MODULATION OF SPATIAL COGNITION IN ADULT RATS BY BIOLOGICAL SEX, GONADAL STEROIDS, AFFECTIVE CONDITIONS, AND CHOLINERGIC NEUROTRANSMISSION

AN ABSTRACT

SUBMITTED ON THE ELEVENTH DAY OF JANUARY, 2013

TO THE DEPARTMENT OF PSYCHOLOGY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF THE SCHOOL OF SCIENCE AND ENGINEERING

OF TULANE UNIVERSITY

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

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Abstract

In rodents, spatial learning and memory is contingent upon examining the relationships between cues in a three-dimensional environment. Although multiple brain structures are involved, the hippocampus serves as the epicenter of spatial information processing. Alternatively, on certain dual-solution navigational tasks, reliance on either a hippocampus-dependent *place* learning strategy or striatum-dependent *response* or *stimulus-response* learning strategy can be used to locate a goal. However, factors that dysregulate hippocampus function result in both poorer performance on spatial tasks and a shift toward adopting striatum-dependent learning strategies. Because gonadal hormones, affective states, and cholinergic neurotransmission modulate hippocampus function, the goal of the current study was to gain a greater understanding of how these factors impact spatial cognition and learning strategy preference. Relative to female rats, male rats performed better during the learning phase of a dual-solution learning task and exhibited a greater preference for a place learning strategy. Notably, in female rats, ovarian hormones modulated both spatial cognition and learning strategy preference, effects that likely involved activation of the putative estrogen membrane receptor GPR30. Alternatively, in male rats, spatial learning and memory, but not the preference for a place learning strategy, was attenuated by the removal of testicular hormones, an effect abrogated by testosterone treatment. Furthermore, for male rats, higher levels of trait anxiety and exposure to multiple reminders of a stressor were associated with a greater

reliance on striatum-based learning strategies. From a neurochemical standpoint, the preference for a striatum-based learning strategy in male rats was associated with lower levels of choline acetyltransferase in the hippocampus, which indicates that the cholinergic system is involved in learning strategy preference. However, antagonism of muscarinic receptors in the hippocampus caused a learning impairment in male rats on the same dual-solution learning task, which indicates that cholinergic neurotransmission in the hippocampus is necessary for learning a task in which the striatum also provides solution. Identifying factors such as stress exposure or gonadal hormones that alter the cholinergic integrity of the hippocampus is an important step in generating therapeutic strategies designed to treat individuals suffering from cognitive decline.

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Acknowledgments

I would like to especially thank my wife, Kelly, for her never ending support, and for her continued willingness to do whatever it takes to help me become the scientist that she believes I can be. I owe a great deal of my success in graduate school to her, and to the love and support of the rest of my family.

In addition to my family, I would like to express my deepest gratitude to my dissertation advisor Dr. Gary Dohanich for the knowledge he has shared with me and for guidance he has given me over the last several years. I am forever indebted to him for his commitment to all aspects of my graduate student training and professional development. I would also like to thank the members of my dissertation committee Dr. Jill Daniel, Dr. Laura Schrader, and Dr. Thomas Hebert, as well as Christine Faust-Witty, Dr. Chantelle Ferland, Dr. Nandini Vasudevan, and Dr. Dave Corey, who helped in the formulation, execution, and interpretation of numerous research projects, many of which have been presented in this manuscript. In addition, I would like to acknowledge the generous financial support provided for my research by the Department of Psychology and the Robert E. Flowerree Award. Lastly, it is important to note the impact that my friend and fellow graduate student Elin Grissom has made, and recognize that a great deal of my success can be attributed to my motivation to just keep up with her.

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Introduction

Spatial Learning and Memory

In both humans and rodents, distinct brain regions subserve learning and memory processes for qualitatively different types of information (Eichenbaum, 2002). Accordingly, in humans, procedural memory, which is modulated by the striatum, refers to the unconscious recollection of skills and habits. Alternatively, declarative memory, which is modulated by the hippocampus, refers to the conscious recollection of facts and experiences. Notably, the declarative memory system can be further subdivided into the semantic and the episodic memory systems. Semantic memory is concept-based, and refers to the conscious recollection of facts and meanings, which exist independent of experiences. The recollection of elements that comprise a given experience fall within the realm of the episodic memory system.

Mazes that require rodents to assess spatial relationships between three-dimensional extramaze objects in order to achieve a goal are often used to examine specific elements of episodic memory. Behavioral tasks designed to assess spatial memory in rodents have been characterized as either working or reference memory tasks (O'Keefe and Dostrovsky, 1971; Olton, 1977; Becker et al., 1980). Spatial working memory tasks assess knowledge for relational information that is relevant only for a particular sequence of the trials. On the other hand, spatial reference memory refers to knowledge for relational information that does not change from trial to trial. As would be

expected, impairments to spatial learning and memory follow from neurobiological manipulations that dysgregulate the function of the hippocampus. In the hippocampus, permanent lesions, temporary inactivation, or transection of subcortical inputs, result in learning and memory impairments on a variety of different hippocampus-dependent learning and memory tasks, including those that assess spatial learning and memory. Rodents with damage to the hippocampus exhibit deficits in contextual fear conditioning (Maren et al., 1997) and poorer spatial performance on reference and working memory versions of the water maze task (Schenk and Morris, 1985; Nilsson et al., 1987; Moser et al., 1995; Cassel et al., 1998; Steele and Morris, 1999), the radial-arm maze task (Olton et al., 1982; Meck et al., 1984; Cassel et al., 1998; Lee and Kesner, 2003a, b), and the continuous alternation task (Kim and Frank, 2009). Further, following damage to the hippocampus, performance also suffers on other types of spatial tasks, such as the Y-maze task (Conrad et al., 1996) and the object location task (Ennaceur et al., 1997), which capitalize on the natural preference of rodents to gravitate towards novelty and do not require food deprivation or water escape as motivating factors. Importantly, although impairments to the structure and function of the hippocampus compromise performance on spatial learning and memory tasks, performance on cognitive tasks that engage the striatum-dependent procedural memory system are typically spared (Packard et al., 1989; Jackson-Smith et al., 1993; McDonald and White, 1993; Compton, 2004; Lee et al., 2008).

Learning Strategy

A defining concept of modern cognitive theory focuses on *how* rather than *how much* information is learned (Tolman, 1948). In a three-dimensional spatial environment, mammals navigate toward a goal by executing distinct cognitive strategies, which are mediated by discrete regions of the brain (Packard, 1999; White and McDonald, 2002; Compton, 2004). A place strategy is mediated by the hippocampus and relies on the relationships between cues in the extra-maze environment and a given goal. Alternatively, both response and stimulus-response strategies are mediated by the striatum and rely on either proprioceptive cues that signal a specific sequence of body turns toward a goal (Packard and McGaugh, 1996), or by discrete cues proximal to the goal that signal its location (McDonald and White, 1994), respectively. Notably, the two brain systems process their respective types of information in parallel and, under certain conditions, interact to control the type of information learned (Knowlton and Squire, 1993; White and McDonald, 2002; Gold, 2004; Mizumori et al., 2004).

The interactive nature of the brain systems can be observed on a probe trial, which occurs after a series of training trials. Variations in training protocols aside, learning the location of the goal can be accomplished in some situations by adopting either a hippocampus-dependent or a striatum-dependent learning strategy. On a probe trial, rodents that adopt a hippocampus-dependent strategy return to the location in the maze where the goal was located during training, whereas those that adopt a response or stimulus-response strategy execute the same body turn used during training or navigate by attending to a single cue that marks the location of the goal during training, respectively. With some exceptions (Martel et al., 2007), adult male rats typically prefer a

place strategy early in the learning process (Packard and McGaugh, 1996; Packard, 1999). As training progresses, the hippocampus-dependent strategy gives way to the striatum-dependent strategy and a response learning strategy is adopted (Packard, 1999; McIntyre et al., 2003; Lex et al., 2011). However, lesions of the fimbria-fornix pathway (M'Harzi and Jarrard, 1992), neurotoxic lesions (McDonald and White, 1994) or temporary inactivation of the hippocampus (Packard and McGaugh, 1996) cause a striatum-dependent strategy to assume control over learning earlier than would be expected. Conversely, although a response strategy is typically expressed after extended training, temporary deactivation of the striatum allows a place strategy to be expressed at times when rats typically exhibit a preference for a response strategy (Packard and McGaugh, 1996).

Although the two brain systems interact cooperatively to control learning, additional research indicates that the two systems also work in a competitive fashion (Packard et al., 1989; McDonald and White, 1993; Compton, 2004; Lee et al., 2008). Dysregulation of hippocampus function by either transection of the fimbria-fornix pathway, neurotoxic lesions, or administration of sodium-channel blockers actually facilitates performance on learning strategy tasks that can only be solved by employing a striatum-dependent strategy (Packard et al., 1989; McDonald and White, 1993; Schroeder et al., 2002; Chang and Gold, 2003a; Compton, 2004; Lee et al., 2008). Moreover, some evidence indicates a bi-directional competition between the memory systems (Lee et al., 2008). Consistent with previous research, Lee and colleagues (2008) reported that performance on striatum-dependent tasks was facilitated by dysregulation of hippocampus function. Strikingly, in the same study, performance on a hippocampus-

dependent task improved when the integrity of the striatum was compromised (Lee et al., 2008). It is also important to note that although the systems work together, and at times compete, to coordinate learning, they also can operate independently of one another. Damage to the hippocampus or striatum impairs performance on tasks mediated by each system, respectively, while sparing performance on tasks that are mediated by the other brain system (Packard et al., 1989; Packard and McGaugh, 1992). Taken together, performance on spatial tasks and the preference for a place learning strategy are contingent upon optimal functioning of the hippocampus. Factors that modulate hippocampus function and spatial learning also correspond with changes in learning strategy preference.

Biological Sex and Spatial Cognition in Adult Rodents

In the dentate gyrus, adult male rats have a thicker and wider granule cell layer than females (Roof and Havens, 1992), a difference likely due to a greater density of granule cells in males (Madeira et al., 1991b), which is reflected in a greater number of mossy fibers extending from the granule cells within the dentate gyrus to area CA3 of Ammon's horn (Madeira et al., 1991a). In area CA3 and CA1, the volume, as well as the number and soma size of pyramidal neurons, are greater in males than females (Madeira et al., 1992; Isgor and Sengelaub, 1998). Conceivably, the complexity of synaptic connections within the hippocampus underlies the enhancement in synaptic plasticity in area CA1 of male rats relative to females (Maren et al., 1994; Yang et al., 2004). Therefore, differences in the size, morphology, and function of the hippocampus between adult male and female rats may be responsible for the differences in spatial memory

between the sexes, in which adult male rats typically outperform females on a variety of spatial tasks (Jonasson, 2005; Luine and Dohanich, 2008).

Relative to female rodents, males tend to make fewer errors on the radial-arm maze task (Luine and Rodriguez, 1994), swim shorter distances to locate a hidden platform in the water maze task (Markowska, 1999), and spend more time with an object in a novel location on an object location task (Beck and Luine, 2002; Bisagno et al., 2003). Arguably, the advantage exhibited by adult male rats on spatial learning and memory tasks is contingent upon the organizing effects of gondal hormones on the hippocampus during critical periods of brain development, which occur both prenatally (Isgor and Sengelaub, 1998, 2003) and neonatally (Dawson et al., 1975; Williams et al., 1990; Williams and Meck, 1991; Roof and Havens, 1992; Roof, 1993a; Mitsushima et al., 2008). As adults, the performance of neonatally-gonadectomized male rats on hippocampus-dependent tasks parallels the performance of female rats (Dawson et al., 1975), which is worse than that of male rats that are gonadally intact. Conversely, administration of gonadal hormones to female rats during the first few days of life facilitates hippocampus-dependent learning and memory (Williams and Meck, 1991; Roof and Havens, 1992; Roof, 1993b). Collectively, these studies indicate that the presence of gonadal hormones during critical periods of brain development, which occur both prior to and shortly after birth, contribute to the male spatial advantage that often expresses in adult rats.

Only a handful of studies to date have been conducted to determine whether the advantage exhibited by adult male rodents on hippocampus-based spatial tasks corresponds with a difference in learning strategy preference. The results from the few

studies to date suggest that the male advantage in spatial performance emerges from a greater reliance on a place learning strategy (Kanit et al., 1998; Kanit et al., 2000a; Kanit et al., 2000b). However, inconsistencies in results between studies, which have employed similar learning paradigms (Kanit et al., 1998; Lehmann et al., 1999; Kanit et al., 2000a; Kanit et al., 2000b), as well as ambiguous learning strategy classification techniques (Jonasson et al., 2004), make drawing definitive conclusions about the relationship between biological sex and learning strategy preference difficult at to this point.

Gonadal Hormones and Spatial Cognition in Adult Female Rodents

Although the powerful organizing effects of gonadal hormones contribute to the male advantage in spatial learning exhibited by adult rats, a growing body of research over the last three decades indicates that fluctuations in gonadal hormones during adulthood, and the corresponding effects on the hippocampus, also play a role (Dohanich, 2002). Much of the work done to date examining the effects of estradiol on spatial learning was inspired in large part by an elegant series of studies, which indicated that estradiol regulated the morphology of pyramidal cells in area CA1 of the hippocampus (Woolley and McEwen, 1992, 1994). The seminal study by Woolley and colleagues (Woolley et al., 1990) revealed that rats in the proestrous stage of the estrous cycle, when estradiol titers were highest, exhibited a 30 percent increase in dendritic spine densities relative to rats that were in estrus, when levels of estradiol were lowest. Follow-up studies conducted with ovariectomized rats revealed that the effect on spine morphology across the estrous cycle is mediated by estradiol (Woolley and McEwen, 1992). Subsequently, although there have been some inconsistencies across species with regard

to the effects of ovarian hormones on spatial reference learning and memory on water maze tasks (Berry et al., 1997; Warren and Juraska, 1997; Frick and Berger-Sweeney, 2001; Rubinow et al., 2004), rodents in proestrus typically exhibit better spatial memory on a variety of other tasks that assess working memory, such as the object location task (Frye et al., 2007; Paris and Frye, 2008), the radial-arm maze task (Pompili et al., 2010), and the Y-maze task (Conrad et al., 2004).

Paralleling the memory enhancement observed during proestrus, additional studies conducted with ovariectomized rodents indicate that rodents treated with estradiol also exhibit an enhancement in spatial learning and memory (Luine and Rodriguez, 1994; Fader et al., 1999; Gibbs, 1999; Daniel and Dohanich, 2001; Sandstrom and Williams, 2001; Markham et al., 2002; Sinopoli et al., 2006; Rodgers et al., 2010; Conrad et al., 2012; Inagaki et al., 2012; Kiss et al., 2012; Phan et al., 2012). Further, the spatial learning and memory enhancement in ovariectomized rodents following estradiol treatment involves activation of the classic intracellular estrogen receptors. For example, antagonism (Zurkovsky et al., 2006) or genetic deletion (Walf et al., 2008; Walf et al., 2009) of estrogen receptors disrupted spatial cognition in ovariectomized rodents treated with estradiol. Alternatively, delivery of a lentivirus that over expressed estrogen receptors in the hippocampus (Foster et al., 2008; Witty et al., 2012), or administration of selective estrogen receptor modulators (Rhodes and Frye, 2006; Frye et al., 2007; Walf et al., 2008; Hammond et al., 2009; Inagaki et al., 2010; Jacome et al., 2010; Phan et al., 2011), was sufficient to enhance spatial cognition in ovariectomized rodents. Notably, both the estrogen receptor alpha (ERα) and beta (ERβ) subtypes modulate intracellular regulators of synaptic plasticity in the hippocampus (Spencer-Segal et al., 2012).

However, ERβ may play a larger role in modulating spatial cognition than ERα (Rhodes and Frye, 2006; Liu et al., 2008; Jacome et al., 2010), although not all studies agree (Frye et al., 2007; Phan et al., 2011).

Importantly, enhancements in spatial learning and memory induced by activation of the classic estrogen receptors located in the cytoplasm of neurons within the hippocampus occur through relatively slow genomic processes that involve translocation from the cytoplasam to the nucleus, dimerization, and the subsequent activation of transcriptional processes (Nelson, 2005). However, in addition to the classic estrogen receptors, the memory enhancing effects of estradiol on spatial learning and memory also are modulated by activation of the putative estrogen membrane receptor GPR30, which is located, among other brain structures, in the hippocampus (Brailoiu et al., 2007; Hazell et al., 2009; Hammond et al., 2011). In ovariectomized rats, chronic administration of G-1, a specific agonist for the estrogen membrane receptor GPR30, mimicked the enhancing effects on spatial learning that occurred following chronic administration of either estradiol, or selective agonists for ERα or ERβ (Hammond et al., 2009). Alternatively, administration of G-15, which is a GPR30 antagonist, abrogated the enhancing effects of estradiol treatment in ovariectomized rats on spatial cognition (Hammond et al., 2012).

Through a non-genomic mechanism that involves increases in intracellular Ca^{2+} (Wu et al., 2005; Brailoiu et al., 2007; Wu et al., 2011) and activation of specific signaling kinases (Zadran et al., 2009; Sarkar et al., 2010; Wu et al., 2011), GPR30 is positioned to modulate the previously documented spatial memory enhancements, which occurred following administration of estradiol during the critical period of memory consolidation (Luine and Frankfurt, 2012). Accordingly, posttraining administration of

estradiol resulted in enhanced performance on both an object placement task (Luine et al., 2003; Frye et al., 2007) and a reference memory version of the water maze task (Packard et al., 1996; Packard and Teather, 1997a, b; Gresack and Frick, 2006), whereas delayed administration of estradiol beyond 2 h following training did not enhance memory. Taken together, the enhancement in cognition that occurs following estradiol treatment during the period of memory consolidation likely involves activation of the putative estrogen membrane receptor GPR30 in the hippocampus (Fernandez et al., 2008; Fan et al., 2010).

Alterations in the morphology and neurochemistry of the hippocampus, which occur across the estrous cycle, correspond with a shift in the way information is learned (Korol et al., 2004; McElroy and Korol, 2005; Tunur et al., 2010). On dual-solution navigational tasks, female rats in proestrus exhibit a preference for a place learning strategy, whereas those in estrus exhibit a preference for a response strategy (Korol et al., 2004). In addition to exhibiting a preference for a place learning strategy (Daniel and Lee, 2004; Quinlan et al., 2008), ovariectomized rats treated with estradiol also exhibit better performance on learning tasks that can only be solved by executing a place strategy (Korol and Kolo, 2002; Davis et al., 2005; Zurkovsky et al., 2006). Conversely, estradiol treatment actually impairs performance on navigational tasks that can only be solved by executing a striatum-dependent strategy (Galea et al., 2001; Korol and Kolo, 2002; Davis et al., 2005). Further, intrahippocampus, but not intrastriatum, administration of estradiol enhances place learning through the involvement of the classic estrogen receptors (Zurkovsky et al., 2006; Zurkovsky et al., 2007). Notably, within a few hours after administration directly into the hippocampus or the striatum, estradiol treatment has been shown to enhance spatial memory (Sinopoli et al., 2006) and impair response learning

(Zurkovsky et al., 2011), respectively. Considering that the striatum does not contain classic estrogen receptors (Shughrue et al., 1997; Shughrue and Merchenthaler, 2001), these results together suggest that the effects of estradiol on spatial cognition are possibly mediated by GPR30, which is located in both the hippocampus, as well as the striatum (Hammond et al., 2011).

