localization of the bilateral cannulae for the respective targeted brain region were considered for statistical analysis.

2.4. Behavioral Testing: Radial-arm maze tests were conducted on a black, wooden maze, elevated 31 cm off the floor, and consisting of a central platform (50 cm diameter) with 16 arms (10 cm x 60 cm) extending radially from the central platform. Food cups were located 2 cm from the distal end of each arm. Shapes in the form of cardboard cutouts were placed on the walls of the testing room to serve as visual cues and to permit spatial orientation. Habituation to the maze occurred in two 10 min sessions. During habituation, each rat was placed inside a large, round, opaque cylinder and allowed to consume 12 halves of sugar-coated cereal (Froot Loops[®]; Kellogg's, Battle Creek MI, USA). Once habituated, the rats began training on the radial-arm maze task. During testing, 12 arms of the 16-arm maze were baited to test for working memory, while the 4 remaining arms were left unbaited and used to test for reference memory. The choice of baited arms was randomized among the animals, but the baited arms for each animal were kept constant for the duration of the study. Repeated entries into a baited arm of the maze were scored as working memory errors, and entries into unbaited arms were scored as reference memory errors. Latency was calculated as the total session time in seconds divided by the total number of arms entered by the animal. Each trial began by placing the rat on the central platform inside the opaque cylinder for 10 seconds, after which the cylinder was removed

and the rat was allowed to freely explore the maze. Testing sessions lasted 10 min or until the rat had entered all 12 baited arms, whichever came first. After every session, the maze apparatus was wiped clean with a damp paper towel. Acceptable training criteria consisted of a rat performing 18 sessions of the radial-arm maze task wherein the animal entered at least 8 of the 12 baited arms of the maze.

After meeting criteria for maze training, the rats underwent brain cannulation surgery (see Section 2.3). After recovery from surgery, radial-arm maze sessions began preceded by infusions of either SCOP or MEC into the MfC or VH (depending on the animal/cohort). SCOP and MEC were both infused at a rate of 0.133 $\mu\text{l}/\text{min}$ for three minutes, at doses of 10, 20, and 50 $\mu\text{g}/\text{side}$. After the three minute infusion the rats were given an additional 10 min inside their home cages before testing to allow for diffusion of drug. Doses of SCOP and MEC were randomized for each animal in the study, and each animal underwent two rounds of dosing: one round of SCOP followed by a five day washout period followed by one round of MEC. During the study, one day of washout was allowed between drug testing sessions.

2.5. Statistical Analysis: The working and reference memory errors and choice latency data were evaluated by analysis of variance with sex as the between subjects factor and drug dose and error type as within subjects factors. As recommended by Snedecor and Cochran (Snedecor and Cochran, 1967) interactions of $p < 0.10$ were followed up by tests of the simple main effects. A threshold of $p < 0.05$, two-tailed was used for determining significance for all tests. Only subjects with histologically verified bilateral cannula placements for infusions within the target areas were included in the analysis.

3. Results

3.1. Pre-cannulation Training

The acquisition data for working and reference memory are shown in figure 1. There were significant main effects of sex ($F_{1,43}=9.49$, $p<0.005$), error type ($F_{1,43}=55.76$, $p<0.0005$) and session block ($F_{2,86}=49.11$, $p<0.0005$). There was a significant interaction of sex x error type ($F_{1,43}=6.02$, $p<0.025$). Follow-up tests of the sex with the different error types showed that males had significantly ($p<0.05$) fewer working and reference ($p<0.01$) memory errors than females,.

Working memory errors during the final phase of training (sessions 13-18) averaged 4.5 ± 0.4 and during the aCSF vehicle sessions during drug testing was 5.6 ± 0.6 . Reference memory errors during the final phase of training (sessions 13-18) averaged 4.3 ± 0.2 and during the aCSF vehicle sessions during drug testing was 4.0 ± 0.6 .

3.2. Cannulations

Figure 2 shows the placements of the bilateral local infusion cannulae for the ventral hippocampal and medial frontal cortical infusion cannulae. Only the subjects with infusion sites within the target area on both sides were included in analysis. For the study of ventral hippocampal infusion effects there were 9 males and 16 females. For the study of medial frontal cortical infusion effects there were 10 males and 10 females.

3.3. Local infusions of Scopolamine into the Ventral Hippocampus. There was a significant overall effect of sex on total working and reference memory errors in the radial arm maze task, with females (14.64 ± 1.18) committing significantly ($F_{1,23}=13.81$, $p<0.005$) more total errors than males (8.44 ± 0.69). There was also a significant main effect of working/reference memory errors ($F_{1,23}=18.26$, $p<0.0005$) with more working memory errors (7.57 ± 0.74) being

committed in the task than reference memory errors (4.84 ± 0.59). Furthermore, there was an interaction between working/reference memory errors x sex ($F_{1,23}=5.59$, $p < 0.05$), with females committing significantly more errors than males with both error types, but with a greater effect with working (male= 4.75 ± 0.43 , female= 9.16 ± 0.92 , $p < 0.005$) than reference memory errors (male= 3.69 ± 0.48 , female= 5.48 ± 0.37 , $p < 0.01$). There was also a SCOP dose x sex interaction ($F_{3,69}=2.31$, $p < 0.09$) that prompted tests of the simple main effects of SCOP effects in each sex. In male rats, there was no significant effect of SCOP into the VH on total working and reference memory errors (Fig. 3). In contrast, with female rats infusions of SCOP into the VH caused significantly more total working and reference errors ($F_{1,15}=3.62$; $p < 0.025$). In females, VH local infusion SCOP doses of 10 ($p < 0.05$) and 20 ($p < 0.005$) $\mu\text{g}/\text{side}$ caused significant increases in total working and reference memory errors compared to performance after infusions of the aCSF vehicle (Fig. 3). There was no significant effect of SCOP treatment on latency in the task for either males or females.

3.4. Local infusions of Mecamylamine into the Ventral Hippocampus. The results of MEC infusions into the VH are presented in figure 4. There were no significant differences on working memory errors, reference memory errors, or latency in the task for either males or females.

3.5. Local infusions of Scopolamine into the Medial Frontal Cortex. There was a significant main effect of sex ($F_{1,18}=16.15$; $p < 0.001$) on errors, with females (11.25 ± 0.65) committing more total errors than males (6.92 ± 0.86). There was also a significant main effect of working/reference memory type ($F_{1,18}=11.57$; $p < 0.005$) with more working (5.46 ± 0.59) than reference (3.62 ± 0.31) memory errors. Significant interactions were observed of working/reference memory error type x sex ($F_{1,18}=6.36$; $p < 0.025$) with a significant ($p < 0.001$)

sex difference with working (male=3.70±0.67, female=7.22±0.59) but not reference memory errors (male=3.22±0.32, female=4.02±0.52). There was also a significant interaction of SCOP x working/reference memory error type ($F_{3,54}=3.50$; $p<0.025$). As shown in figure 5, SCOP infusions into the MfC had a significant ($p<0.05$) effect increasing working memory errors, with the 50 µg/side dose causing more working memory errors in the maze compared to vehicle treatment regardless of sex ($p<0.05$). There was no significant MfC SCOP effect observed on reference memory errors. There was no effect of SCOP infusions into the MfC on task latency.

3.6. Local infusions of Mecamylamine into the Medial Frontal Cortex. Figure 6 shows the effect of local infusions of MEC into the MfC on radial-arm maze performance. There was a significant main effect of working/reference memory type ($F_{3,18}=5.80$; $p<0.05$) with more working (5.42±0.56) than reference memory errors (4.30±0.27). There was a significant interaction between the dose of MEC and working/reference memory errors ($F_{3,18}=2.89$; $p<0.05$). The 20 µg/side MEC dose into the MfC caused a significant ($p<0.025$) increase in working memory errors regardless of sex. Since this study used a repeated measures design, in which each animal is given each of the drug treatments, the overlap of standard error bars is not necessarily indicative of the lack of significance of drug treatment. Rather, it is the consistency of the effect between doses that drives the significance determination. Infusions of MEC into the MfC had no significant effect on reference memory errors or response latency.

4. Discussion

The results of this study reinforce the findings from previous literature showing that male rats outperform female rats during memory tasks that carry a spatial component. Male rats in our study clearly committed fewer working memory errors than females while performing the 16-

arm radial-arm maze task as shown in figure 1, which displays choice accuracy for the rats prior to drug treatment. We found significant differences in radial-arm maze performance during the SCOP phase of the experiments in the VH, with females having a significant memory impairment due to SCOP treatment whereas the males were not affected by the same dose range of SCOP. There were no statistically significant differential sex effects on response to MEC or in response to the SCOP in the MfC. Sex differences in choice accuracy with vehicle infusions intercurrent with the drug challenges was less robust possibly due to carryover effects of the drug actions in the same animals before the vehicle infusion. Overall, our results from this study confirm what has typically been reported regarding an advantage for male rodents in spatial memory tasks (Jonasson, 2005) and give insight into the cholinergic component and brain region that may underlie the sexually dimorphic performance.