Gonadal Hormones and Spatial Cognition in Adult Male Rodents

In addition to the role of gonadal hormones in females, the male advantage in spatial learning and memory in adulthood might also be due to the higher levels of testosterone in males relative to females. Similar to the effects of estradiol in female rodents, in males, a number of studies conducted both *in vitro* and *in vivo* have revealed that testosterone, or its 5-alphase reductase metabolite dihydrotestosterone, regulates a variety of morphological and neurochemical endpoints in the hippocampus that are involved in learning and memory. As estradiol does in female rats (Galea et al., 2006), in male rats, testosterone regulates neurogenesis (Spritzer and Galea, 2007; Benice and Raber, 2010; Spritzer et al., 2011a; Okamoto et al., 2012) and cell proliferation in the dentate gyrus (Wainwright et al., 2011), as well as the density of synaptic spines in area CA1 (Kovacs et al., 2003; Leranth et al., 2003) and the morphology of spines in areas CA1 and CA3 (Hatanaka et al., 2009; Li et al., 2012a). In the hippocampus, orchidectomy dysregulates and testosterone replacement restores, acetylcholine release (Mitsushima et al., 2008, 2009), as well as levels of choline acetyltransferase (Nakamura et al., 2002) and acetylcholinesterase (Leonard et al., 2010), the enzymes that synthesize and degrade acetylcholine, respectively. Further, within the hippocampus, androgens

have also been shown to modulate NMDA receptor binding (Kus et al., 1995; Romeo et al., 2005b), phosphorylation of extracellular-signal regulated kinases (Nguyen et al., 2005; Rossbach et al., 2007; Hatanaka et al., 2009; Carrier and Kabbaj, 2012), levels of cAMP response element binding protein (Nguyen et al., 2009), the expression of growth factors (Bimonte-Nelson et al., 2003; Li et al., 2012a), the duration of action potentials (Pouliot et al., 1996), and the induction of long-term potentiation (Sakata et al., 2000; Smith et al., 2002). As would be expected, many of these cellular responses are dependent upon activation of androgen receptors (Nguyen et al., 2005; Hatanaka et al., 2009; Okamoto et al., 2012).

Many of the earlier studies that examined the activational effects of testosterone on spatial learning and memory were largely inconclusive (Goudsmit et al., 1990; Smith et al., 1996). Alternatively, recent studies have begun to uncover a beneficial role for androgens on spatial learning and memory. The most consistent effects of testosterone and dihydrotestosterone on spatial cognition in orchidectomized rodents have been observed on working memory tasks, such as the radial-arm maze task (Harrell et al., 1990; Bimonte-Nelson et al., 2003; Daniel et al., 2003; Gibbs and Johnson, 2008; Spritzer et al., 2008; Hasegawa and Mochizuki, 2009; Spritzer et al., 2011b), the delayed matching-to-position T-maze task (Gibbs, 2005), the delay-dependent version of the water maze task (Sandstrom et al., 2006; Benice and Raber, 2009), and the novel object location task (McConnell et al., 2012). Additional findings suggest that testosterone, by acting at its cognate intracellular androgen receptor in the hippocampus, also impacts performance on spatial reference memory versions of the water maze task (Naghdi et al., 2001; Jones and Watson, 2005; Rizk et al., 2005; Edinger and Frye, 2007). Interestingly,

despite the limited effects of estradiol treatment in male rodents on many of the neurochemical endpoints in the hippocampus that are involved in memory (Leranth et al., 2003; Romeo et al., 2005a; Tsurugizawa et al., 2005; Spritzer and Galea, 2007; Hatanaka et al., 2009; Mitsushima et al., 2009), estradiol, also a metabolite of testosterone, is as effective as a spatial memory enhancer in male rats as testosterone or dihydrotestosterone (McConnell et al., 2012).

Unlike the effects of estradiol in female rodents (Conrad et al., 2004; Frye et al., 2007; Walf et al., 2008; Inagaki et al., 2010; Jacome et al., 2010; Phan et al., 2012), the effects of testosterone in males on spatial learning and memory tasks that take advantage of the preference of rodents to gravitate toward novelty have received very little attention (McConnell et al., 2012). Importantly, in male rats, gonadal hormones and activation of androgen receptors regulate appetite (Gentry and Wade, 1976), as well as anxiety (Frye and Seliga, 2001; Zuloaga et al., 2011) and stress reactivity (Handa et al., 1994; McCormick et al., 2002), non-mnemonic factors which may have influenced performance on spatial tasks motivated by either food reward (Spritzer et al., 2008; Spritzer et al., 2011b) or the stress associated with water escape (Khalil et al., 2005; Sandstrom et al., 2006). Therefore, it is important to examine the effects of testosterone on a Y-maze task, which is a spatial learning and memory task that capitalizes on the propensity of rodents to gravitate towards novel spatial environments, and is not contingent upon either the motivation to obtain food reward or the stress associated with water escape.

Further, because lower levels of estradiol in female rodents are associated with both poorer spatial learning and memory (Dohanich, 2002), as well as a bias toward striatum-based learning strategies (Korol et al., 2004), it is important to characterize the

role of testicular hormones in male rodents on learning strategy preference. Although the results of a study by Gibbs (2005) suggest that testosterone may not impact learning strategy preference in male rats, the matching-to-position T-maze task that was not specifically intended to differentiate learning strategy preference (Packard and McGaugh, 1996). Accordingly, subsequent studies that have employed the matching-to-position T-maze task to examine the effects of estradiol on spatial cognition in ovariectomized rats failed to differentiate learning strategy preference as a function of estradiol treatment (Gibbs, 2007). Therefore, it is still unclear whether the previously reported deficits in spatial cognition following removal of testicular hormones translate into a greater reliance on striatum-dependent learning strategies.

Affective Modulators of Spatial Cognition

Broadly defined, psychological stress occurs when the magnitude of threat to well-being that presents within a given situation outweighs the perceived resources to effectively cope with the situation (Lazarus and Folkman, 1984). Physiologically, exposure to a stressor results in activation of the hypothalamic-pituitary-adrenocortical axis (HPA) and the subsequent release of neurotransmitters and hormones that coordinate allostatic processes to cope with the stressor, which ultimately culminate in the reinstatement of a state of internal stability (McEwen, 2000). In response to stress, the paraventricular nucleus (PVN) of the hypothalamus releases corticotropin releasing hormone (CRH) into the portal vessels located at the base of the brain. CRH then acts at the anterior pituitary gland to stimulate the release of adrenocorticotropin hormone (ACTH), which travels peripherally to the adrenal gland, stimulating release of

glucocorticoids to prepare the organism to cope with the threat. To terminate the stress response and attenuate the subsequent release of high levels of glucocorticoids, glucocorticoids feedback onto mineralocorticoid and glucocorticoid receptors located in the PVN, the anterior pituitary, and the hippocampus. Importantly, prolonged exposure to stress or corticosterone induces a down-regulation of both mineralocorticoid and glucocorticoid receptors in the hippocampus, which consequently induces a prolongation of the stress response by reducing negative feedback (Jacobson and Sapolsky, 1991; de Kloet, 1992; Joels et al., 2003).

Following exposure to chronic stressors, and the concomitant increase in levels of corticosterone, the hippocampus undergoes significant changes, which are believed to underlie the corresponding deficits in spatial learning and memory (McEwen, 1999). Exposure to chronic stress induces retraction of the dendrites in the CA3 subfield (Vyas et al., 2002; McLaughlin et al., 2007), a reduction in synaptic plasticity in the CA1 subfield (Alfarez et al., 2003; Holderbach et al., 2007), and a decrease in both cell proliferation and survival in the dentate gyrus (Gould et al., 1997; Heine et al., 2004). As would be expected given the importance of the hippocampus in spatial cognition, chronic exposure to stressors impairs performance on spatial learning and memory tasks, and thereby limits *how much* information is learned (Conrad, 2010). In male rodents, chronic stress-induced impairments in spatial performance have been documented on the Y-maze task (Conrad et al., 1996; Conrad et al., 2003; Wright and Conrad, 2005; Wright et al., 2006; Wright and Conrad, 2008; Chen et al., 2010), novel object location tasks (Luine, 2002; Bowman et al., 2003), reference memory versions of the water maze task (Sandi et al., 2003; Wright and Conrad, 2008), and working memory versions of both the

appetitively motivated radial-arm maze task (Srikumar et al., 2006; Veena et al., 2009) and the water version of the radial-arm maze task (Hoffman et al., 2011; Hutchinson et al., 2012).

Similar to the effects of chronic stress, exposures to single episodes of acute stress also alter hippocampus function and correspondingly compromise spatial learning and memory (Cazakoff et al., 2010). Whether prior to the learning phase or prior to the testing phase, brief exposures to either immobilization stress (Conrad et al., 2004), predator stress (Diamond et al., 1999; Diamond et al., 2006), inescapable shock (de Quervain et al., 1998; Kim et al., 2001; Kim et al., 2005), or an elevated platform (Xiong et al., 2003; Yang et al., 2003; Wong et al., 2007; Howland and Cazakoff, 2010) reliably impair spatial cognition. Acute stress-induced disruptions in spatial cognition have been reported on novel object placement tasks (Howland and Cazakoff, 2010; Li et al., 2012b), a Y-maze task (Conrad et al., 2004), reference memory versions of the water maze task (de Quervain et al., 1998; Kim et al., 2001; Xiong et al., 2003; Yang et al., 2003; Kim et al., 2005; Wong et al., 2007), and working memory versions of both the appetitively motivated radial-arm maze task (Diamond et al., 1996) and the water version of the radial-arm maze task (Woodson et al., 2003; Sandi et al., 2005; Diamond et al., 2006; Park et al., 2008). In addition to higher levels of corticosterone (de Quervain et al., 1998; Woodson et al., 2003; Li et al., 2012b), disruptions in spatial cognition following acute stress have been directly linked to a variety of changes in the hippocampus, such as alterations in dendrite morphology (Diamond et al., 2006), NMDA receptor subtypes (Wong et al., 2007; Howland and Cazakoff, 2010), the expression of specific kinases and

growth factors (Sandi et al., 2005; Conboy et al., 2009), and synaptic plasticity (Diamond and Rose, 1994; Kim et al., 2005; Wong et al., 2007).

Although exposure to chronic and acute stress powerfully impact spatial learning and memory, disruptions in both spatial cognition and affective outcomes also arise from changes in emotionality that are arguably more subtle in nature (Korte et al., 1999; Zoladz et al., 2010). In animal models of posttraumatic stress disorder (PTSD), exposure to reminders of an aversive event, which are designed to simulate the intrusive thoughts that characterize PTSD (Association, 2000), elevate plasma corticosterone (Hagewoud et al., 2011), alter synaptic plasticity (Li et al., 2005), heighten anxiety (Korte et al., 1999; Louvart et al., 2005; Siegmund and Wotjak, 2007; Hawley et al., 2011a; Hawley et al., 2012b) and disrupt retrieval of a previously formed spatial memory (Zoladz et al., 2010). Behaviorally, rodents recently exposed to a reminder of an aversive event spend less time on the open arms of an elevated plus-maze (Korte et al., 1999), which is an indicator of elevated anxiety, and commit more errors on a retrieval trial of a spatial working memory task (Zoladz et al., 2010). Interestingly, aberrant manifestations in affective behavior (Maier, 2001; Louvart et al., 2005) and alterations in spatial cognition (Zoladz et al., 2010) can be maintained indefinitely by repeated exposures to reminders of a stressor.

Independent of stressor manipulation, performance on learning and memory tasks also is modulated by subtle individual differences in trait anxiety (Herrero et al., 2006; Muigg et al., 2008). Unlike state anxiety, which is defined as a transient increase in emotionality characterized by increased tension and apprehension, trait anxiety is considered a relatively stable personality characteristic that refers to the propensity to experience higher levels of emotionality (Spielberger, 1972). With regard to spatial

cognition, rats categorized as high in trait anxiety exhibited poorer performance on a reference memory version of the water maze task than rats with lower levels of trait anxiety (Herrero et al., 2006). Moreover, rats categorized as high in trait anxiety are especially susceptible to the disruptive effects of stress, such that exposure to chronic stress selectively impaired spatial cognition in rats that exhibited higher levels of anxiety well before exposure to the stressors (Bellani et al., 2006). Therefore, although spatial cognition is impacted by a variety of adverse experiences that activate the stress response and alter hippocampus function, certain affective phenotypes may be particularly vulnerable to disruptions in spatial cognition (Mueller et al., 2009).

Notably, there is an intimate link between emotionality, spatial learning and learning strategy (Packard, 2009). In accordance with the notion that impairments to the hippocampus function shift control over learning to the striatum (McDonald and White, 1994; Packard and McGaugh, 1996), heightened levels of stress and anxiety, which impair spatial learning and memory, shift *how* information is learned (Packard, 2009). Specifically, exposures to stressors or administration of pharmacological agents that heighten anxiety-like behavior, impair performance on hippocampus-dependent learning tasks (Schwabe et al., 2008; Wingard and Packard, 2008; Sadowski et al., 2009), facilitate performance on striatum-dependent tasks (Wingard and Packard, 2008; Sadowski et al., 2009), and ultimately bias rodents toward a striatum-dependent learning strategy (Elliott and Packard, 2008; Schwabe et al., 2008; Ferragud et al., 2010). As would be expected given the role of the amygdala in modulating emotionality (Killcross et al., 1997; LeDoux, 2003), administration of an anxiogenic agent directly into the amygdala impairs place learning, facilitates response learning (Wingard and Packard,

2008), and bias rats toward a response learning strategy (Packard and Wingard, 2004). Furthermore, inactivation of the amygdala with a sodium channel blocker attenuates the impairing and enhancing effects of anxiety on hippocampus-dependent and striatum-dependent place and response learning, respectively (Packard and Gabriele, 2009). Through the actions of corticosterone, and the modulatory role of the amygdala, stress and anxiety compromise performance on hippocampus-dependent tasks and shift control over learning toward the striatum-based memory system. However, the effects of reactivation of an aversive memory, or differences in trait anxiety, on these processes still remain to be determined.

Cholinergic Modulation of Spatial Cognition

The cholinergic system of the hippocampus is dynamic in that it regulates a variety of cognitive processes (Pepeu and Giovannini, 2004), which include spatial learning and memory (Deiana et al., 2010) and the balance between place and response learning strategies (Chang and Gold, 2003b; McIntyre et al., 2003). To date, there have been numerous studies conducted examining the relationship between cholinergic function and spatial cognition (Everitt and Robbins, 1997; Deiana et al., 2010; Klinkenberg and Blokland, 2010). In the hippocampus, better performance on spatial learning tasks is associated with a greater number of muscarinic receptors (Van der Zee et al., 1995), higher levels of acetylcholine release (Fadda et al., 1996; Ragozzino et al., 1996; Ragozzino et al., 1998; Stancampiano et al., 1999; Darnaudery et al., 2000; Roland and Savage, 2007; Vetreno et al., 2008; Anzalone et al., 2009), and an increase in choline acetyltransferase (ChAT) protein expression (Wang et al., 2009a; Rodgers et al., 2010)

and activity (Ingram et al., 1981; Dunbar et al., 1993; Tarricone et al., 1993). Consonant with the notion that acetylcholine sets the dynamics of memory encoding and memory retrieval (Hasselmo, 2006), levels of high-affinity choline uptake (HACU), the rate limiting step in acetylcholine synthesis (Sarter and Parikh, 2005), were elevated immediately following spatial training (Marighetto et al., 1993), whereas HACU was decreased 15 minutes following training (Decker et al., 1988; Marighetto et al., 1993). Interestingly, both levels of acetylcholine release (Fadda et al., 2000), and ChAT activity (Ingram et al., 1981; Tarricone et al., 1993) in the hippocampus have been shown to positively predict spatial performance, which suggests that the integrity or density of cholinergic fibers can also serve as an indicator of spatial cognition.

With regard to muscarinic receptors, earlier evidence indicates that the use of place learning strategies is attenuated following systemic administration of either atropine or scopolamine, which prevents acetylcholine from acting at muscarinic receptors (Sutherland et al., 1982; Whishaw, 1985; Cassel and Kelche, 1989; Day and Schallert, 1996; Cain et al., 2000). Interestingly, under certain conditions, the place learning deficit in rats treated systemically with a muscarinic receptor antagonist does not correspond with impaired striatum-dependent cued or egocentric learning (Whishaw, 1985; Paylor and Rudy, 1990). Likewise, systemic administration of a low dose of scopolamine (0.2 mg/kg) impaired spatial memory, but not the memory for a discrete motor response that was required to solve the spatial task (von Linstow Roloff et al., 2007). Further, on tasks that can be solved by using either strategy, rodents revert from using a place navigational strategy to a response strategy following systemic administration of scopolamine (McFarland, 1989). Notably, in humans, administration of scopolamine shifts neural

activity from the hippocampus to the striatum during learning of a virtual version of the Morris water maze (Antonova et al., 2011). Unfortunately, up to this point, the systemic route by which muscarinic receptor antagonists have been administered somewhat limits interpretation of these results. However, it is important to note that place learning is particularly vulnerable to the effects of cholinergic dysfunction and that such impairments are associated with shifts toward using the striatum-based memory system.

Results from studies using central administration of muscarinic receptor antagonists to examine the relationship between spatial cognition and cholinergic function in the hippocampus have important implications for understanding how the hippocampus-based and striatum-based memory systems operate in relation to one another (Packard and McGaugh, 1992). Accordingly, intrahippocampus infusion of scopolamine impairs spatial performance on reference memory versions of the water maze task (Carli et al., 1995, 1997; Riekkinen and Riekkinen, 1997; Carli et al., 1998; Carli et al., 1999; Herrera-Morales et al., 2007), the Hebb-Williams maze task (Rogers and Kesner, 2003; Hunsaker et al., 2007), and working memory versions of both the radial-arm maze task (Kim and Levin, 1996; Mishima et al., 2000; Mikami et al., 2007; Xu et al., 2009) and the T-maze task (Fader et al., 1998; Gibbs, 1999). Poorer spatial learning and memory also result from intrahippocampus administration of the selective muscarinic receptor antagonist pirenzepine, which directly implicates the involvement of the postsynaptic M1 receptor subtype in spatial learning and memory processes (Herrera-Morales et al., 2007). Importantly, although intrahippocampus administration of scopolamine impairs spatial learning, performance on non-spatial tasks is typically spared (Brito et al., 1983; Carli et al., 1997).

Using *in vivo* microdialysis to measure levels of acetylcholine in the hippocampus and the striatum, an elegant series of studies by Gold and colleagues have identified a role for the neurotransmitter in regulating place and response learning strategies. In accordance with the idea that the hippocampus and the striatum compete for control over learning, higher levels of acetylcholine release in the hippocampus both prior to and during training trials were indicative of an innate preference for a place strategy on the probe trial of a dual-solution learning strategy task (McIntyre et al., 2003). In a follow-up study, higher levels of acetylcholine release in the hippocampus essentially maintained the preference for a place learning strategy even after extensive training (Chang and Gold, 2003b), which is a time point that typically corresponds with a preference for a response strategy (Packard, 1999). Furthermore, optimal performance on tasks that can be solved by employing either memory system corresponds with both a shift toward adopting response learning strategies and an increase in the amount of acetylcholine released in the striatum (Pych et al., 2005a). Likewise, on striatum-dependent tasks, the ratio of acetylcholine release between the hippocampus and striatum changes in accordance with performance. Specifically, levels of acetylcholine initially increase in the hippocampus at the onset of training, when performance is poor, and slowly begin to increase in the striatum, as performance begins to improve (Pych et al., 2005b). Taken together, levels of acetylcholine release in the hippocampus and striatum correspond with the relative engagement of each memory system.

In addition to the results from microdialysis studies, control over learning has also been shown to shift toward the striatum-dependent memory system following elimination of the primary source of acetylcholine to the hippocampus (Janis et al., 1998).

Impairments to place learning following medial septum administration of the saporin neurotoxin, which destroys cholinergic cell bodies, corresponded with a preference for a stimulus-response learning strategy. However, more recent studies that have investigated the relationship between acetylcholine and learning strategy using similar lesioning techniques have yielded incongruent results (Cahill and Baxter, 2001; Bizon et al., 2003; Jonasson et al., 2004; Fitz et al., 2008). Differences in behavioral protocols designed to assess learning strategy and subtle variations in lesioning techniques may underlie the discrepant findings. In addition, although levels of acetylcholinesterase and choline acetyltransferase were virtually eliminated in both the basal forebrain and the hippocampus, selective lesions of the basal forebrain with the saporin toxin tended to increase the density of M1 receptors in the hippocampus (Levey et al., 1995). Conceivably, on some learning tasks, the upregulation of post synaptic receptors muscarinic receptors may be sufficient to compensate for the loss of cholinergic input following such lesions. Furthermore, on other hippocampus-dependent tasks, such as contextual fear conditioning tasks, selective cholinergic lesions of the basal forebrain (Frick et al., 2004) do not mimic the memory impairing effects of intrahippocampus scopolamine (Gale et al., 2001; Rogers and Kesner, 2004). Taken together, lesions of the cholinergic cell bodies in the basal forebrain, which are designed to induce cholinergic hypofunction, may not entirely explain the relationship between acetylcholine in the hippocampus and learning strategy. Administration of cholinergic antagonists directly into the hippocampus, which alter the relationship between acetylcholine activity in the hippocampus and striatum, would overcome compensatory mechanisms in the

hippocampus that occur following lesions to the cholinergic cell bodies in the medial septum (Levey et al., 1995).

Modulators of Cholinergic Neurotransmission in the Hippocampus

A variety of factors that impact spatial cognition also impact cholinergic neurotransmission in the hippocampus. In accordance with the previously documented male spatial advantage, adult male rats release higher levels of acetylcholine in the dorsal hippocampus than adult females (Masuda et al., 2005). Furthermore, both acetylcholine release in the hippocampus (Mitsushima et al., 2009), as well as mRNA and protein levels of ChAT in both the hippocampus (Nakamura et al., 2002; Bohacek et al., 2008; Rodgers et al., 2010) and medial septum (Gibbs, 1996, 1997; Nakamura et al., 2002), are elevated in gonadectomized male and female rats treated with either testosterone or estradiol, respectfully. Consistent with the relationship between acetylcholine release and learning strategy preference, estradiol treatment of ovariectomized rats enhances the release of acetylcholine within the hippocampus during learning on a dual-solution learning strategy task (Marriott and Korol, 2003). Notably, in ovariectomized rats, the enhancement in acetylcholine release in the hippocampus following estradiol treatment is mimicked by administration of G-1, which indicates that activation of the estrogen membrane receptor GPR30 is sufficient to enhance acetylcholine release in the hippocampus (Hammond et al., 2011). Conceivably, the male spatial advantage and the effects of gonadal hormones on spatial recognition memory and learning strategy preference are mediated by cholinergic neurotransmission within the hippocampus.

In addition to gonadal hormones, exposures to stressors also exert powerful effects on cholinergic neurotransmission in the hippocampus in rodents. Much like the effects of repeated exposure to stressors on cholinergic endpoints in the hippocampus (Gonzalez and Pazos, 1992; Sunanda et al., 2000), exposure to a single traumatic event also alters levels of acetylcholine (Mark et al., 1996; Mitsushima et al., 2008), the density of muscarinic receptors (Brand et al., 2008), ChAT activity (Wahba and Soliman, 1992), and the expression of genes that regulate cholinergic activity (Kaufer et al., 1998). Interestingly, exposure to the context in which a footshock was previously administered produces a robust increase in acetylcholine release within hippocampus that is comparable to the amount released upon exposure to the initial stressor (Nail-Boucherie et al., 2000; Calandreau et al., 2006). Although higher levels of acetylcholine in the hippocampus before and during training are indicative of enhanced spatial cognition (Ragozzino et al., 1996) and a bias toward a place learning strategy (McIntyre et al., 2003), enhancing hippocampus cholinergic neurotransmission just prior to the retrieval trial of a spatial task impairs retrieval of a recently formed memory (Rogers and Kesner, 2003). It is tempting to consider that elevated levels of acetylcholine in the hippocampus may have contributed to the inability to retrieve a recently-formed spatial memory, which was observed following exposure to a reminder of an inescapable footshock (Zoladz et al., 2010). Taken together, alterations in the cholinergic system of the hippocampus may underlie the effects of stress exposure and emotionality on spatial recognition memory and learning strategy preference.

As illustrated in Figure 1, cholinergic neurotransmission in the hippocampus is influence by biological sex, gonadal hormones, and stress exposure. With this in mind,

the male spatial advantage, as well as the effects of gonadal hormones and emotionality on spatial recognition memory and learning strategy preference, may be mediated by alterations in cholinergic neurotransmission in the hippocampus. Accordingly, the objective of the following set of experiments was to gain a greater understanding of the neorobiological factors that dictate spatial cognition in adult rats.

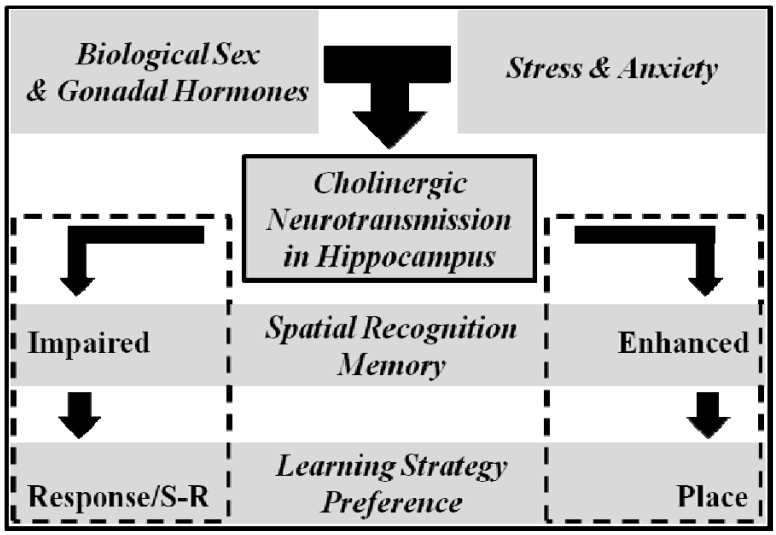

Figure 1. Model depicting modulators of cholinergic function in the hippocampus, which in turn impact spatial recognition memory and learning strategy preference. S-R = stimulus-response learning strategy.

General Methods

General procedures

All procedures were approved by the Tulane University Institutional Animal Care and Use Committee in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* (1996). Long-Evans rats were used in all experiments and were provided free access to food (Teklad Inc., Madison, WI) and water in animal care facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Rats were acclimated to the vivarium for approximately 1 week upon arrival to the facility and to procedural and behavioral testing rooms for at least 30 min before procedures or behavioral testing began.

With the exception of Experiments 4 and 5, all procedures were conducted during the light phase of the reversed 12:12 h light-dark cycle (lights on at 07:00 h). All procedures were conducted during the dark phase of the 12:12 light-dark cycle (lights off at 11:00 h) in Experiments 4 and 5.

Experiment 1

Primary aim: *To determine the effects of biological sex and ovarian hormones on learning strategy preference in adult rats*. On dual-solution navigational tasks, the preference for a hippocampus-dependent learning strategy exhibited by male rodents is contingent upon optimal functioning of the hippocampus (McDonald and White, 1994; Packard and McGaugh, 1996). Male rodents typically outperform females on a variety of spatial learning and memory tasks, which rely on the hippocampus (Luine and Dohanich, 2008). Interestingly, both spatial learning and memory and the preference for a place learning strategy in females are influence by levels of ovarian hormones, such that during the proestrous stage of the estrous cycle, when titers of ovarian hormones are elevated, spatial cognition is improved (Frye et al., 2007) and a place learning strategy is preferred (Korol et al., 2004). While examining the role of ovarian hormones in female rats, we expected the previously reported male spatial advantage to manifest as a greater reliance on a place learning strategy in Experiment 1. ***Summary of findings:*** *On the dual-solution water T-maze task in Experiment 1, male rats exhibited enhanced learning relative to females during training and subsequently exhibited a preference for a place learning strategy on the probe trial. Stage of the estrous cycle did not impact learning or learning strategy preference in female rats.*

Animals and estrous cycle monitoring

Animals. Male and female rats were obtained from litters (*N* =7) bred in the vivarium of Tulane University. Litters consisted of 8-10 rats that were weaned from their dam at 21 days of age and housed in groups of 2-3 by sex. However, only 2-4 rats from each litter were used in the current experiment.

Estrous cycle monitoring. Beginning at 52 days of age, vaginal lavage was conducted prior to behavioral testing between 07:00-09:00 h in order to establish regular estrous cyclity and to examine the effects of stage of the estrous cycle on learning strategy (Korol et al., 2004; McElroy and Korol, 2005; Pleil and Williams, 2010). Lavage samples were transferred to slides and stained with toluidine blue for microscopic examination to confirm at least 2 regular cycles of 4 or 5 days (Menard and Dohanich, 1989). Proestrus was indicated by a prevalence of clustered nucleated epithelial cells that was greater than the proportion of cornified epithelial cells. Estrus was indicated by the presence of cornified epithelial cells in the absence of nucleated epithelial cells. One rat was excluded from the study because of irregular cycling. Male rats in both experiments were handled for 1 min each day, 7-10 days prior to behavioral testing, to control for the effects of handling during estrous cycle tracking in female rats. At approximately 65 days of age, female rats that were in either estrus (n = 9) or proestrus (n = 9), as well as their male counterparts (n = 22), were tested on a dual-solution water-T maze task.

Behavioral testing procedures

Dual-solution water T-maze task. Learning strategy was assessed using a free-standing version of a dual-solution water T-maze, which is illustrated in Figure 2. The maze was constructed of clear Plexiglas that formed three arms of equal size (60 length x 30 width x 45 height cm), which were filled to a level of 20 cm with water (25° C) made opaque by white non-toxic tempura paint. A circular escape platform (11 cm in diameter), submerged 2 cm below the surface of the water, was located in either the east or west arm of the maze across all training trials and the probe trial. The platform position was counterbalanced across conditions for both experiments. The maze was located in the center of a room that consisted of an array of three-dimensional spatial cues, none of which were proximal to the goal arms. Additionally, after placing the rat into the maze, experimenters immediately stepped behind one of two black curtains, affixed with additional cues, which hung behind the north and south start arm positions.

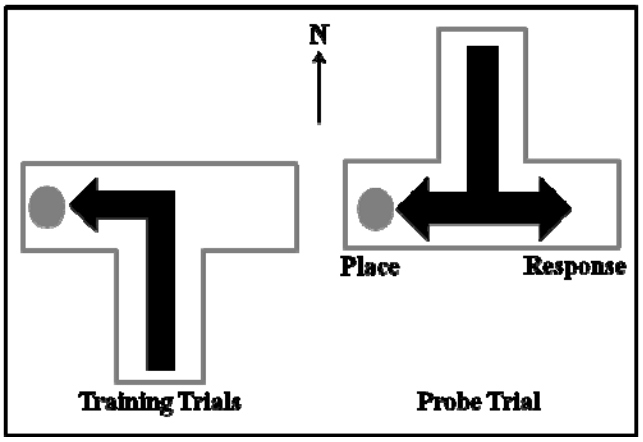

Figure 2. Training and probe trials of a dual-solution version of the water T-maze task, which categorizes learning strategy preference.

During training, rats were placed in the arm of the maze located in the south position and allowed 60 s to escape to the platform. Once the platform was mounted, rats were left undisturbed for an additional 15 s. Rats that failed to locate the platform within

60 s were guided to the platform by an experimenter. Eight training trials were conducted consecutively and separated by an inter-trial interval of 60 s. Following training, the maze was rotated 180 degrees and the platform was repositioned to the designated goal arm during training. Rats were then placed into the maze from the arm in the north position and a probe trial was conducted. During training trials, performance was assessed by recording the first full body entry into either the arm that contained the platform or the opposite arm. Learning was indicated by the percentage of initial entries into the arm that contained the platform as training progressed (Packard and Wingard, 2004; Elliott and Packard, 2008). Arm choice accuracy during training, which was not confounded by chance performance on the first trial, was indicated by a greater percentage of correct initial arm choices summed across trials 2-8. On the probe trial, rats that returned to the arm that contained the escape platform during training were identified as place learners. Alternatively, on the probe trial, rats that executed the same body turn required to successfully locate the platform on training trials initially entered the opposite goal arm on the probe trial, and were identified response learners.

Statistical analyses

Analyses of variance (ANOVA) with between-subjects effects of stage of the estrous cycle (proestrus, estrus) or sex and within-subjects effect of trial block (1-4) were conducted to examine learning during training on the dual-solution water T-maze task. Independent samples *t*-tests were conducted to examine the effects of stage of the estrous cycle and sex on performance collapsed across trials 2-8, which was independent of chance performance on the first trial. Statistical significance was indicated by $p < 0.05$.

Behavioral testing results

Dual-solution water T-maze task. There was an effect of trial [$F(3,48) = 6.50$, $p < 0.01$], but no effect of estrous stage [$F(1,16) = 0.00$, $p = 1.00$], or interactive effect [$F(3,48) = 0.26$, $p = 0.85$], on learning across training trials (Figure 3A). As training progressed, correct first arm choices increased independent of stage of the estrous cycle. The results depicted in Figure 3B indicate that stage of the estrous cycle did not impact performance collapsed across trials 2-8 [$t(16) = \pm 0.38$, $p = 0.71$], which was independent of chance performance on the first trial. The results shown in Figure 3C indicate that stage of the estrous cycle did not impact learning strategy as indicated on the probe trial [$\chi^2(1) = 0.90$, $p = 0.34$]. Neither rats in proestrus [$\chi^2(1) = 0.11$, $p = 0.74$], nor those in estrus [$\chi^2(1) = 1.00$, $p = 0.32$], exhibited a learning strategy bias.

Females were compared to males independent of the stage of the estrous cycle. There was a effect of trial [$F(3,114) = 21.18$, $p < 0.001$], and sex [$F(1,38) = 4.90$, $p < 0.05$], but no interactive effect [$F(3,114) = 1.76$, $p = 0.16$], on learning during training trials (Figure 3A). Although both male and female rats exhibited an increase in correct first arm as training progressed, males outperformed females. Moreover, the results depicted in Figure 3B indicate that female rats made more incorrect initial arm choices than males when performance was collapsed across trials 2-8 [$t(38) = \pm 2.65$, $p < 0.05$]. Figure 3C illustrates the results of the probe trial in which the preferred learning strategy of male and female rats tended to differ [$\chi^2(1) = 3.30$, $p = 0.07$]. Accordingly, male rats exhibited a preference for a place strategy [$\chi^2(1) = 4.55$, $p < 0.05$] and female rats did not exhibit a strategy preference [$\chi^2(1) = 0.22$, $p = 0.64$].

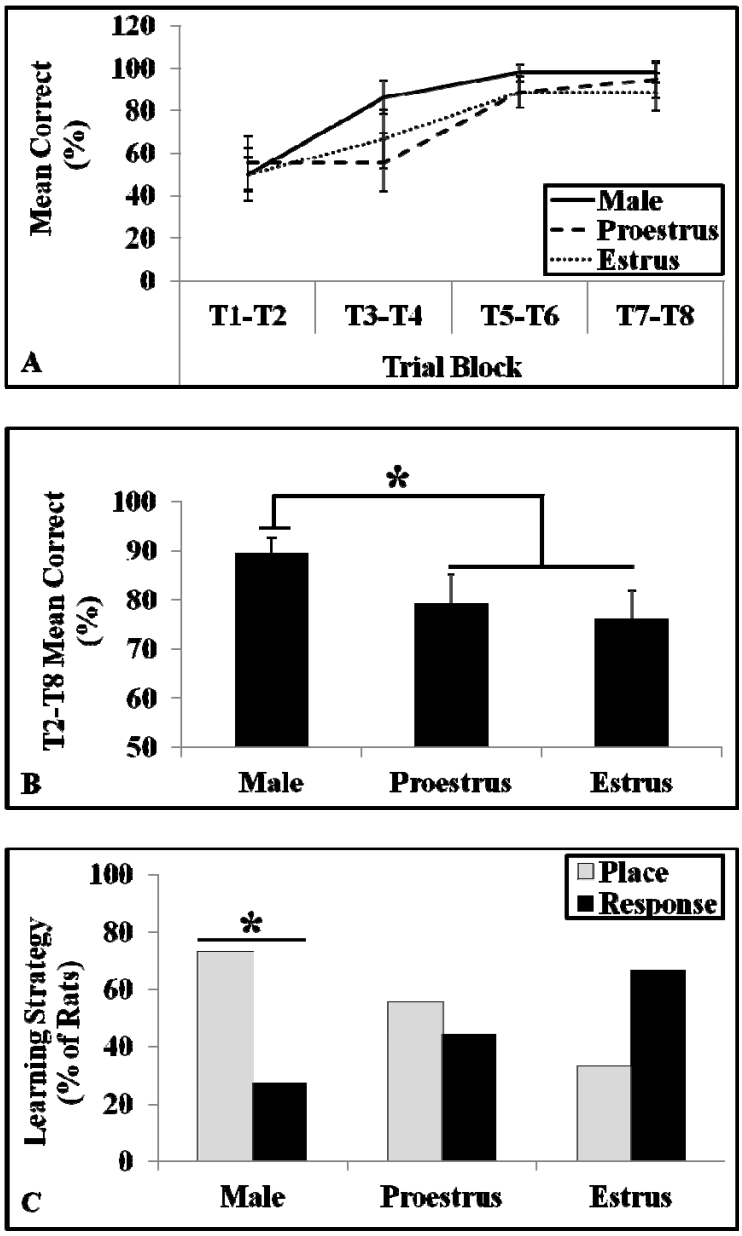

Figure 3. (**A**) Results from the training trials of a dual-solution water T-maze task in which learning was reflected as a greater percentage of correct first arm choices as training progressed. (**B**) Correct first arm choices collapsed across training trials 2-8, which was not impacted by chance performance on the first trial (*$p < 0.05$, male compared to female rats independent of stage of estrous cycle). (**C**) Results from the probe trial of a dual-solution water T-maze task designed to categorize learning strategy preference, which was indicated by the initial arm entry (*$p <$ 0.05, male rats that preferred a place strategy compared to a response strategy). Adapted from (Hawley et al., 2012a)

Experiment 2

Primary aim: *To determine the effects of orchidectomy on learning strategy preference in adult rats.* Gonadal hormones modulate spatial cognition in both sexes (Dohanich, 2002). With some exceptions (Martel et al., 2007), male rats typically prefer a place learning strategy early in the learning process (Packard, 1999). Interestingly, in female rodents, preference for a place learning strategy is dependent upon higher levels of gonadal hormones, specifically estradiol (Korol et al., 2004). However, the role of testicular hormones in male rats on learning strategy preference has not been examined on a task exclusively designed for that purpose. Given that removal of testicular hormones disrupts cognition on various spatial tasks (Daniel et al., 2003; Sandstrom et al., 2006; Hasegawa and Mochizuki, 2009; Spritzer et al., 2011b; McConnell et al., 2012), we expected removal of testicular hormones to increase the preference for a response learning strategy in Experiment 2. ***Summary of findings:*** *On the dual-solution water T-maze task in Experiment 2, orchidectomy attenuated learning during training, but only minimally impacted preference for a place learning strategy on the probe trial.*

Animals and surgery

Animals. Thirty-two adult male rats, purchased from Harlan Laboratories Inc. (Indianapolis, IN), arrived at the animal care facility at approximately 55 days of age and were individually housed throughout the experiment.

Surgery. Four weeks prior to behavioral testing, at approximately 60 days of age, half (n = 16) of the rats were orchidectomized bilaterally under anesthesia induced by intraperitoneal injections of ketamine (100 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (7 mg/kg, Miles Laboratories, Shawnee, KS). The sac of the scrotum and the underlying tunica were incised for both orchidectomy (ORX) and sham (SHAM) surgeries. The testicular artery and vein were ligated bilaterally and the testes of rats that received orchidectomy were removed. Ligation of vas deferens and the closing of the surgical site were done with 4.0 silk sutures (Teleflex Medical, Coventry, CT). For 3 days after surgery, rats were provided free access to drinking water containing ibuprofen (25 mg/kg) to reduce post-surgical pain during their recovery. To minimize the potential for stress associated with surgery and behavioral testing, rats were handled for 1 min each day for 1 week prior to behavioral testing.

Behavioral testing procedures

Learning during training trials and learning strategy on the probe trial were assessed on a dual-solution water T-maze task (Figure 2) in accordance with the procedures described for Experiment 1.

Statistical analyses

Learning and learning strategy preference as a function of orchidectomy (SHAM, ORX) were analyzed in accordance with Experiment 1.