The most significant finding of this study is that infusions of SCOP into the VH resulted in significantly more working and reference memory errors in females, but not in males, during the 16-arm maze task. We have previously found similar results in the radial-arm maze in female rats using SCOP (Kim and Levin, 1996), and the compound is a commonly used tool to induce working memory impairments in rodent models (Buccafusco, 2009; Klinkenberg and Blokland, 2010). However, this is the first time we have directly compared both male and female performance using this paradigm, and it is very interesting that SCOP infusions did not cause memory impairments in males when infused into the VH. These results suggest that mAChR mechanisms in the VH may play a more significant role in females for spatial memory processing. Moreover, infusions of MEC into the VH had no observed effect on maze performance in either males or females, suggesting that mAChR mechanisms in this brain area may be a key factor in the differences observed between males and females during spatial

memory tasks. It should be noted that in our previously published study neither administration of mAChR nor nAChR agonists into the VH resulted in any improvement in female radial-arm maze performance (Kim and Levin, 1996). Perhaps there is some optimal level of VH cholinergic function below which causes memory impairments, and above which has no discernable benefit. Importantly, there were no significant effects on latency observed during the task for either sex as a result SCOP infusions into the VH. Therefore, it is unlikely that the increases in working and reference memory errors we found in this study were the result of off-target effects such as sedation.

Significant increases in working memory errors were observed in the radial-arm maze as a result of both SCOP (50 $\mu\text{g}/\text{side}$) and MEC (20 $\mu\text{g}/\text{side}$) infusions into the MfC. However, these effects were seen in both males and females, suggesting that these neural mechanisms are likely shared between the sexes. It is interesting, however, that infusions into the MfC had effects on working memory performance, but not reference memory, and this was true for both SCOP and MEC treatments. The MfC contributes greatly to memory processes, and the MfC-VH circuit is particularly crucial to spatial working memory (Burton et al., 2009; Kyd and Bilkey, 2003). Furthermore, cholinergic activity is critical to MfC function. The MfC is densely innervated with cholinergic neurons, and receives projections from the basal forebrain (Eckenstein et al., 1988). It has been shown that both tonic and phasic cholinergic signaling in the MfC are involved in cognitive function (Parikh et al., 2007). Based on our results and others, it may be that the MfC contains an essential cholinergic circuit shared by both sexes that is important for working memory function.

As previously stated, the functional connections between the MfC and hippocampus are crucial to working memory processes. Studies have shown synchronized activity between these

brain regions during the performance of working memory tasks (Benchenane et al., 2010; Jones and Wilson, 2005). It has been suggested that the flow of information runs from hippocampus to prefrontal cortex, as the prefrontal firing lags behind that of the hippocampus (Hyman et al., 2005, 2010). A recent study found that VH to prefrontal projections were responsible for encoding task relevant information regarding spatial cues that were important for performing a spatial working memory task (Spellman et al., 2015). Taken with the evidence that males seem to rely more heavily on hippocampal activity during cognitive tasks while females may rely more heavily on PFC mechanisms (Gur et al., 2002), this may explain, in part, the “male advantage” consistently observed in spatial working memory tasks. In light of the results from our current study, these differences may also include a significant mAChR component to the neural processing of spatial cognitive information. Another possibility may be that the differential response by males and females to hippocampal infusions of SCOP could be due to differences in emotional response rather than cognitive response. However, we did not see any of drug infusion effects on response latency in the radial-arm maze indicative of alterations in freezing behavior, indicative of emotional effects of SCOP infusions into the hippocampus.

In summary, the results of our study suggest that mAChR mechanisms in the VH may play a significant role in the sex differences observed during the performance of spatial working memory tasks. Our results also demonstrate that both mAChR and nAChR mechanisms in the MfC are critically important to working memory processes in both males and females. Our findings are consistent with previously published studies demonstrating a “male advantage” in the performance of working memory tasks containing a significant spatial component. Future studies are needed to more fully explore the extent of the differences observed in our study, including which mAChRs may be playing the largest role.