Behavioral testing results

Dual-solution water T-maze task. There was a significant effect of trial [$F(3,90) = 8.75$, $p < 0.001$], and an effect of orchidectomy (ORX) that approached significance [$F(1,30) = 4.10$, $p = 0.052$], but no interactive effect of orchidectomy and trial [$F(3,90) = 2.17$, $p = 0.10$] on learning during the training phase of the water T-maze task (Figure 4A). Although the performance of both ORX and SHAM rats improved as training progressed, ORX rats tended to make fewer initial arm entries into the arm that contained the platform than SHAM rats. More importantly, the results illustrated in Figure 4B indicate that ORX rats made significantly more incorrect initial arm choices when performance was collapsed across trials 2-8 [$t(30) = \pm 2.51$, $p < 0.05$]. Although the removal of testicular hormones decreased arm choice accuracy on training trials, SHAM and ORX rats did not differ in their preferred learning strategy as indicated by the results of a probe trial shown in Figure 4C [$\chi^2(1) = 0.16$, $p = 0.69$]. SHAM rats exhibited a preference for a place strategy [$\chi^2(1) = 4.00$, $p < 0.05$], while ORX rats also exhibited a tendency to prefer a place strategy [$\chi^2(1) = 2.25$, $p = 0.13$].

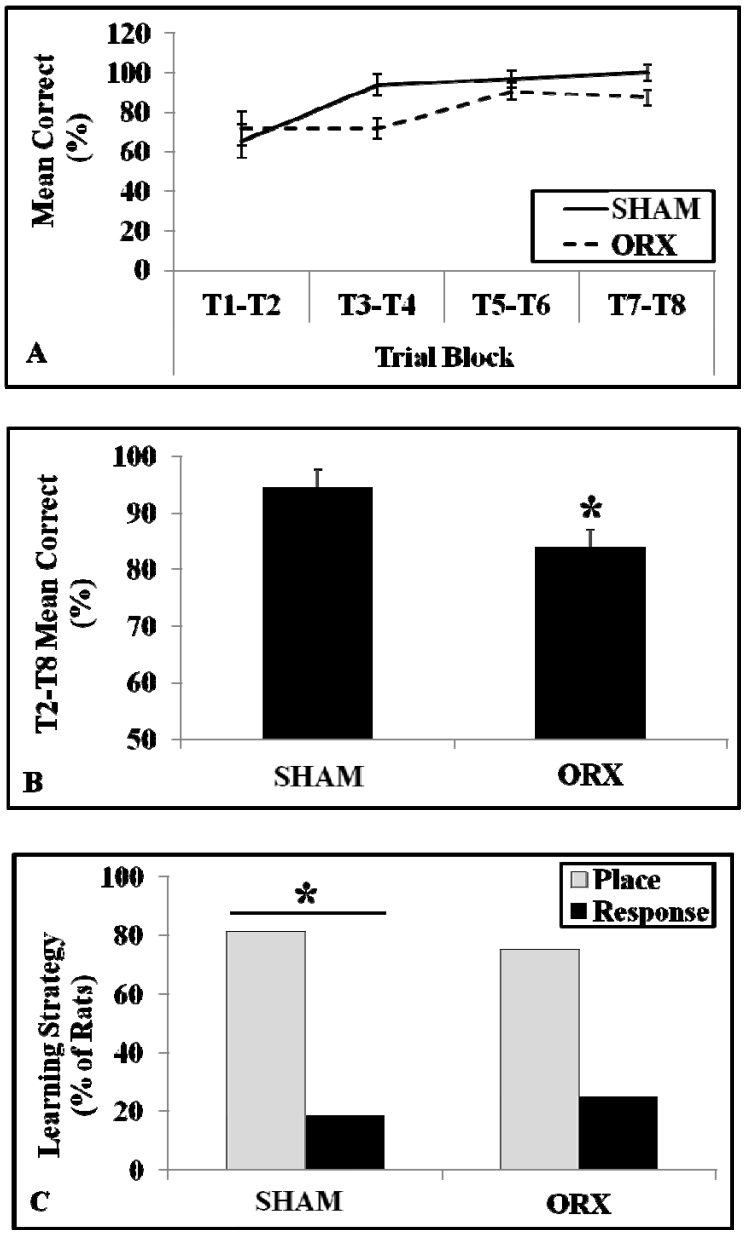

Figure 4. (**A**) Results from the training trials of a dual-solution water T-maze task in which learning was reflected as a greater percentage of correct first arm choices as training progressed. (**B**) Correct arm choices collapsed across training trials 2-8, which was not impacted by chance performance on the first trial (*p < 0.05, SHAM compared to ORX rats). (**C**) Results from the probe trial of a dual-solution water T-maze task designed to categorize learning strategy preference, which was indicated by the initial arm entry (*$p <$ 0.05, SHAM rats that preferred a place strategy compared to a response strategy). Adapted from (Hawley et al., 2012a).

Experiment 3

Primary aim: *To determine the effects of orchidectomy and testosterone treatment on spatial recognition memory in adult male rats*. Although ovarian hormones have been shown to modulate spatial cognition on tasks that capitalize on the propensity of rodents to gravitate toward novelty, such as the object placement task (Frye et al., 2007) and the Y-maze task (Conrad et al., 2004), the effects of testosterone on such tasks have gone virtually unexplored (McConnell et al., 2012). Importantly, performance on these types of tasks is not influenced by appetite or the stressful nature of the water, two non-mnemonic factors which are also regulated by testosterone (Gentry and Wade, 1976; Viau, 2002). Therefore, because estrogen enhances spatial memory in female rats on tasks that capitalize on the propensity of rats to seek out novel spatial environments, and because testosterone enhances spatial memory in male rodents in a delay-dependent fashion on certain water maze tasks (Sandstrom et al., 2006; Benice and Raber, 2009), we expected testosterone to modulate spatial recognition memory on a Y-maze task in a delay-dependent fashion. Additionally, because higher levels of testosterone have been associated with enhanced spatial memory in orchidectomized male rats on certain water-based task (Spritzer et al., 2011b), the second aim of Experiment 3 was to determine if higher levels of testosterone are necessary to restore orchidectomy-induced deficits in spatial recognition memory. ***Summary of findings:*** *In Experiment 3, orchidectomy impaired spatial recognition memory on the Y-maze task that featured a 48-h delay, but*

not a 24-h delay, between the information and retention trials, an effect that was abrogated by treatments producing higher levels of testosterone. Orchidectomy attenuated the normal increase in body weight and the relative weight of the androgen-sensitive ischiocavernosus muscle, effects which were prevented by testosterone treatment. Correlational analyses confirmed that higher levels of testosterone were indicative of heavier ischiocavernosus muscle weights and better spatial recognition memory on the Y-maze task that featured a 48-h delay.

Animals, surgery and hormone treatment

Animals. Male rats (N = 48) were obtained from Harlan Laboratories, Inc. at approximately 70 days of age and were individually housed throughout the experiment. To further minimize the potential for stress associated with surgery and behavioral testing, rats were handled for 1 min each day for 2 weeks prior to behavioral testing.

Surgery and hormone treatment. At approximately 75 days of age, rats were subjected to sham surgery or orchidectomized bilaterally in accordance with the procedures in Experiment 2. Immediately following orchidectomy or sham surgery, rats received two subcutaneous implants of Silastic capsules constructed of medical tubing (Dow Corning Corporation, Midland, MI) into the dorsal region of the neck. Capsules were filled with either free testosterone (Sigma-Aldrich Co., St. Louis, MO) or remained empty. Each capsule (1.47 mm ID x 1.96 mm OD) was cut 20 cm long and the open ends were sealed with small pieces of beveled dowels covered in waterproof silicone sealant (General Electric Co., Huntersville, NC), which resulted in 15 cm of exposed tubing. The group of rats subjected to sham surgery (n = 12; SHAM) and a group of 12

orchidectomized rats (ORX) were implanted with two empty capsules. The remaining orchidectomized rats were implanted with either two testosterone-filled capsules (*n* = 12; ORX-TT) or one testosterone-filled and one empty capsule (*n* = 12; ORX-T).

Behavioral testing procedures

Y-maze task. Spatial recognition memory was characterized on the hippocampus-dependent version of the Y-maze task (Conrad et al., 1996), which is illustrated in Figure 5. The maze was constructed from grey opaque Plexiglas that formed three identical arms (50 length x 10 width x 20 height cm; Stoelting ANY-maze, Wood Dale, IL), and was surrounded by a variety of two and three dimensional extramaze cues.

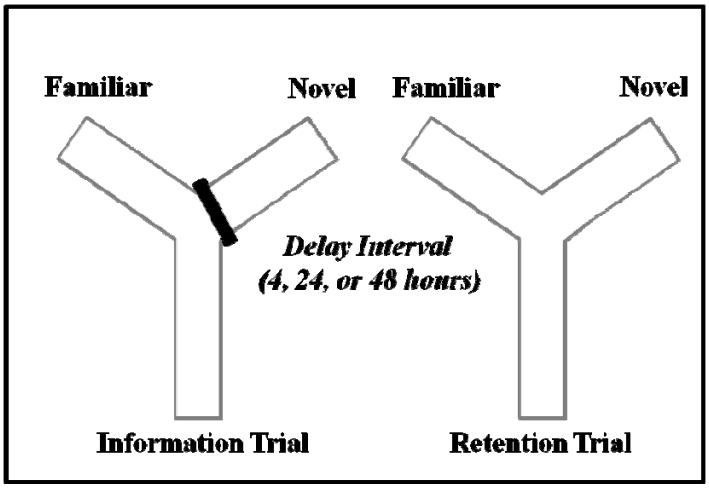

Figure 5. Information and retention trials on a Y-maze task, which categorizes spatial recognition memory.

Rats were placed into the maze for a 15-min information trial and were allowed to freely explore the start arm and a second arm, but access to the third arm was blocked by an opaque plastic partition. Rats were returned to their home cages and transported back to the vivarium following the information trial for a delay period. After the delay, rats were placed back into the same start arm and allowed to freely explore all three arms for

a 5-min retention trial. Retention trials were video-recorded by an overhead camera for future scoring. Entry into an arm was defined as all four paws crossing into the arm proper by an experimenter blind to the conditions. The maze was cleaned thoroughly with 70% ethanol and air-dried after each trial to remove olfactory cues. The first Y-maze task was conducted 1 month after surgery and featured a 24-h delay between the information and retention trials. For the second Y-maze test, which was conducted 1 week later and featured a 48-h delay between trials, the maze was relocated to a different spatial environment. Consistent with previous studies, spatial recognition memory was indicated by the percentage of entries into the novel arm relative to the familiar arm on the retention trial (Wright and Conrad, 2005). In addition, the difference between the percentage of entries into the novel relative to the familiar arm was calculated for correlational analyses (Conrad et al., 2004). Greater preference for the novel arm indicated better spatial recognition memory. The total number of arms entered during the retention trial served as an indicator of general activity.

Procedures to determine efficacy of hormone treatment

Approximately 1 week after the second Y-maze test, rats were weighed, placed into a DecapiCone (Braintree Scientific Inc., Braintree, MA) and rapidly decapitated. Trunk blood was collected and allowed to coagulate for 90 min at room temperature and samples were centrifuged at 2000g for 15 min. Samples of serum were extracted and stored at -20° C prior to shipment to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. Testosterone titers were determined by radioimmunoassay, which was conducted using a commercially available kit

(Testosterone Coat-a Count®; Siemens, Tarrytown, NY). The average intra-assay and inter-assay variations (% CV) were 3.6 and 7.0, respectively.

In addition to determination of testosterone levels, a midline ventral incision was made to expose the perineal musculature and the androgen-sensitive ischiocavernosus muscle was excised and weighed by an experimenter blind to the conditions to confirm the efficacy of orchidectomy and hormone treatments (Collins et al., 1992). Gain in body weight was calculated as a percentage increase from the time of surgery and capsule implantation to the time of sacrifice, which was approximately 6 weeks. Ischiocavernosus muscle weights were expressed as a percentage of body weight at the time of sacrifice.

Statistical analyses

Differences between hormone conditions in activity on the Y-maze task, increases in body weights, relative weights of the ischiocavernosus muscle, and testosterone levels were analyzed by ANOVA. Post-hoc analyses were conducted using Fisher's Least Significant Difference tests. Paired-samples *t*-tests were conducted to determine within-condition preference for the novel arm on the retention trial of the Y-maze task, which is an indicator of spatial recognition memory (Wright and Conrad, 2008). Bivariate correlations were performed to explore the relationships between testosterone levels, cognitive outcomes, and physiological endpoints. Statistical significance was indicated by $p < 0.05$.

Behavioral testing results

Y-maze task: 24-h delay. No differences in activity, as indicated by total arm entries, were found between hormone conditions on the retention trial of the Y-maze task with a 24-h delay between the information and retention trials [Figure 6A; $F(3,44) = 0.47$, $p = 0.71$]. With regard to spatial recognition memory, rats in all hormone conditions exhibited a preference for the novel relative to the familiar arm (Figure 6B). Accordingly, the SHAM [$t(11) = \pm 3.06$, $p < 0.05$], ORX [$t(11) = \pm 2.24$, $p < 0.05$], ORX-T [$t(11) = \pm 3.86$, $p < 0.01$], and the ORX-TT [$t(11) = \pm 4.60$, $p < 0.01$] conditions all exhibited a preference for the novel arm relative to the familiar arm.

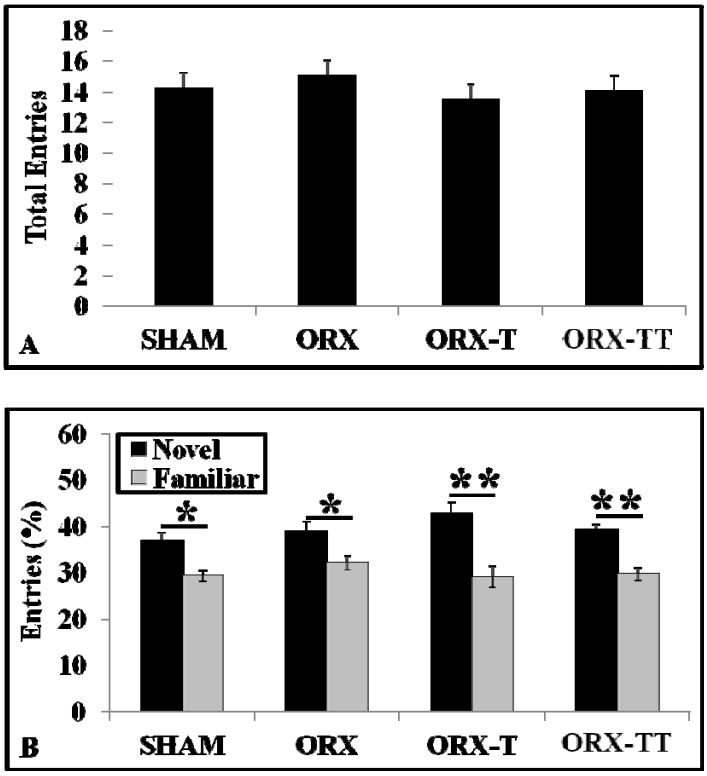

Figure 6. Performance on the retention trial of a Y-maze task, which followed 24 h after the information trial. (**A**) Neither orchidectomy nor testosterone treatment affected level of activity, as indicated by total arm entries. (**B**) Neither orchidectomy nor testosterone treatment affected spatial recognition memory, as indicated by a greater percentage of entries into the novel arm. Rats in all conditions exhibited a preference for the novel arm relative to the familiar arm (*$p < 0.05$; **$p < 0.01$; percentage of entries into the novel arm vs. the familiar arm).

Y-maze task: 48-h delay. As depicted in Figure 7A, no differences in activity were found between conditions on the retention trial of the Y-maze test that employed a 48-h delay between the information and retention trials [$F(3,44) = 0.60$, $p = 0.62$]. With regard to spatial recognition memory, preference for the novel arm relative to the familiar arm was exhibited by rats in the SHAM [$t(11) = \pm 2.71$, $p < 0.05$] and ORX-TT [$t(11) = \pm 4.58$, $p < 0.01$] conditions, but not those in either the ORX [$t(11) = \pm 0.03$, $p = 0.98$] or the ORX-T [$t(11) = \pm 0.03$, $p = 0.98$] conditions (Figure 7B).

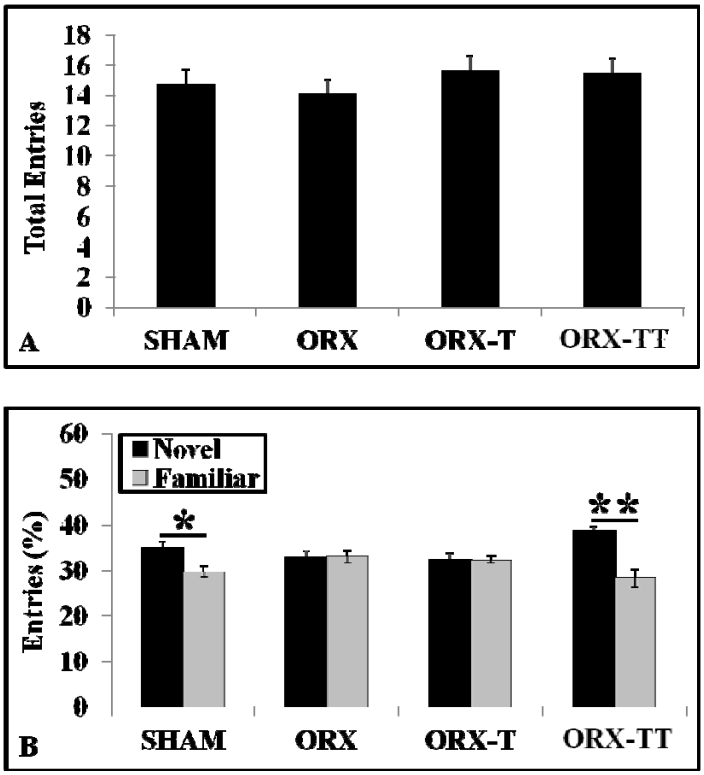

Figure 7. Performance on the retention trial of a Y-maze task, which followed 48 h after the information trial. (**A**) Neither orchidectomy nor testosterone treatment affected level of activity, as indicated by total arm entries. (**B**) Orchidectomy impaired, and treatment with higher levels of testosterone rescued, spatial recognition memory, as indicated by a greater percentage of entries into the novel arm. Rats in the SHAM and ORX-TT conditions exhibited a preference for the novel arm relative to the familiar arm (*$p < 0.05$; **$p < 0.01$; percentage of entries into the novel arm vs. the familiar arm).

Efficacy of hormone treatment: Physiological outcomes

Body weight. As indicated in Figure 8A, orchidectomzed rats gained less body weight over the 6 weeks of the experiment, an effect prevented by implantation of testosterone capsules [$F(3,44) = 10.49$, $p < 0.001$]. Rats in the ORX condition gained significantly less weight than those in the SHAM, ORX-T, and ORX-TT conditions (all p-values < 0.01). Rats in the ORX-TT condition tended to gain less weight than both rats in the SHAM ($p = 0.07$) and the ORX-T ($p = 0.09$) conditions. No difference in body weight gain was found between rats in the SHAM and ORX-T conditions ($p = 0.91$).

Ischiocavernosus muscle. As illustrated in Figure 8B, implantation of testosterone capsules also prevented atrophy of the androgen-sensitive ischiocavernosus muscle [$F(3,44) = 19.22$, $p < .001$]. The weight of the muscle, which was adjusted for body weight, was significantly lighter in rats in the ORX condition relative to those in the SHAM, ORX-T, and ORX-TT conditions (all p-values < 0.01). Notably, the relative weight of the ischiocavernosus muscle was significantly heavier in the ORX-TT condition relative to both the SHAM ($p < 0.001$) and ORX-T ($p < 0.01$) conditions. No difference in the adjusted ischiocavernosus muscle weight was found between rats in the SHAM and ORX-T conditions ($p = 0.68$).

Testosterone levels. The reportable range for the testosterone assay was 0.09-7.47 ng/ml. Only one rat in the ORX condition had levels of testosterone within the reportable range and was included in correlational analyses. Two rats, one from the SHAM and the other from the ORX-TT condition, had levels of testosterone above the reportable range and were excluded from correlational analyses. Samples for each rat were analyzed in duplicate and the mean value was used as the representative testosterone level.

Significant differences in testosterone levels were found between hormone conditions [$F(2,31) = 64.87$, $p < 0.001$], such that levels of testosterone in the ORX-TT condition were elevated relative to both the SHAM ($p < 0.001$) and the ORX-T ($p < 0.001$) conditions (Figure 8C). No differences in testosterone levels were found between rats in the SHAM and ORX-T conditions ($p = 0.79$).

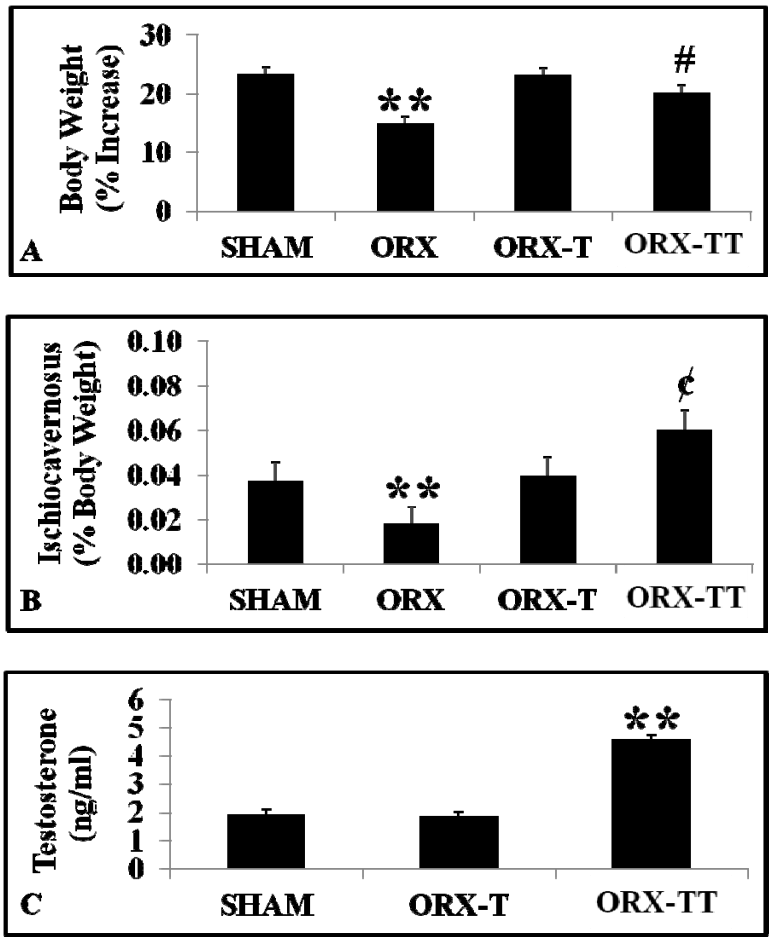

Figure 8. Physiological effects of orchidectomy and testosterone treatment. (**A**) Orchidectomy reduced, and testosterone treatment restored, the increase in body weight, which occurred over 6 weeks (**$p < 0.01$, ORX vs. all other condition; #$p < 0.10$, SHAM and ORX-T conditions vs. ORX-TT condition) and (**B**) the relative weight of the ischiocavernosus muscle (**$p < 0.01$,ORX vs. all other conditions; ¢$p < 0.01$, SHAM and ORX-T conditions vs. ORX-TT condition). (**C**) Implantation of one testosterone-filled capsule (ORX-T) mimicked testosterone levels in SHAM rats. Implantation of two testosterone-filled capsules (ORX-TT) resulted in higher levels of testosterone in ORX rats (**$p < 0.01$, SHAM and ORX-TT conditions vs. ORX-TT condition).

Correlations

Testosterone levels and spatial cognition. On the retention trial of the Y-maze task that employed a 24-h delay between the information and retention trials, testosterone levels were not correlated with either total arm entries [$r(35) = -0.15$, $p = 0.39$; *data not shown*] or preference for the novel relative to the familiar arm [$r(35) = -0.05$, $p = 0.78$; *data not shown*]. However, the results illustrated in Figure 9 indicate that on the retention trial of the Y-maze that employed a 48-h delay between the information and retention trials, higher levels of testosterone were predictive of a greater preference for the novel arm relative to the familiar arm [$r(35) = 0.47$, $p < 0.01$], but not predictive of total arm entries [$r(35) = -0.04$, $p = 0.83$; *data not shown*].

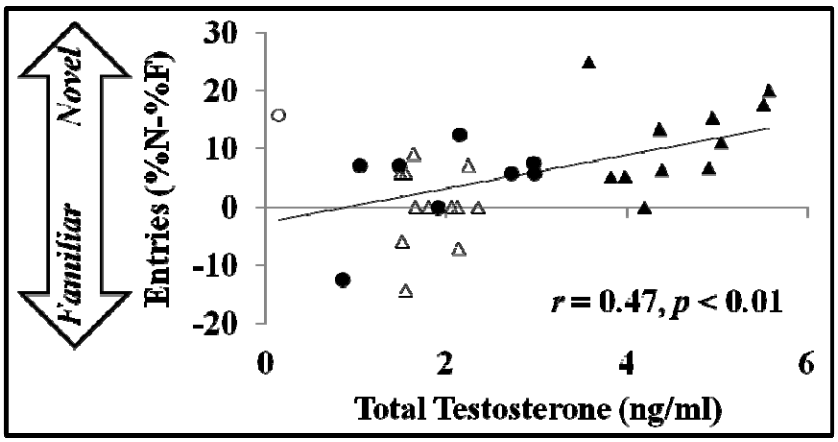

Figure 9. Relationship between levels of testosterone and spatial recognition memory on the retention trial of the Y-maze task that featured a 48-h delay. Higher levels of testosterone were associated with better spatial recognition memory, as indicated by a greater preference for novel arm relative to the familiar arm. SHAM= filled circle; ORX= open circle; ORX-T= open triangle; ORX-TT= filled triangle.

Testosterone levels and physiological outcomes. Figure 10 illustrates the relationship between level of testosterone and relative weight of the ischiocavernosus muscle. Higher levels of testosterone were associated with heavier muscle weights [$r(35) = 0.62$, $p < 0.001$], which confirmed the relative weight of the muscle as an indicator of

testosterone levels. Levels of testosterone were not correlated with the increase in body weight from the time of surgery and capsule implantation to the time of sacrifice [$r(35) = -0.26$, $p = 0.14$; *data not shown*].

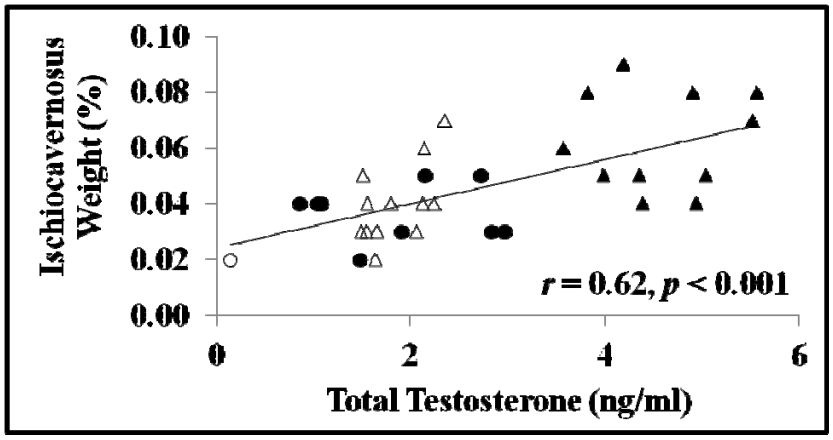

Figure 10. Relationship between levels of testosterone and weight of the ischiocavernosus muscle weight, which was adjusted for body weight. Higher levels of testosterone were associated with heavier ischiocavernosus muscle weights. SHAM= filled circle; ORX= open circle; ORX-T= open triangle; ORX-TT= filled triangle.

Experiment 4

Primary aim: *To determine if short-term treatment to ovariectomized rats with G-1, a GPR30 agonist, would mimic the memory enhancement that typically occurs during proestrus, when levels of ovarian hormones are naturally elevated.* Higher levels of ovarian hormones, which occur during the proestrous stage of the estrous cycle, facilitate performance on spatial tasks, such as the object placement task (Frye et al., 2007) and Y-maze task (Conrad et al., 2004), which take advantage of the preference of rodents to seek out novelty. In ovariectomized rats, short-term treatment with drugs that selectively activate either the ERα or the ERβ receptor subtype mimic the effects of estradiol and facilitate spatial performance on object placement tasks (Frye et al., 2007; Hammond et al., 2009; Jacome et al., 2010). In addition, chronic administration of G-1, an agonist at the putative estrogen membrane receptor GPR30, also enhances spatial performance on tasks motivated by food reward (Hammond et al., 2009). Given that chronic administration of G-1 has been shown to mimic the memory-enhancing effects of chronic estradiol treatment on spatial tasks motivated by food reward, in Experiments 4a and 4b, we expected short-term treatment with G-1 to parallel the memory-enhancing effects that occur on the Y-maze when levels of endogenous gonadal hormones are naturally elevated during proestrus. ***Summary of findings:*** *Collectively, the results from the Y-maze tasks in Experiments 4a and 4b indicated that rats that were in the proestrous stage of the estrous cycle during the information trial subsequently exhibited a preference for the novel on*

the retention trial. The enhancement in spatial recognition memory that occurred in rats with naturally elevated levels of ovarian hormones was paralleled in ovariectomized rats administered G-1, an estrogen receptor GPR30 agonist, at 48 and 24 h prior to the information trial.

Animals, estrous cycle monitoring, surgery and drug treatment

Animals. Female rats (*N* = 18) in Experiment 4a were obtained from litters (*N* = 7) bred in the vivarium of Tulane University. Shortly after weaning, 2-3 female rats from each of the 7 liters were randomly selected for the current experiment. Litter sizes ranged between 8-13 rats. Female rats (*N* = 20) in Experiment 4b were purchased from Harlan Laboratories Inc. and arrived at the animal care facility at approximately 60 days of age. Rats were housed in groups of 2-3 throughout both experiments.

Estrous cycle monitoring (Experiment 4a). Beginning at 55 days of age, vaginal lavage was conducted prior to behavioral testing between 10:00-12:00 h in order to establish regular estrous cyclity and to examine the effects of stage of the estrous cycle on spatial recognition memory. The procedure for estrous cycle monitoring was performed in accordance with Experiment 1. Levels of ovarian hormones during the information trial were categorized as either high (proestrus) or low (estrus/diestrous).

Surgery and drug treatment (Experiment 4b). At approximately 65 days of age, rats were ovariectomized under anesthesia induced by intraperitoneal injections of ketamine (100 mg/kg, Fort Dodge Animal Health) and xylazine (7 mg/kg, Miles Laboratories). Dorsal ventral incisions were made bilaterally approximately 5 cm from the most posterior point of the rib cage. After the ovaries were excised, ovarian blood

vessels were ligated, the muscle wall was closed with 4.0 silk sutures (Teleflex Medical) and the skin was closed with 2-3 titanium wound clips (Mercer Glassware Inc., New York, NY). Rats were provided free access to drinking water containing ibuprofen (25 mg/kg) to reduce post-surgical pain during their recovery for 3 days after surgery and handled for 1 min each day for 7 consecutive days beginning 1 week after surgery. On the last 2 days of handling, which occurred 48 and 24 h prior to behavioral testing, rats were administered intramuscular injections of either sesame oil (0.1 ml; Sigma-Aldrich Co.) or G-1 (25 µg; Sigma-Aldrich Co.), the estrogen membrane receptor agonist, suspended in sesame oil (0.1 ml).

Behavioral testing procedures

Y-maze task. The dimensions of the Y-maze used in Experiment 4a and 4b were identical to that described for Experiment 3 (Figure 5). The testing procedure, which was conducted in accordance with Experiment 3, featured only a 48-h delay between information and retention trials. Unlike male rats, some female rats will occasionally climb on top of the walls of the Y-maze briefly before climbing back down into the maze. Therefore, only rats that did not exhibit climbing behavior were included in Experiment 4a (proestrus, $n = 5$; estrus/diestrus, $n = 11$) and Experiment 4b (G-1, $n = 7$; oil, $n = 9$).

Statistical analyses

For both Experiment 4a and 4b, independent-samples *t*-tests were conducted to determine between-group differences in activity, which was indicated by total arm entries on the retention trial of the Y-maze. Paired-samples *t*-tests were conducted to determine within-condition preference for the novel arm on the retention trial of the Y-maze task, which is an indicator of spatial recognition memory (Wright and Conrad, 2008). Statistical significance was indicated by $p < 0.05$.

Behavioral testing results

Y-maze task: Effect of stage of the estrous cycle. In Experiment 4a, no differences in activity, as indicated by total arm entries on the retention trial, were found between rats that were in proestrus and those in the estrus or diestrus stages of the estrous cycle during the information trial [Figure 11A; $t(14) = \pm 0.53$, $p = 0.61$]. With regard to spatial recognition memory, rats in proestrus during the information trial [$t(4) = \pm 3.51$, $p < 0.05$], but not those in estrus or diestrus [$t(10) = \pm 0.32$, $p = 0.75$], exhibited a preference for the novel arm relative to the familiar arm (Figure 11B).

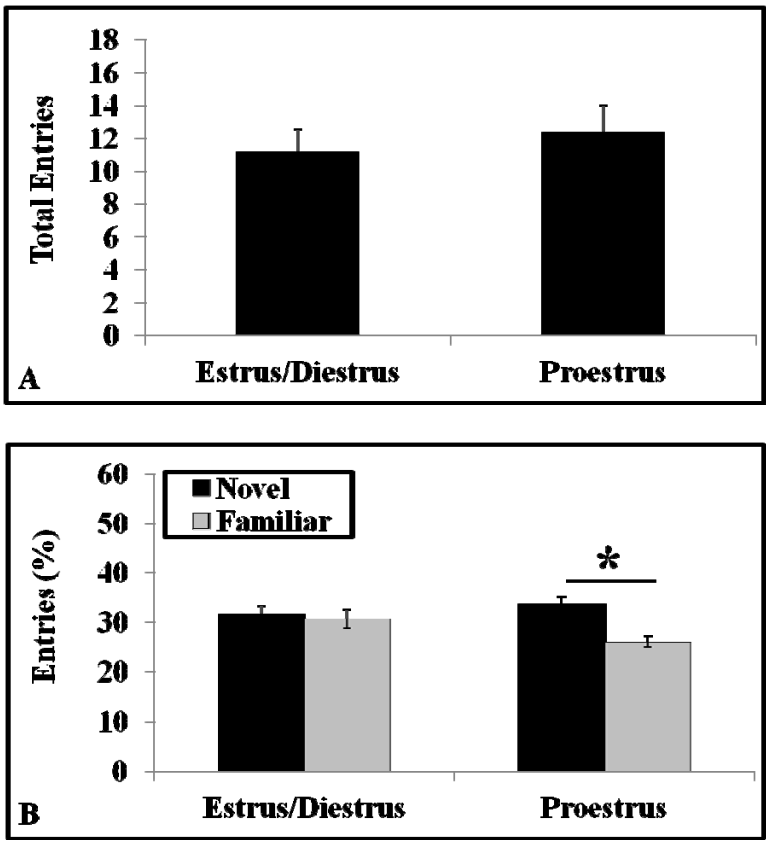

Figure 11. Effects of stage of the estrous cycle at the time of the information trial on activity and spatial recognition memory on the retention trial of a Y-maze task. (**A**) No differences in activity, as indicated by total arm entries, were found between rats in proestrus and those in estrus/diestrus at the time of the information trial. (**B**) However, only rats in proestrus exhibited recognition of the novel arm after a 48-h delay, as indicated by a greater preference for novel arm relative to the familiar arm (*$p < 0.05$; comparison of the percentage of entries into the novel arm vs. the familiar arm).

Y-maze task: Effect of the GPR30 agonist G-1. In Experiment 4b, no differences in activity, as indicated by total arm entries on the retention trial, were found between rats administered the estrogen membrane receptor agonist G-1 and those administered oil 24 and 48 h prior to the information trial [Figure 12A; $t(14) = \pm 1.20$, $p = 0.25$]. With regard to spatial recognition memory, rats treated with G-1 prior to the information trial [$t(6) = \pm 7.10$, $p < 0.001$], but not those treated with oil [$t(8) = \pm 0.36$, $p = 0.78$], exhibited a preference for the novel arm relative to the familiar arm (Figure 12B).

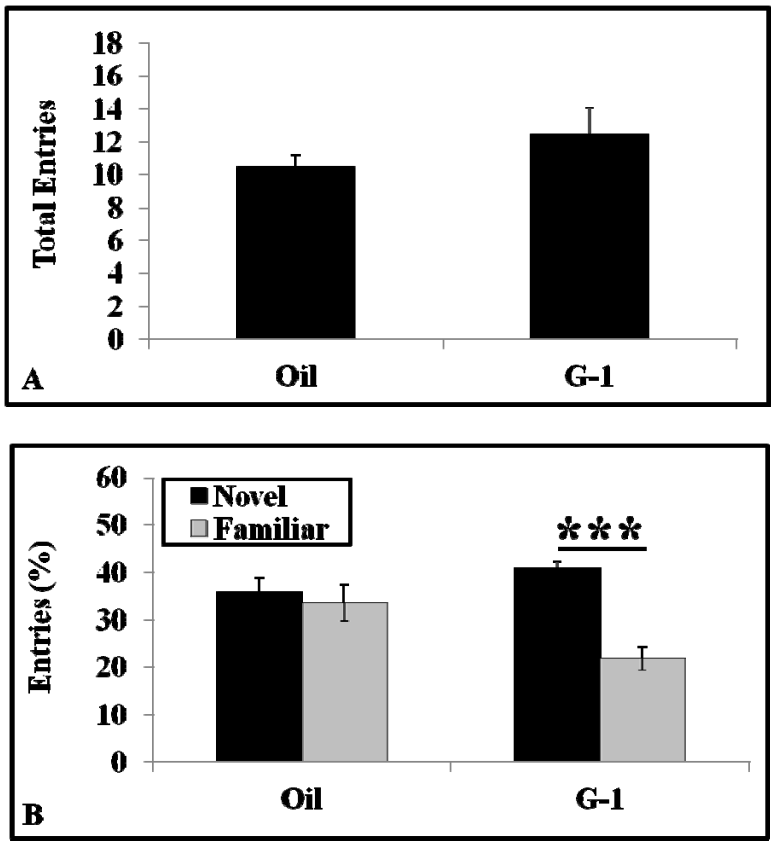

Figure 12. Effects of the estrogen membrane receptor agonist G-1 on activity and spatial recognition memory on the retention trial of a Y-maze task. (**A**) No differences in activity, as indicated by total arm entries, were found between administered oil or G-1 at 24 and 48 h prior to the information trial. (**B**) However, only rats administered G-1 exhibited recognition of the novel arm, as indicated by a greater preference for novel arm relative to the familiar arm (***$p < 0.001$; comparison of the percentage of entries into the novel arm vs. the familiar arm).

Experiment 5

Primary aim: *To determine the effects of posttraining administration of estradiol to ovariectomized rats on learning strategy preference.* In female rodents, higher levels of estradiol result in a preference for a hippocampus-dependent place learning strategy over a striatum-dependent response learning strategy (Korol et al., 2004). Interestingly, in ovariectomixed rats, administration of estradiol enhances memory when administered immediately after completion of training on hippocampus-dependent tasks, such as the Morris water maze task and the object location task (Packard and Teather, 1997b; Luine et al., 2003; Inagaki et al., 2010; Jacome et al., 2010), which suggests that estradiol can impact spatial cognition during the period of memory consolidation, possibly though the involvement of the GPR30 receptor. Therefore, because higher levels of estradiol during training biases rats toward a place learning strategy, and because posttraining administration of estradiol enhances spatial memory, we expected posttraining administration of estradiol to bias ovariectomized rats toward a place learning strategy on a task that can be solved by relying on either a hippocampus or a striatum dependent learning strategy. ***Summary of findings:*** *Results from the dual-solution cued-platform water maze (CPWM) task in Experiment 5 indicated that posttraining administration of estradiol to ovariectomized rats resulted in a greater preference for a place learning strategy across a series of probe trials conducted 24 h later.*

Animals, surgery and hormone treatment

Animals. Female rats (*N* = 14), purchased from Harlan Laboratories Inc., arrived to the animal care facility at approximately 60 days of age and were housed in groups of 2-3 throughout the experiment.