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Figure Legends

- Figure 1. Sex differences in pretreatment acquisition of working and reference memory on the 16-arm radial maze (mean±sem). Males had significantly fewer working memory errors ($p<0.005$) and reference memory errors ($p<0.01$) than females, errors±sem.
- Figure 2. Placements of bilateral cannula in each animal used for data analysis in the study. Animals with incorrect cannula placement were excluded from analysis. A) Medial Frontal Cortex (MfC); B) Ventral Hippocampus (VH)
- Figure 3. Effects of local infusions of SCOP into the VH on radial-arm maze performance in male and female rats (mean±sem). Doses of SCOP (10 and 20 µg/side) caused significant increases in working and reference memory errors in females, but not in males, when compared to aCSF vehicle ($p<0.05$ and $p<0.005$ respectively). There was also a significant sex effect, with females committing significantly more errors overall than males ($p<0.05$). errors±sem (n=9 males and n=16 females)
- Figure 4. Local infusions of MEC into the VH had no effect on radial-arm maze performance in either males or females. errors±sem (n=9 males and n=16 females)
- Figure 5. Local infusions of SCOP (50 µg/side) into the MfC caused significant increases in working memory error in both male and female rats in the radial-arm maze compared to aCSF vehicle ($p<.05$). No effect was observed on reference memory

errors as a result of SCOP treatment. Females also committed significantly more errors overall in the task ($p < 0.001$). errors \pm sem (n=10 males and n=10 females)

Figure 6. Effects of local infusions of MEC into the MfC on radial-arm maze performance. At a dose of 20 μ g/side, infusions of MEC caused significant increases in working memory errors in both males and females, but had no effect on reference memory errors ($p < 0.05$). errors \pm sem (n=10 males and n=10 females)