Surgery and hormone treatment. Rats were ovariectomized at approximately 65 days of age in accordance with the procedure outlined in Experiment 4b. Following recovery from surgery, rats were used 1-2 times per week over the following 4 months as stimulus rats to examine male rat sexual behavior. To induce sexual receptivity for this procedure, rats were administered an injection of 10 µg of estradiol benzoate (Sigma-Aldrich Co., St. Louis, MO) followed 42-44 h later by an injection of 500 µg of progesterone (Sigma-Aldrich Co.). Both steroids were delivered intramuscularly in 0.1 ml of sesame oil (Sigma-Aldrich Co.). One week after their final sexual experience, rats were administered a subcutaneuous injection of either vehicle (100% EtOH; *n* = 7) or estradiol (60 µg/kg; *n* = 7) suspended in vehicle immediately following completion of the final training trial on the dual-solution cued-platform water maze task described below.

Behavioral testing procedures

Dual-solution cued-platform water maze (CPWM) task. The CPWM consisted of a white circular pool, 180 cm in diameter, filled to a depth of 26 cm with water maintained at a temperature of approximately 25° C made opaque by the addition of non-toxic white paint (Figure 13). During all trials, an escape platform (15 length x 15 width x 30 height cm) marked by a salient visual cue was submerged 2 cm below the surface of the water, 30 cm from the wall of the pool. The cue consisted of a black plastic ball (3 cm

in diameter) affixed to a piece of threaded rod constructed of galvanized steel (1 cm diameter x 11 cm height), bolted to the center of the platform, and covered horizontally with black and pink electrical tape (each stripe approximately 2 cm).

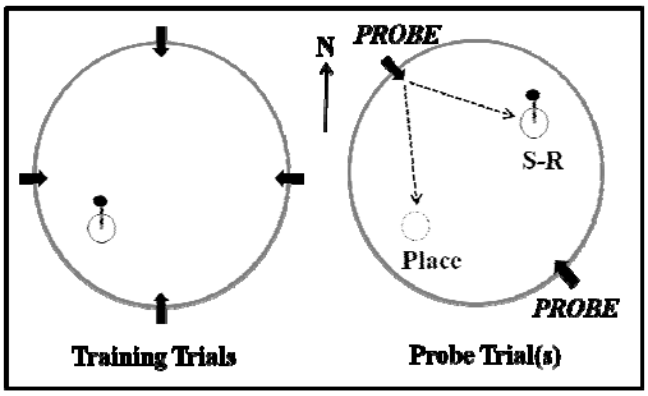

Figure 13. Training and probe trials on dual-solution version of a cued-platform water maze task, which assesses learning strategy preference. S-R = stimulus-response.

Eight training trials were conducted from each of the four cardinal points in a pseudorandomized order with the escape platform located in the southwest quadrant of the pool. Rats were allowed 60 s to locate the platform where they remained for an additional 15 s. Rats were guided to the platform if they did not locate it within 60 s. Twenty-four hours following completion of training, four probe trials were conducted in which the platform was moved to the opposite quadrant of the pool (northeast). Rats were entered into the pool twice from the northwest (probe trials 1 and 3) and twice from the southeast location. All trials, including the probe trials, were separated by an inter-trial interval of 5-15 min. Additionally, to minimize carry-over effects from previous probe trials, rats were immediately removed from the platform upon mounting it. The path length to locate the escape platform during training and probe trials were recorded by tracking software (HVS Image, Ltd., United Kingdom) interfaced to an overhead camera. During training, increasingly shorter escape path lengths as training progressed served as

an indicator of learning. During the probe trials, rats that initially swam to within 5 cm of the training location of the escape platform were categorized as place learners and those that swam directly to the newly relocated cued platform were categorized as stimulus-response learners. The percentage of place responses across the four probe trials was calculated as an additional indicator of place learning strategy preference.

Statistical analyses

Learning on the training trials of the CPWM was analyzed by ANOVA with a between-subjects effect of hormone treatment and a within-subjects effect of trial (1-8). Between group differences and within group learning strategy preference on the probe trials of the CPWM task were determined by conducting χ^2 analyses. Between group differences in learning strategy preference across all probe trials were determined by independent samples *t*-tests. Statistical significance was indicated by $p < 0.05$.

Behavioral testing results

Dual-solution CPWM task. Importantly, between rats that received estradiol and those that received vehicle following the final training trial, there was no main [$F(1,12) = 0.82$, $p = 0.38$] or interactive [$F(7,84) = 1.01$, $p = 0.43$] effect on learning during training trials (Figure 14A). However, there was a significant effect of trial during training [$F(7,84) = 9.75$, $p < 0.001$], which indicated that path lengths to locate the escape platform decreased as training progressed independent of hormone treatment. On the first probe trial, rats treated with estradiol following training tended to prefer a place learning strategy, whereas rats treated with vehicle following training tended to adopt a stimulus-

response learning strategy [Figure 14B; $\chi^2(1) = 2.57$, $p = 0.11$]. When the percentage of place responses was analyzed across all four probe trials, rats treated with estradiol following training exhibited a greater percentage of place learning strategies than rats treated with vehicle following training [Figure 14C; $t(12) = \pm 3.33$, $p < 0.01$].

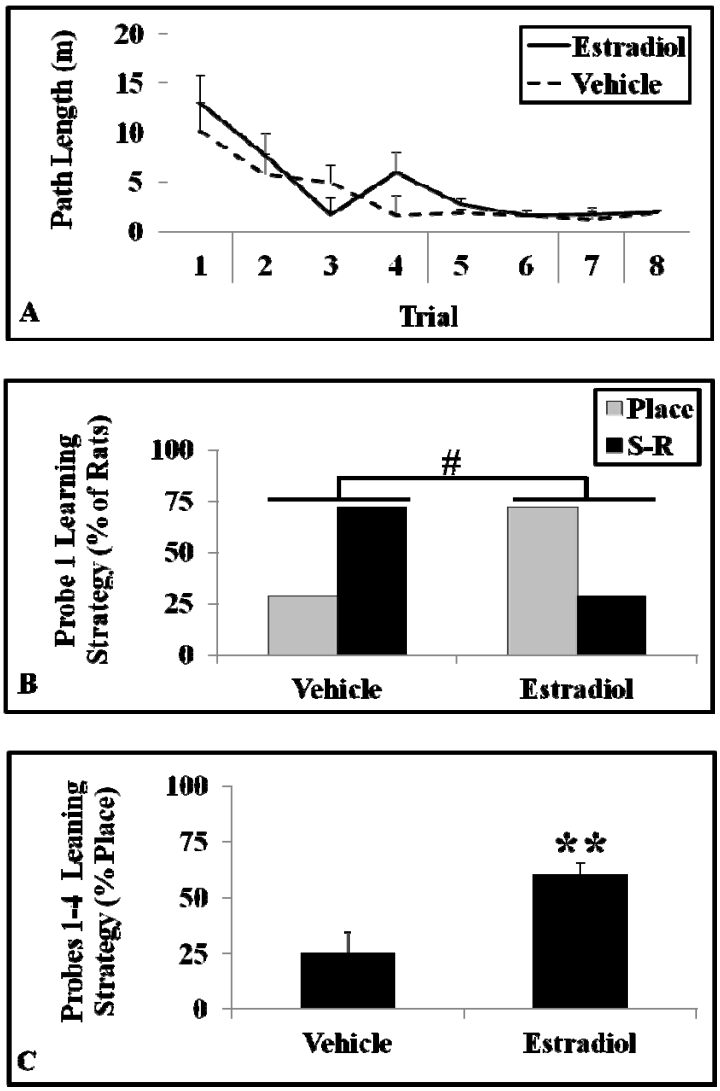

Figure 14. Effects of posttraining administration of estradiol on learning strategy preference 24 h later. (**A**) No difference in learning, as indicated by path length, found between rats that received estradiol or vehicle immediately following the final training trial of a dual-solution learning task. (**B**) On the first probe trial, rats treated with estradiol tended to exhibit a place learning strategy bias relative to vehicle treated rats (#$p < 0.10$) . (**C**) Across all four probe trials, rats treated with estradiol exhibited a place learning strategy more often than vehicle-treated rats (**$p < 0.01$). S-R = stimulus-response.

Experiment 6

Primary aim: *To determine the relationships between trait anxiety, spatial recognition memory, and learning strategy preference in adult male rats.* Pharmacological agents that transiently elevate state anxiety impair spatial learning and memory and correspondingly shift control over learning toward the striatum-dependent memory system (Packard and Wingard, 2004). Interestingly, higher levels of trait anxiety, which occur independent of state anxiety, also are associated with poorer performance on spatial tasks (Herrero et al., 2006). Because higher levels of trait anxiety are predictive of poorer spatial learning and memory, and because elevated levels of state anxiety bias rats toward striatum-dependent learning strategies, in Experiment 6, we expected that adult male rats expressing higher levels of trait anxiety would exhibit poorer spatial recognition memory on a Y-maze task and a bias toward a striatum-dependent learning strategy on a dual-solution learning task**.**

Summary of findings: *In Experiment 6, better spatial recognition memory on the Y-maze task, indicated by a greater percentage of entries into the novel arm, was positively correlated with a place learning strategy on the dual-solution visible-platform water maze (VPWM) task, which was indicated by longer path lengths to reach the platform. More importantly, lower levels of anxiety, indicated by a greater percentage of time spent in the center of the open field, was correlated with better spatial recognition memory on the Y-maze task and a place learning strategy on the dual-solution VPWM task.*

Animals

Adult male rats (*N*=14), purchased from Harlan Laboratories Inc,. arrived to the animal care facility at approximately 55 days of age and were housed in groups of 2 throughout the experiment. Rats were allowed to acclimate to the facility for approximately 1 week and handled for 1 min each day for 5 consecutive days prior to behavioral testing.

Behavioral testing procedures

Y-maze task. A 4-h delay was implemented between the information and retention trial of the Y-maze task (Figure 5). The dimensions and all other procedures for the Y-maze task were identical to that outlined for Experiment 3. Due to a recording malfunction, data for one subject were lost and Y-maze analyses were based on 13 rats.

Dual-solution visible-platform water maze (VPWM) task. One week after completion of the Y-maze task, rats were tested on a VPWM (Figure 15), which consisted of a white circular pool, 180 cm in diameter, filled to a depth of 26 cm with water maintained at a temperature of approximately 25° C and made opaque by the addition of non-toxic white paint. During training trials and the probe trial, a visible black platform measuring 9.5 cm in diameter and projecting 3 cm above the water surface was located 30 cm from the wall of the pool. Eight training trials were conducted from each of the four cardinal points in a pseudorandomized order with the visible escape platform located in the southwest quadrant of the pool. Rats were allowed 60 s to locate the platform where they remained for an additional 30 s. Rats were guided to the platform if they did not locate it within 60 s. Immediately following completion of training, a probe

trial was conducted in which the platform was relocated to the opposite quadrant of the pool (northeast). During the probe trial, rats were placed into the pool from the south location, which is the position most distal from the relocated platform (Kanit et al., 1998). All trials were separated by an inter-trial interval of 5-15 min. The path length to locate the escape platform during training and the probe trials were recorded by tracking software (HVS Image, Ltd., United Kingdom) interfaced to an overhead camera.

During training, increasingly shorter escape path lengths as training progressed served as an indicator of learning. However, longer path lengths during the probe trial served as an indicator of place learning, as rats first returned to the location in the maze where the platform was located during training before orienting to and escaping to the newly relocated platform (McDonald and White, 1994; Kim et al., 2001).

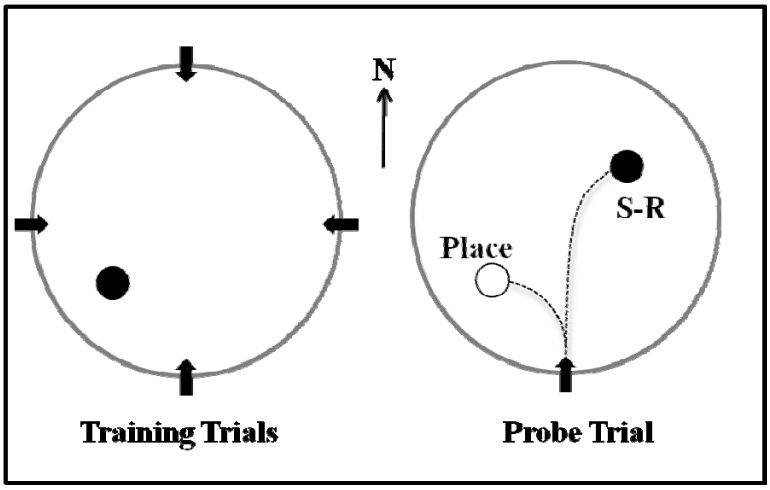

Figure 15. Training and probe trials on a dual-solution version of a visible-platform water maze task, which assesses learning strategy preference. S-R = stimulus-response.

Open field test. Four days after completion of the VPWM, rats were tested once in an open field (90 length x 90 width x 45 height cm) divided into sixteen equal-sized squares (four center squares) for 5 min. The number of total squares crossed and the time spent in the center were recorded by both video monitoring system and a computerized

tracking system, which were interfaced to an overhead camera. The open field was cleaned thoroughly with 70% ethanol and air-dried after each trial to remove olfactory cues. Higher levels of anxiety were indicated by less time spent in the center squares of the field. A greater number of squares crossed indicated greater activity.

Statistical analyses

A bivariate correlation was conducted to explore the relationship between spatial recognition memory, which was indicated by a greater percentage of entries into the novel arm of the Y-maze on the retention trial, and learning strategy preference, which was indicated by the path length on the probe trial of the VPWM task. Bivariate correlations were then conducted to examine the relationships between anxiety, indicated by the time spent in the center of the open field, and both spatial recognition memory and learning strategy preference. Statistical significance was indicated by $p < 0.05$.

Behavioral testing results

Correlations. A greater percentage of entries into the novel arm versus the familiar arm on the Y-maze task was correlated positively with the path length to reach the platform on the dual-solution VPWM task [Figure 16A; $r = 0.74$, $p < 0.01$], which indicates that better spatial recognition memory on the Y-maze task was associated with a place learning strategy preference on the dual-solution VPWM task. With regard to trait anxiety, a greater percentage of time spent in the center of the open field was positively correlated with both a greater percentage of entries into the novel arm versus the familiar arm on the Y-maze task [Figure 16B; $r = 0.64$, $p < 0.05$] and longer path lengths to reach

the platform on the dual-solution VPWM task [Figure 16C; $r = 0.72$, $p < 0.01$]. Therefore, lower levels of trait anxiety were associated with both better spatial recognition memory and a preference for a place learning strategy.

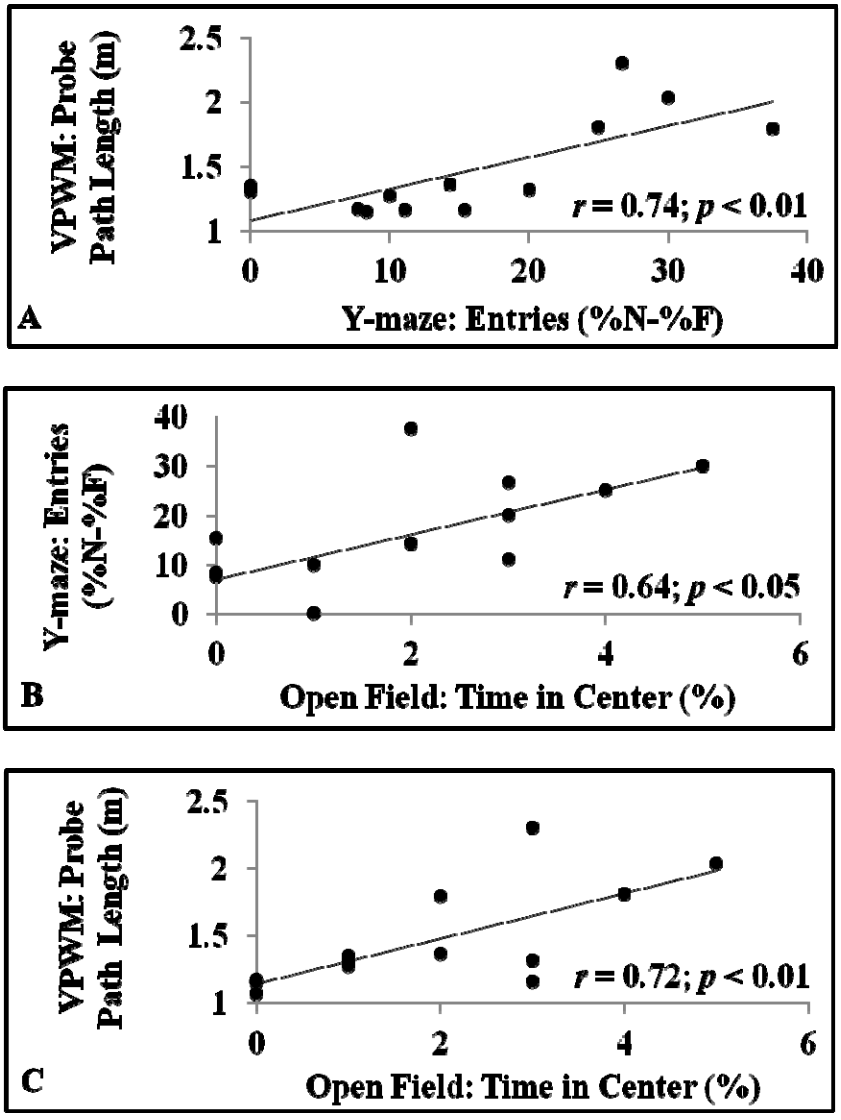

Figure 16. Relationships between spatial recognition memory, learning strategy, and trait anxiety. (**A)** A greater percentage of entries into the novel arm of the Y-maze, an indicator of better spatial recognition memory, was associated with longer path lengths on the dual-solution water maze task ($r = 0.74$; $p < 0.01$), which is an indicator of place learning. More time in the center of the open field, an indicator of lower levels of trait anxiety, was associated with (**B**; $r = 0.64$; $p < 0.05$) a greater percentage of entries on the Y-maze and (**C**; $r = 0.72$; $p < 0.01$) longer path lengths on the probe trial of the dual-solution water maze task. Adapted from (Hawley et al., 2011b).

Experiment 7

Primary aim: *Determine the effects of reminders of an aversive event on spatial recognition memory and learning strategy preference in adult male rats.* Exposure to an acute stressor heightens anxiety (Mitra et al., 2005), disrupts retrieval of recently-learned spatial information (Diamond et al., 2006), and biases rodents toward striatum-dependent learning strategies (Kim et al., 2001). After the effects of the initial stressor subside, exposure to subsequent reminders of an aversive event, which are designed to simulate the intrusive thoughts that characterize posttraumatic stress disorder (PTSD), potentiate anxiety (Korte et al., 1999) and disrupt retrieval of recently-learned spatial information (Zoladz et al., 2010). Therefore, we predicted in Experiment 7 that exposure to a reminder of an aversive event would disrupt retrieval of recently-formed spatial memory on a Y-maze task and correspondingly bias adult male rats toward striatum-dependent learning strategies. ***Summary of findings:*** *Rats exposed to footshock, independent of additional reminders, exhibited lower levels of activity on the Y-maze task conducted 1 week later. However, neither exposure to footshock nor additional reminders of the stressor impacted spatial recognition memory, indicated by a greater preference for the novel arm. One month after exposure to footshock, rats exposed to the stressor exhibited a response learning strategy bias on the dual-solution water T-maze task. However, two months after the footshock, only rats exposed to additional reminders of the stressor exhibited a bias toward a stimulus-response learning strategy on the dual-solution visible*

platform water maze (VPWM) task, an effect that corresponded with heighten emotionality in the open field, which was indicated by shorter path lengths.

Animals

Male Long-Evans rats (*N* = 47), purchased from Harlan Laboratories Inc., arrived to the animal care facilities at approximately 55 days of age and were housed individually throughout the experiment. Rats were acclimated to the housing conditions for approximately 2 weeks prior to the onset of behavioral procedures. During that time, rats were handled for 1 min each day on 4 different days in order to habituate to experimenters. A timetable for the behavioral procedures is outlined in Figure 17.

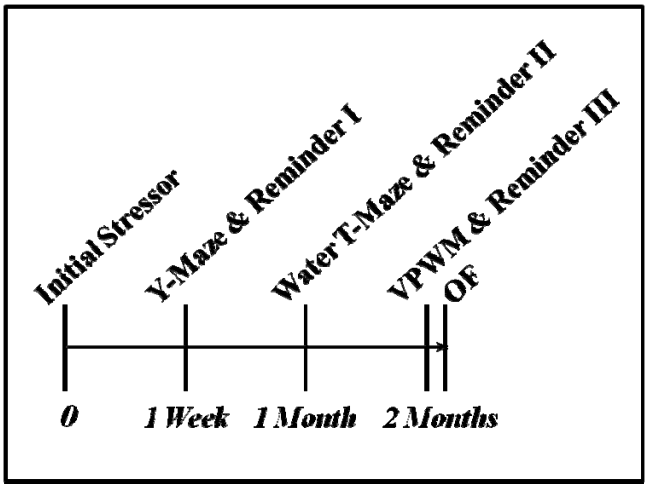

Figure 17. Timeline for behavioral testing. The initial stressor consisted of exposure to footshock during an inhibitory avoidance (IA) training trial. To re-activate the memory for the aversive event, reminders of the stressor (IA retrieval trials) were performed 30 min prior to the retention trial on the Y-maze task, designed to assess spatial recognition memory, and again 30 min prior to the probe trials on the two dual-solution learning strategy tasks, designed to assess learning strategy preference. VPWM=visible-platform water maze; OF=open field test, used to assess anxiety and activity. Adapted from (Hawley et al., 2013)

Behavioral testing procedures

Inhibitory avoidance (IA). The stress paradigm, which included exposure to reminders of the initial stressor, was adapted from a protocol developed to interfere with the retrieval of hippocampus-dependent memories (Zoladz et al., 2010). The initial stressor was a brief exposure to an inescapable footshock, which was administered in an enclosed chamber (50 length x 25 width x 30 height cm) that was divided into two equal-sized compartments, separated by an automatic guillotine door (Coulborn Instruments, Whitehall, PA). The illuminated compartment had walls lined externally with white posterboard and the dark compartment had walls lined externally with black posterboard. The apparatus, including the drop pan below the grid floor, was cleaned thoroughly with 70% ethanol between trials.

On the day of exposure to the initial stressor, which took place during the inhibitory avoidance (IA) training trial, rats were placed into the illuminated compartment, and after 30 s, the guillotine door was automatically raised allowing access to the dark compartment. Photocells detected entry into the dark compartment, which signaled the guillotine door to close, thereby sequestering the rat. Three seconds after the door closed, a single electrical shock (0.6 mA) was delivered for 3 s though the grid floor. Rats were removed from the dark compartment 15 s after the shock terminated. Control rats underwent an identical procedure, but did not receive footshock. Latency to enter the dark compartment prior to administration of the stressor was scored to account for pre-existing differences in anxiety-like behavior.

Rats were divided into the following groups to examine the effects of the initial stressor (IA training trial) and subsequent reminders (IA retrieval trials) on cognitive

outcomes: *not shocked/reminded* (*n*=15); *shocked/not reminded* (*n*=15); and *shocked/ reminded* (*n*=17). Rats in the *not shocked/reminded* and the *shocked/reminded* groups were re-exposed to the chamber (IA retrieval trial) 30 min prior to each of the retention and probe trials on the cognitive tasks described below while rats in the *shocked/not reminded* group remained in their home cages (Zoladz et al., 2010). The procedure for the IA retrieval trials were identical to the IA training trial with the exception that entry into the dark compartment was not followed by footshock (Zoladz et al., 2010). Longer latencies to enter the dark compartment confirmed re-activation of the aversive memory. Failure to enter the dark compartment during the IA retrieval trial resulted in a maximum latency of 600 s.

Y-maze task. One week after administration of the footshock stressor, rats were tested on a Y-maze task that featured a 4-h delay between the information and retention trials (Figure 5). However, to reactivate the memory for the stressor, the *not shocked/reminded* and the *shocked/reminded* groups were subjected to an IA retrieval trial (Reminder I) 30 min prior to the retention trial, while rats in the *shocked/not reminded* group remained in their home cages. The dimensions and all other procedures for the Y-maze task were identical to Experiment 3.

Dual-solution water T-maze task. One month after administration of the footshock stressor, a dual-solution version of the water T-maze task (Figure 2) was used to determine if a second exposure to a reminder of the stressor impacted learning strategy. The dimensions of the maze and platform, the temperature and color of the water, as well as all testing procedures, were conducted in accordance with that outlined in Experiments 1 and 2. However, in this experiment, the maze was surrounded by a variety of three-

dimensional spatial cues affixed to black curtains that surrounded the maze and hung from the ceiling to the floor. In addition, 1 day prior to training, rats were placed in the water T-maze for a 1-min swim without an escape platform present in order to habituate to the water. Furthermore, rats received six training trials, separated by 30 s, each day for 2 consecutive days followed by a single probe trial on the third day (Packard & Wingard, 2004). Thirty minutes prior to the probe trial, the *not shocked/reminded* and the *shocked/reminded* groups were subjected to an IA retrieval trial (Reminder II).

Dual-solution visible platform water maze (VPWM) task. Two months after the initial stressor, a dual-solution VPWM task (Figure 15) was used to determine if a third exposure to a reminder of the footshock stressor impacted performance on a dual-solution learning strategy task that has been previously shown to be sensitive to the effects of shock (McDonald and White, 1994; Kim et al., 2001). The dimensions of the maze and platform, as well as the temperature and color of the water, were identical to that described for Experiment 6. With the following exceptions, all procedures were conducted in accordance with Experiment 6. Training trials were separated by an inter-trial interval of 30 s and the probe trial was conducted 24 h after training (Kim et al., 2001). However, 30 min prior to the probe trial, the *not shocked/reminded* and the *shocked/reminded* groups were subjected to an IA retrieval trial (Reminder III). During training, increasingly shorter escape path lengths as training progressed served as an indicator of learning. During the probe trial, rats that initially swam to within 5 cm of the training location of the visible platform were categorized as place learners and those that swam directly to the newly relocated platform were categorized as stimulus-response learners by an experimenter blind to conditions (McDonald & White, 1994).

Open field test. Four days after completion of the VPWM task, activity and anxiety were assessed on an open field test that was conducted in accordance with Experiment 6. Activity was indicated by total path length and anxiety was indicated by the percentage of time in the center of the open field.

Statistical analyses

An ANOVA with a between-subjects effect of stress condition (*not shocked/reminded, shocked/not reminded, shocked/ reminded*) was conducted on the latency to enter the dark compartment prior to administration of the footshock stressor during the IA training trial. Reactivation of the aversive memory, which was indicated by the latency to re-enter the dark compartment, was examined by ANOVA with a within-subjects effect of IA retrieval trial (Reminder I, Reminder II, Reminder III), subsequent orthogonal contrasts, and a between-subjects effect of experiencing the initial stressor (*not shocked/ reminded, shocked/ reminded*). Independent sample *t*-tests were conducted to confirm memory for the initial stressor during each IA retrieval trial. Between group differences in activity on the Y-maze task were analyzed by ANOVA. Paired sample *t*-tests were conducted to assess within group spatial memory. Learning on the training trials of the water T-maze and the VPWM was analyzed by ANOVA with a between-subjects effect of stress condition and a within-subjects effect of trial block (1-6) and trial (1-8), respectively. Between group differences and within group learning strategy preference on the probe trial of the water T-maze and the VPWM tasks (place versus response/stimulus-response) were determined by conducting χ^2 analyses. Activity and anxiety on the open field were analyzed by ANOVA. When warranted, post-hoc analyses

were conducted using Fisher's Least Significant Difference tests. Statistical significance was indicated by $p < 0.05$.

Behavioral testing results

Inhibitory avoidance (IA). No differences in anxiety-like behavior between the stress conditions were found prior to administration of footshock (Figure 18A), which was indicated by the latency to enter the dark compartment during the IA training trial [$F(2,44) = 0.07$, $p = 0.94$]. For the IA retrieval trials, the results depicted in Figure 18B indicate an effect of footshock [$F(1,30)=182.79$, $p<0.001$], such that rats exposed to footshock exhibited longer latencies to cross into the dark chamber during all three reminders at 1 week, 1 month, and 2 months, which took place just prior to the retention trial on the Y-maze [$t(30) = \pm 9.03$, $p < 0.001$], and just prior to the probe trials on the water T-maze [$t(30) = \pm 13.31$, $p < 0.001$] and the VPWM [$t(30) = \pm 13.55$, $p < 0.001$]. Additionally, an interactive effect of stress condition by IA retrieval trial (Reminders) was uncovered [$F(2,60) = 3.29$, $p < 0.05$]. Specifically, Helmert's orthogonal contrasts reveled that rats exposed to footshock exhibited longer latencies to enter the dark compartment during the reminders that preceded the probe trials of learning strategy tasks, conducted at 1 month and 2 months (Reminders II and III), when compared to the reminder that preceded the retention trial of the Y-maze task conducted at 1 week [Reminder I; $p < 0.05$], which indicates that the memory for footshock strengthened with the passage of time.

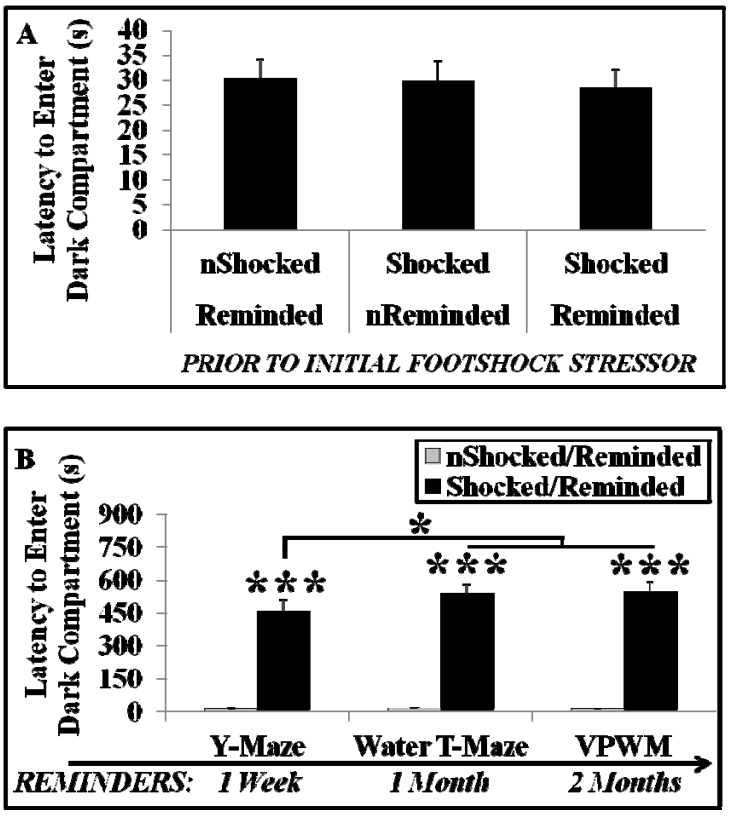

Figure 18. Results from an inhibitory avoidance (IA) paradigm designed to induce the initial stressor (footshock) and subsequently reactivate the aversive memory. (**A**) During the training phase of the IA task, there were no differences in the latency to enter the dark compartment of a shuttle box where a brief footshock would be administered. (**B**) During the IA retrieval trials (reminders), which were conducted 1 week, 1 month, and 2 months after exposure to footshock (IA training trial), longer latencies to enter the dark compartment were exhibited by rats administered the initial footshock relative to rats that were not exposed to footshock during IA training (***$p < 0.001$). For rats exposed to the footshock and exposed to an IA retrieval trial, latencies to enter the dark compartment increased from the first reminder relative to the second and third reminders (*$p < 0.05$). "n" denotes rats that were not administered a footshock during the IA training trial or not reminded of the shock (IA retrieval trials). Adapted from (Hawley et al., 2013).

Y-maze task**.** The results depicted in Figure 19A indicate that rats exposed to footshock during the IA training trial exhibited lower levels of activity on the Y-maze task [$F(2,44) = 7.26$, $p < 0.01$]. Post-hoc analyses confirmed that the *not shocked/reminded* group made more arm entries than both the *shocked/not reminded* ($p < 0.01$) and the *shocked/reminded* ($p < 0.01$) groups during the retention trial. No difference in activity emerged between the two groups of rats exposed to the initial stressor ($p =$

0.78). However, three rats, all of which were in the *shocked/ reminded* stress condition, did not move from the start arm on the retention trial and were therefore excluded from additional Y-maze analyses. The results illustrated in Figure 19B indicate that the *not shocked/reminded* [$t(14) = \pm 6.27$, $p < 0.001$], the *shocked/not reminded* [$t(14) = \pm 4.96$, $p < 0.001$], and the *shocked/reminded* [$t(13) = \pm 7.43$, $p < 0.001$] groups exhibited a preference for the novel relative to the familiar arm. Additionally, no difference in spatial recognition memory between stress conditions was uncovered [$F(2,41) = 1.77$, $p = 0.18$].

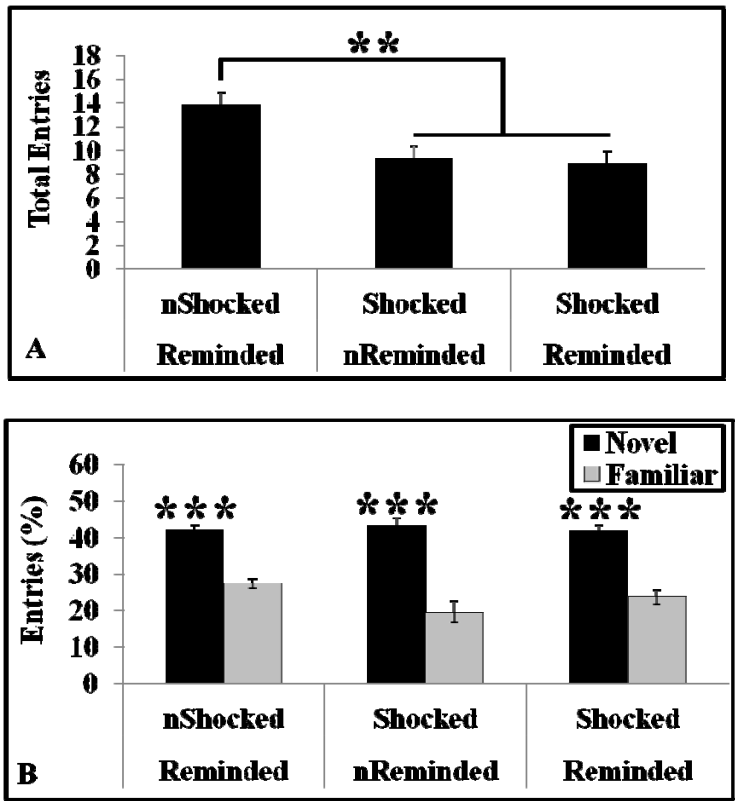

Figure 19. Activity and spatial recognition memory exhibited during the retention trial of a Y-maze task. (A) Both groups of rats exposed to footshock (IA training trial) made fewer arm entries on the retrieval trial of the Y-maze task than the group not exposed to footshock (**$p < 0.01$). (B) Neither footshock alone nor reminder of the footshock impacted spatial memory as all stress conditions exhibited recognition of the novel arm (***$p < 0.001$; comparison of the percentage of entries into the novel arm relative to the familiar arm). “n” denotes rats that were not administered a footshock during the IA training trial or not reminded of the shock (IA retrieval trial). Adapted from (Hawley et al., 2013).

Dual-solution water T-maze task**.** Figure 20A illustrates the results from the training trials on the dual-solution water T-maze task in which there was an effect of training trial block [$F(5,220) = 10.69$, $p < 0.001$], but no effect of stress condition [$F(2,44) = 1.15$, $p = 0.33$], or interactive effect of trial block and stress condition [$F(10,220) = 0.35$, $p = 0.97$] on learning. Therefore, as training progressed, arm choice accuracy increased independent of stress condition. The results from the probe trial depicted in Figure 20B, indicate that the *not shocked/reminded* group did not exhibit a learning strategy preference [$\chi^2(1) = 0.07$, $p = 0.80$], but that both groups of rats exposed to the initial stressor tended to exhibit a bias rats toward a response learning strategy. While the *shocked/not reminded* group exhibited a significant preference for a response learning strategy [$\chi^2(1) = 5.40$, $p < 0.05$], the *shocked/reminded* group also tended to adopt this same strategy [$\chi^2(1) = 2.88$, $p = 0.09$]. No between group difference in learning strategy preference was uncovered [$\chi^2(2) = 2.53$, $p = 0.28$].

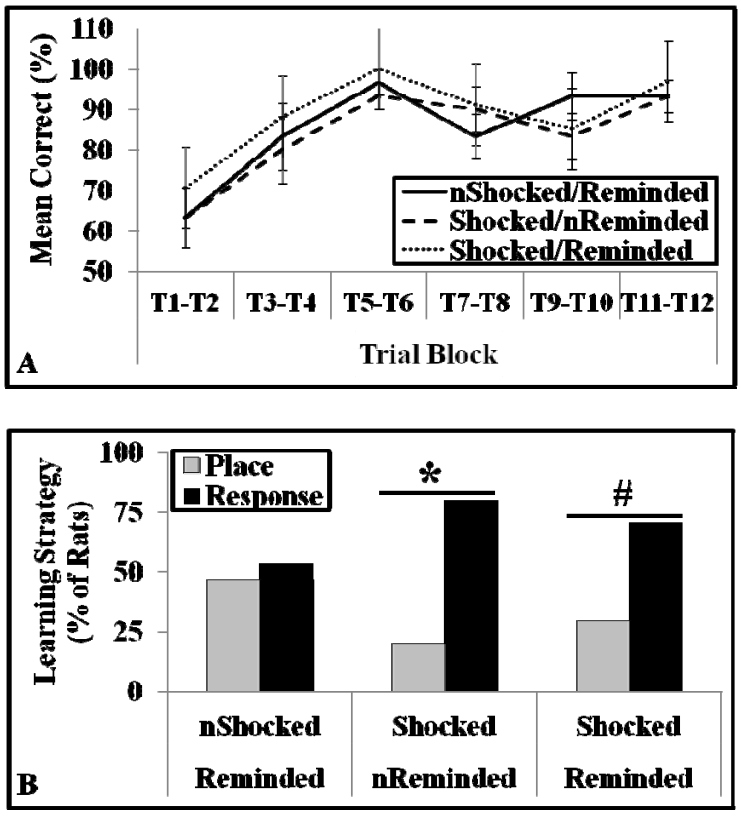

Figure 20. Results from a dual-solution water T-maze task designed to examine learning on training trials and learning strategy on a probe trial. (**A**) No differences in learning were found between conditions during training. (**B**) Rats exposed to footshock (IA training trial), but not reminded of the stressor (IA retrieval trial), preferred a response strategy compared to a place strategy (*$p < 0.05$). Likewise, rats exposed to footshock (IA training trial) and subsequently reminded of the stressor (IA retrieval trial) 30 min prior to the probe trial also tended to prefer a response strategy compared to a place strategy ($^{\#}p = 0.09$). "n" denotes rats that were not administered a footshock during the IA training trial or not reminded of the shock (IA retrieval trial). Adapted from (Hawley et al., 2013).

Dual-solution VPWM task. The results from the training trials on the dual-solution VPWM task illustrated in Figure 21A indicate that there was an effect of training trial [$F(7,308) = 12.57$, $p < 0.001$], but no effect of stress condition [$F(2,44) = 0.24$, $p = 0.78$], or interactive effect of trial and stress condition [$F(14,308) = 1.06$, $p = 0.39$] on learning. Therefore, escape path lengths decreased independent of stress condition as training progressed. The results of the probe trial, which are shown in Figure 21B, indicate that neither the *not shocked/reminded* [$\chi^2(1) = 0.07$, $p = 0.80$] nor the

shocked/not reminded group [$\chi^2(1) = 0.07$, $p = 0.80$] exhibited a learning strategy bias. However, exposure to the reminder of the initial stressor during the IA retrieval trial biased the *shocked/reminded* group toward a stimulus response-strategy [$\chi^2(1) = 4.77$, $p < 0.05$]. No between group difference in learning strategy preference was detected [$\chi^2(2) = 3.30$, $p = 0.19$].