Figure 1

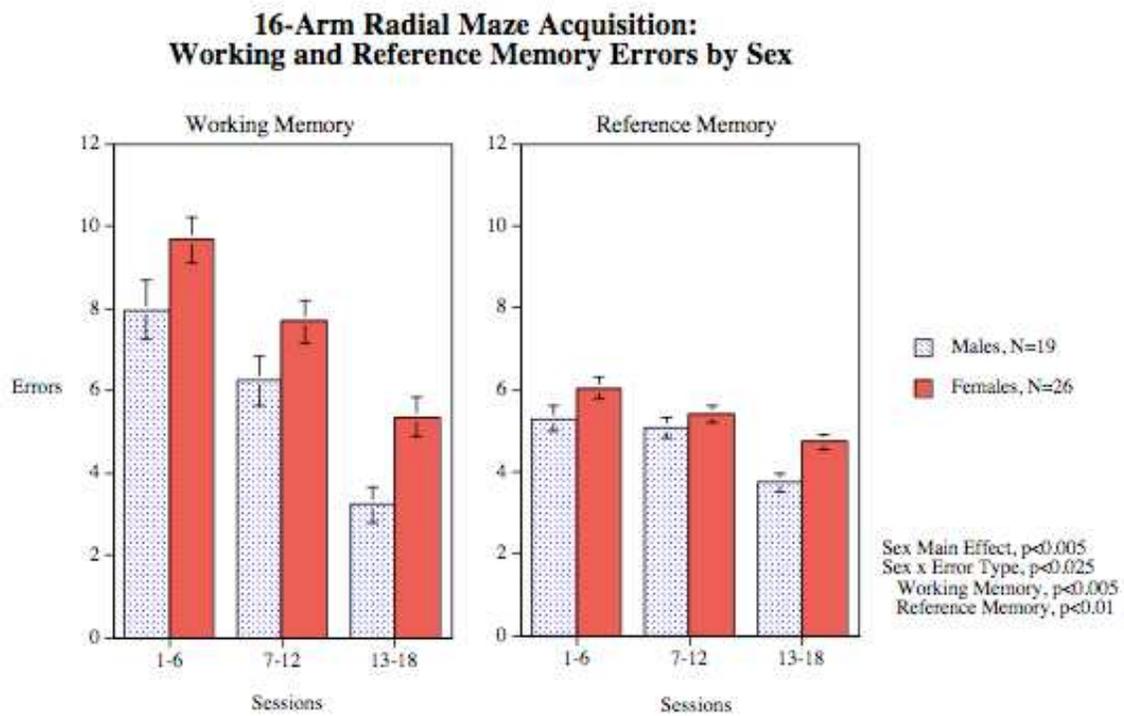


Figure 2

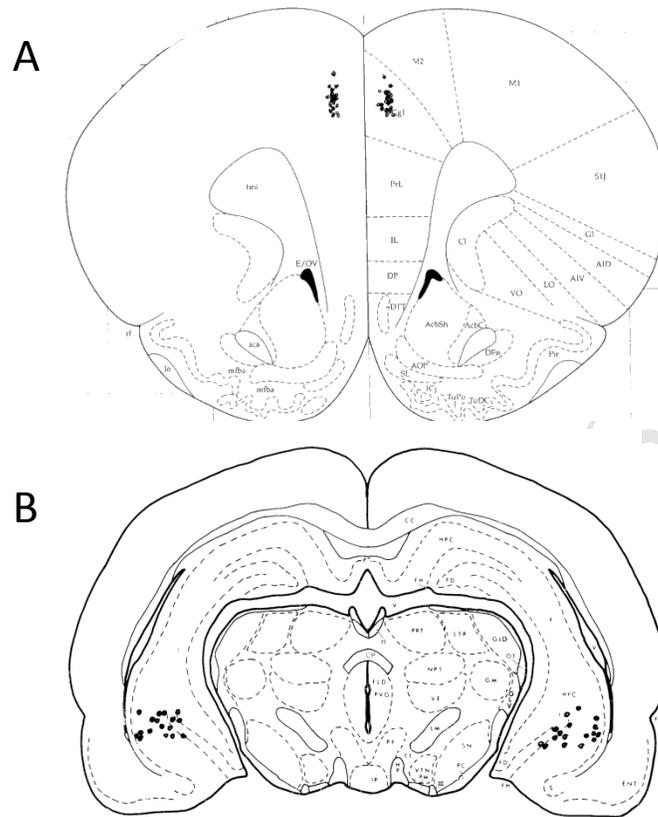


Figure 3

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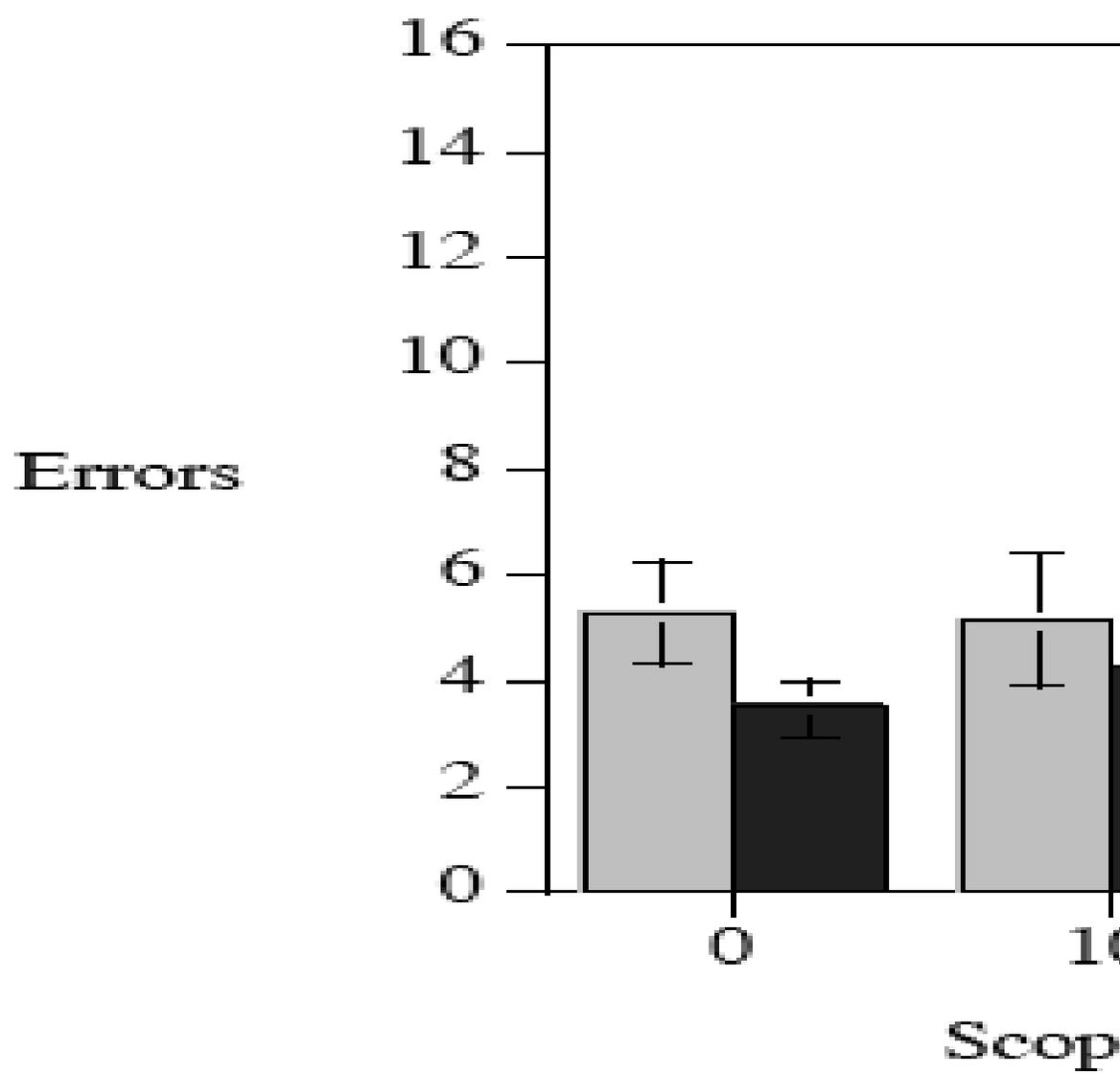


Figure 4

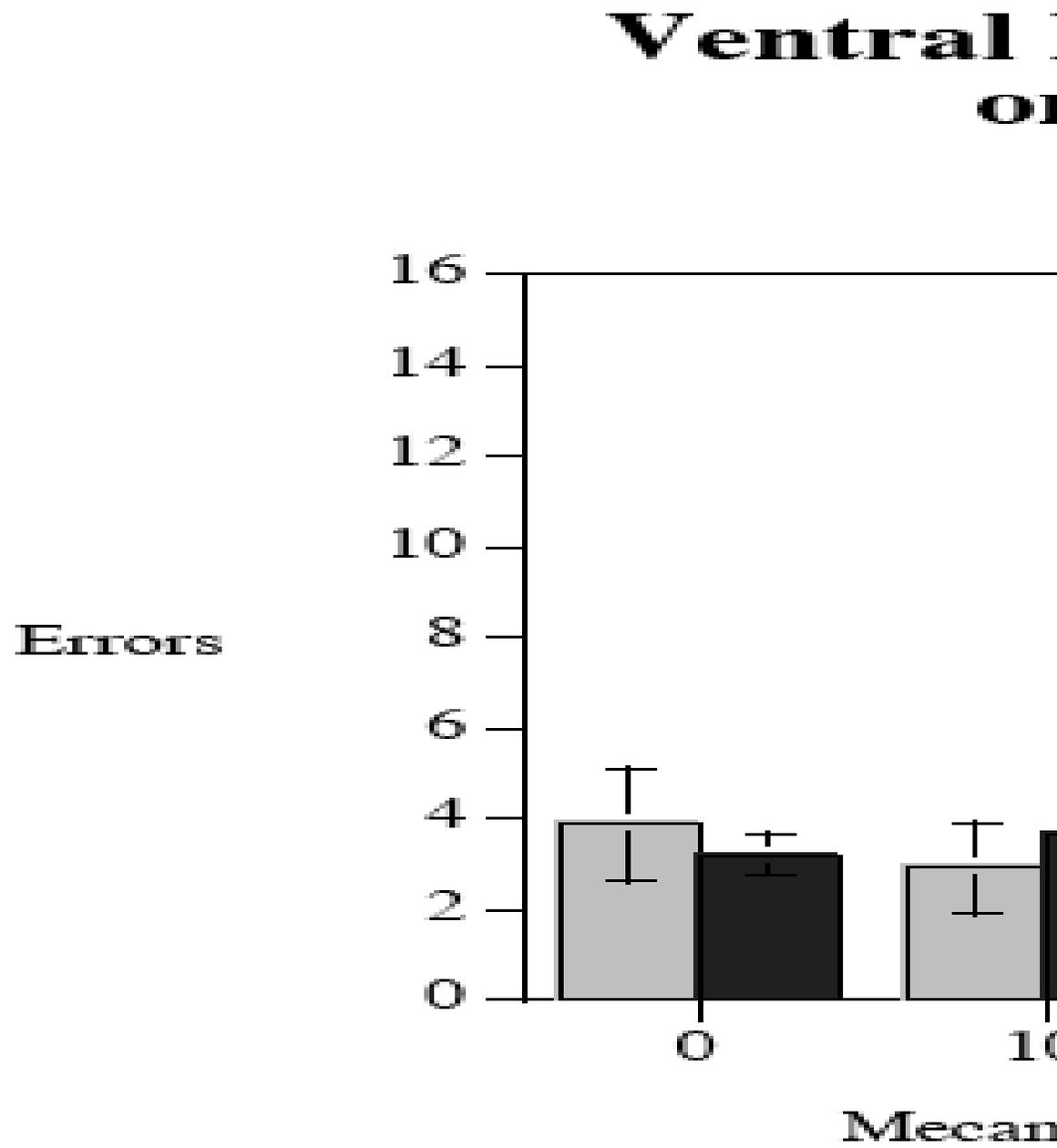


Figure 5

ACCEPTED MANUSCRIPT

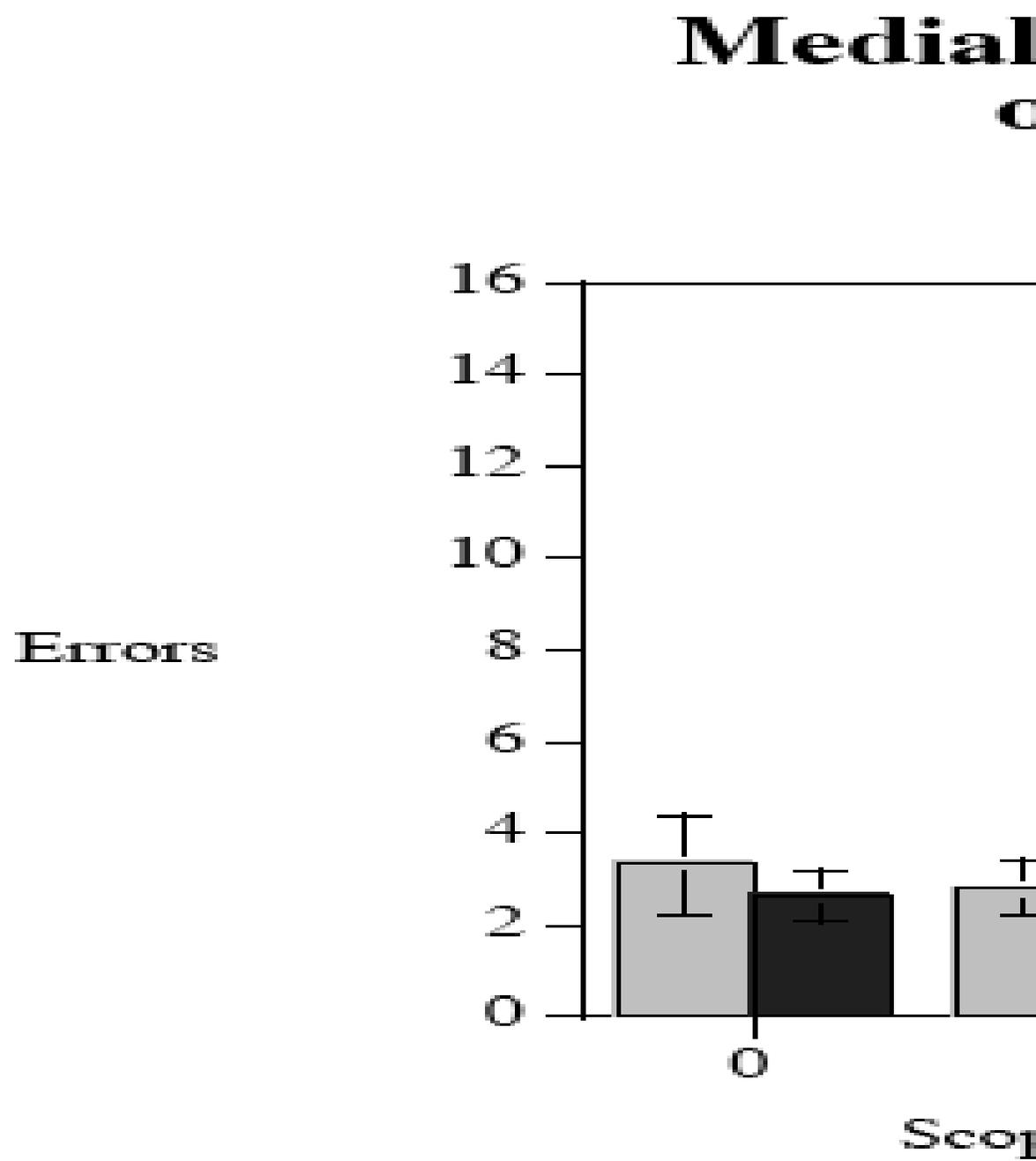
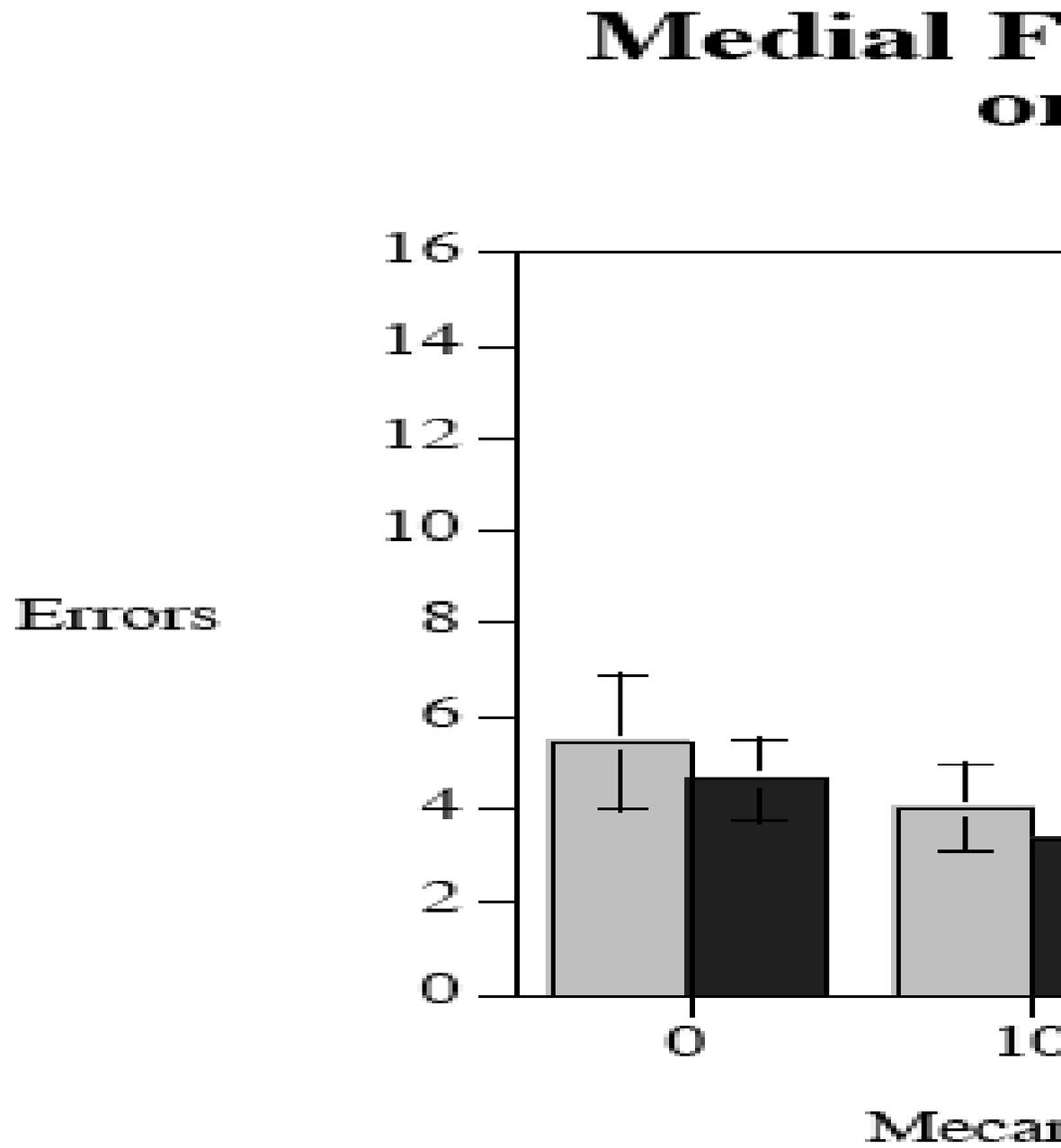


Figure 6



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Highlights

Ventral hippocampal scopolamine (SCOP) impaired memory in female but not male rats.

Ventral hippocampal mecamlamine (MEC) had no effect on memory in either sex.

Medial frontal cortical SCOP or MEC infusions impaired memory in both sexes.

Ventral hippocampal muscarinic receptors may be key in sex differences in memory.