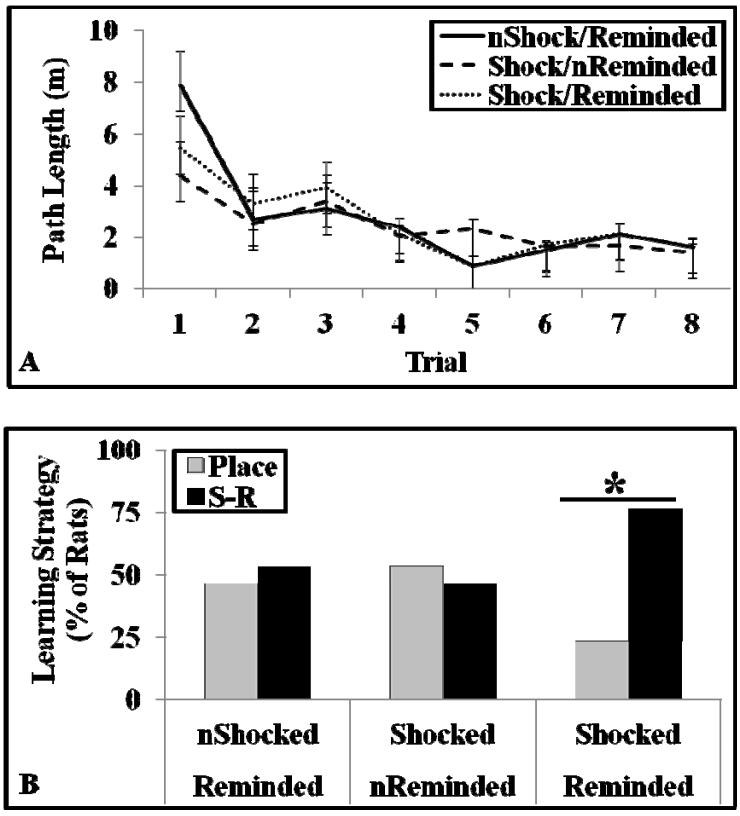

Figure 21. Results from a dual-solution visible-platform water maze task designed to examine learning on training trials and learning strategy on a probe trial. (**A**) No differences in learning were found between conditions during training. (**B**) Rats exposed to footshock (IA training trial) and reminded of the stressor (IA retrieval trial) 30 min prior to the probe trial preferred a stimulus-response (S-R) strategy compared to a place strategy (*$p < 0.05$). "n" denotes rats that were not administered a footshock during the IA training trial or not reminded of the shock (IA retrieval trial). Adapted from (Hawley et al., 2013).

Open field test. Figure 22 illustrates the results from the open field test in which there was an effect of stress condition on total path length [A; $F(2,44) = 7.11$, $p < 0.01$] but not percentage of time in the center squares of the open field [B; $F(2,44) = 0.25$, $p =$

0.78]. Post-hoc tests confirmed that the *shocked/reminded* group exhibited shorter path lengths, which signified lower levels of activity, than both the *not shocked/reminded* ($p < 0.01$) and the *shocked/not reminded* ($p < 0.05$) groups. Although not significant, there was also a trend for the *shocked/not reminded* group to exhibit shorter path lengths than the *not shocked/reminded* group ($p = 0.13$).

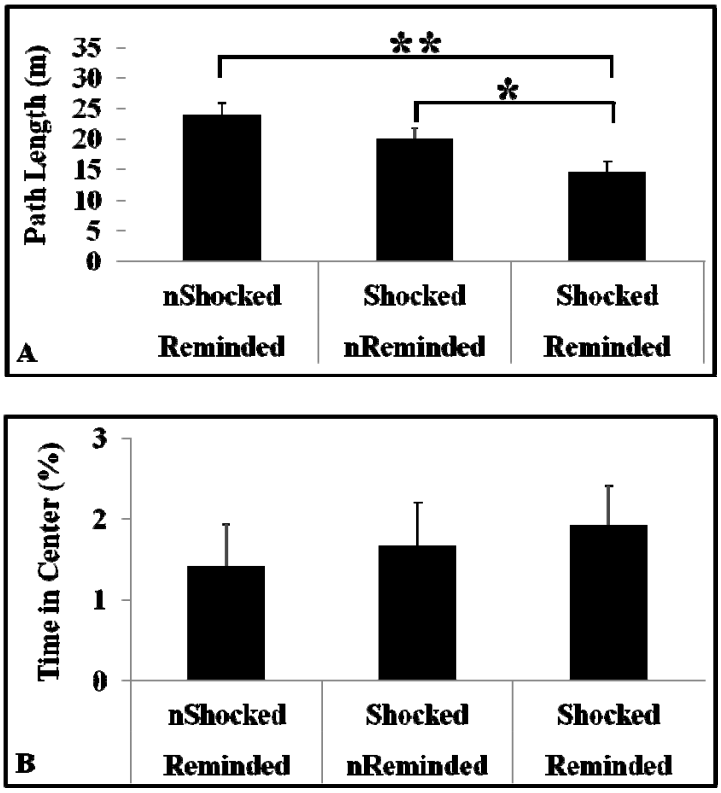

Figure 22. Activity and anxiety exhibited during an open field test. (**A**) Rats repeatedly reminded of the footshock (IA retrieval trials) exhibited shorter path lengths relative to rats that were not exposed to footshock (**$p < 0.01$) and to rats that were never reminded of the footshock (*$p < 0.05$). (**B**) Stress condition did not impact the percentage of time in the center of the open field. "n" denotes rats that were not administered a footshock during the IA training trial or not reminded of the shock (IA retrieval trial). Adapted from (Hawley et al., 2013).

Experiment 8

Primary aim: *To determine the relationship between proteins that regulate cholinergic neurotransmission in the hippocampus and learning strategy preference in adult male rats.* Higher levels of acetylcholine in the hippocampus are associated with a preference for a hippocampus-dependent learning strategy (McIntyre et al., 2003). Therefore, we expected higher levels of choline acetyltransferase (ChAT) and the high-affinity choline uptake transporter (CHT) to be associated with a preference for a hippocampus-dependent learning strategy, given that the proteins modulate acetylcholine synthesis and serve as a predictor of spatial cognition (Dunbar et al., 1993; Tarricone et al., 1993).

Summary of findings: *Rats that displayed a place learning strategy on the dual-solution cued-platform water maze (CPWM) task had elevated protein levels of ChAT, but not CHT, in the dorsal hippocampus, but not the ventral hippocampus, than rats that displayed a stimulus-response learning strategy.*

Animals

Male Long-Evans rats (N = 32), purchased from Harlan Laboratories Inc., arrived at the animal care facility at approximately 65 days of age and were housed individually throughout the experiment. After acclimating to the facilities and housing conditions for 1 week, rats were handled for 1 min each day for 7 consecutive days prior to testing.

Behavioral testing procedures

Dual-solution cued-platform water maze (CPWM) task. The dimensions of the maze, platform, visual cue, and the temperature and color of the water, were identical to that described for Experiment 5 (Figure 13). With the following exceptions, all procedures were conducted in accordance with Experiment 5. Training trials were separated by an inter-trial interval of 60 s, rats were acclimated to the water 24 h prior to training for a 1-min swim without the platform present, and only one probe trial was conducted, which occurred immediately after training from either the northwest or southeast location.

Tissue collection and neurochemical procedures

Tissue dissection and processing. Immediately following completion of the probe trial, rats were rapidly decapitated and the right and left hippocampus were dissected and separated into dorsal and ventral sections on ice. Tissue was then flash-frozen on dry ice, and stored at -80° C until processing. Tissue was homogenized in 15 µl/mg lysis buffer containing 1mM EGTA, 1mM EDTA, 20 mM Tris, 1 mM sodium pyrophosphate tetrabasic decahydrate, 4 mM 4-nitrophenyl phosphate disodium salt hexahydrate, 0.1 µM microcystin, and 1% protease inhibitor cocktail (Sigma-Aldrich Co.). Samples were centrifuged for 15 min at 1000 x g at 4° C, protein concentration of supernatants was determined (Bradford Protein Assay Kit; Pierce, Rockford, IL), and each sample was diluted 1:1 with Laemmli Sample Buffer (Bio-Rad; Hercules, CA) mixed with 350 mM D,L-dithiothreitol, boiled for 5 min, and stored at -80° C.

Electrophoresis and immunostaining for Western blots. For each sample, 25 ug of total protein were loaded and separated at 200 V on 10% SDS-PAGE gels (Bio-Rad) for 60 min. Molecular weight markers (Kaleidoscope; Bio-Rad) were included with each run. Proteins were transferred to nitrocellulose membranes at 100V for 1 h. Membranes were blocked with 5% nonfat dry milk in 0.1% Tween/1 X Tris-buffered saline (TTBS) at room temperature for 1 h. Following this, membranes were incubated with primary antibody overnight at 4° C in TTBS (ChAT) or 1% nonfat dry milk-TTBS (choline transporter; CHT). Primary antibodies used were for ChAT (mouse monoclonal, 1:1500; Millipore) and CHT (rabbit polyclonal, 1:1000; Millipore). Blots were washed three times for 15 min each with TTBS and incubated with 5% nonfat dry milk containing goat antimouse IgG secondary antibody (ChAT, 1:20,000; CHT 1:4000; Santa Cruz) conjugated to horseradish peroxidase for 1.5 h at room temperature. Blots were washed again three times for 15 min each and incubated with the chemiluminescent substrate Pierce ECL (Fisher Scientific) for 1 min (CHT) or SuperSignal West Femto (Fisher Scientific) for 5 min (ChAT) and exposed to film (Kodak Biomax MR) for varying durations to capture optimal signal intensity. To stain for the loading control, β-actin blots were washed and stripped with stripping buffer (RestorePlus Western Blot; Fisher Scientific) for 45 min at 37° C and blocked and incubated with the primary antibodies for β-actin (mouse monoclonal; 1:15,000; Santa Cruz). Secondary antibody for β-actin was goat antimouse IgG (1:10,000; Santa Cruz). Films were imaged using MCID Core imaging software (InterFocus Imaging Ltd., Cambridge, England), and optical density was measured for bands of interest. Samples from each treatment group were equally represented on each blot. Values were computed as a percentage of immunoreactivity of

either ChAT or CHT relative to the β-actin loading control for the particular blot (Sung et al., 2008). Mean values were calculated for the samples taken from rats that adopted a stimulus-response learning strategy. Each value was subsequently represented as a percentage relative to 100%, the average value per blot for rats that adopted a stimulus-response learning strategy.

Statistical analyses

ANOVA with a within-subjects effect of trial (1-8) and a between-subjects effect of learning strategy preference on the probe trial was conducted to examine learning on training trials. Independent samples *t*-tests as a function of learning strategy preference were performed to confirm differences in total path length on the probe trial, as well as the percentage of path traveled on the probe in the quadrant that contained the platform during training and the quadrant that contained the newly relocated platform. One-sample *t*-tests, which compared the percentage of path in each of the two quadrants of interest to chance (25%), were performed to further confirm strategy biases in rats categorized as place and stimulus-response learners. One-sample *t*-tests were conducted to examine differences in cholinergic proteins within each subdivision of the hippocampus in place learners relative to the mean (100%) of stimulus-response learners (Zhou et al., 2007). Statistical significance was indicated by $p < 0.05$.

Behavioral testing results

Dual-solution CPWM task. Importantly, between rats that adopted place or a stimulus-response learning strategy on the probe trial (Figure 23A), there was neither a

main [$F(1,30) = 0.01$, $p = 0.92$] nor an interactive [$F(2,210) = 0.07$, $p = 1.00$] effect on learning the dual-solution CPWM task, which was indicated by increasingly shorter path lengths on training trials 1-8 (Figure 23B). Therefore, the main effect of trial [$F(7,210) = 3.45$, $p = 0.01$] indicates that as training progressed the path length to locate the platform decreased equally between rats that displayed a place strategy and rats that displayed a stimulus-response strategy on the probe trial.

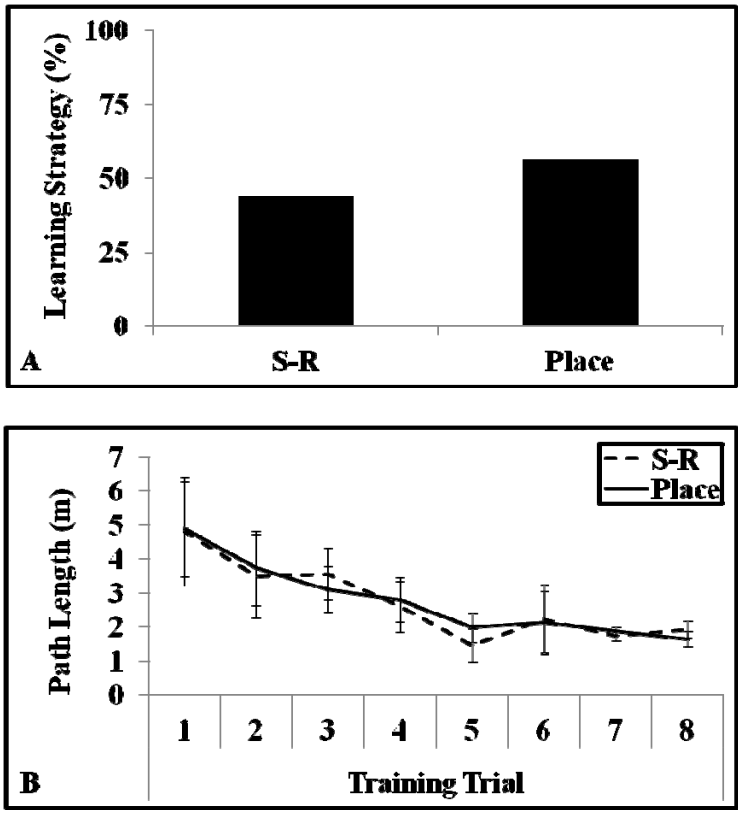

Figure 23. Percentage of rats that displayed a place or stimulus-response (S-R) learning strategy on a probe trial, as well as path lengths during training trials as a function of strategy preference on the probe trial. (**A**) Fourteen rats exhibited a S-R strategy and 18 rats exhibited a place learning strategy on the probe trial. (**B**) No differences in path length during training were found between rats that adopted a place strategy and those that adopted a S-R learning strategy on the probe trial.

On the probe trial, rats categorized as place learners exhibited longer path lengths than rats categorized as stimulus-response learners [$t(30) = \pm 4.57$, $p < 0.001$], which confirmed the categorization of learning strategy preference made by a trained observer

(Figure 24A). Furthermore, the percentage of the path length on the probe trial in the quadrant that contained the platform during training (Figure 24B) was significantly greater for rats categorized as place learners relative to stimulus-response learners [$t(30) = \pm 16.05$, $p < 0.001$] and significantly greater than chance performance [$t(17) = \pm 8.63$, $p < 0.001$]. Additionally, on the probe trial, the percentage of the path length in the quadrant that contained the newly relocated platform was significantly less than chance in rats categorized as place learners [$t(17) = \pm 6.09$, $p < 0.001$], indicating a bias away from a stimulus-response strategy (Figure 24B). Conversely, the percentage of path length on the probe trial in the quadrant that contained the newly relocated platform (Figure 24B) was significantly greater for rats categorized as stimulus-response learners relative to rats categorized as place learners [$t(30) = \pm 10.02$, $p < 0.001$] and significantly greater than chance performance [$t(13) = \pm 7.46$, $p < 0.001$]. In addition, on the probe trial, the percentage of path length in the quadrant that contained the platform during training was significantly less than chance in rats categorized as stimulus-response learners [$t(13) = \pm 69.00$, $p < 0.001$], indicating a bias away from a place strategy (Figure 24B).

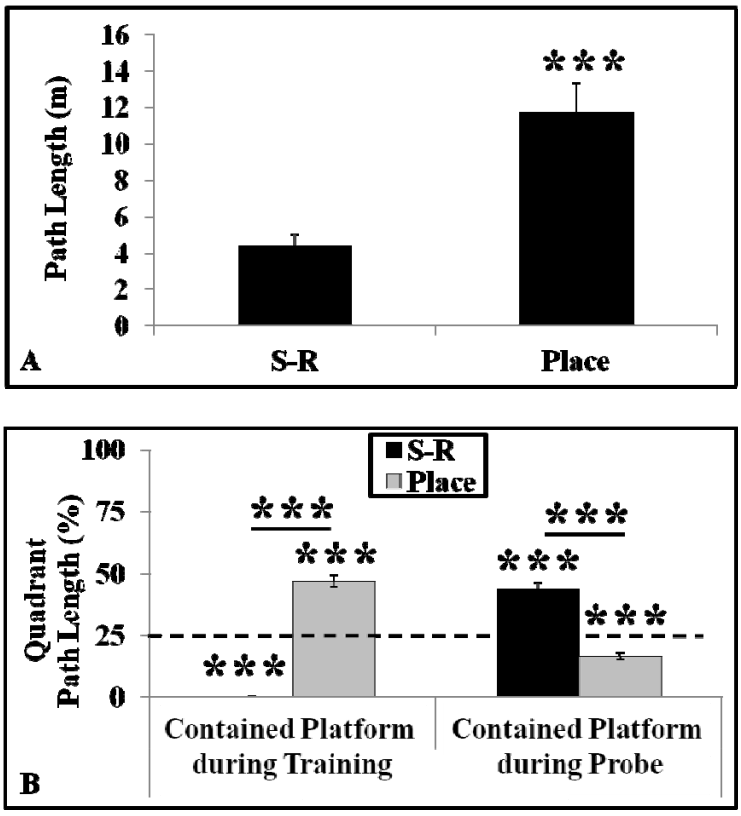

Figure 24. Total and percentage of path length on the probe trial in the quadrant that contained the platform during training and the quadrant that contained the newly relocated platform as a function of strategy choice. (**A**) Place learners exhibited longer path lengths on the probe trial than stimulus-response (S-R) learners (***$p < 0.001$). (**B**) Place learners exhibited a greater percentage of the path on the probe trial in the quadrant that contained the platform during training [***$p < 0.001$ vs. both S-R learners and chance performance (25%)]. Place learners were biased away from the quadrant that contained the platform on the probe trial [***$p < 0.001$ vs. chance performance (25%)]. S-R learners exhibited a greater percentage of the path on the probe trial in the quadrant that contained the newly relocated platform [***$p < 0.001$ vs. both place learners and chance performance (25%)]. S-R learners were biased away from the quadrant that contained the platform during training [***$p < 0.001$ vs. chance performance (25%)].

Neurochemical results

ChAT and CHT levels in hippocampus. Western blots from samples taken from the dorsal and ventral hippocampus of rats tested on a dual-solution CPWM task revealed a single band of ChAT-like immunoreactivity at approximately 70 kDa, CHT-like immunoreactivity at approximately 65 kDa, and immunorectivity for the loading control β-actin at approximately 43 kDa. Rats categorized as place learners had significantly

higher levels of ChAT [Figure 25A; $t(17) = \pm 2.82$, $p < 0.05$], but not CHT [Figure 25B; $t(17) = \pm 0.67$, $p = .51$], in the dorsal hippocampus relative to rats categorized as stimulus-response learners. Neither levels of ChAT [Figure 25C; $t(17) = \pm 0.81$, $p = .43$] nor CHT [Figure 25D; $t(17) = \pm 1.03$, $p = .32$] in the ventral hippocampus were higher in place learners relative to stimulus-response learners. Importantly, levels of the loading control β-actin, which were used to determine relative levels of ChAT [$t(17) = \pm 1.38$, $p = .19$; *data not shown*] and CHT [$t(17) = \pm 1.38$, $p = .19$; *data not shown*] immunoreactivity in the dorsal hippocampus, were no different in place learners relative to stimulus-response learners. Likewise, levels of the loading control β-actin, which also were used to determine relative levels of ChAT [$t(17) = \pm 0.59$, $p = .56$; *data not shown*] and CHT [$t(17) = \pm 1.51$, $p = .15$; *data not shown*] immunoreactivy in the ventral hippocampus, were no different in place relative to stimulus-response learners.

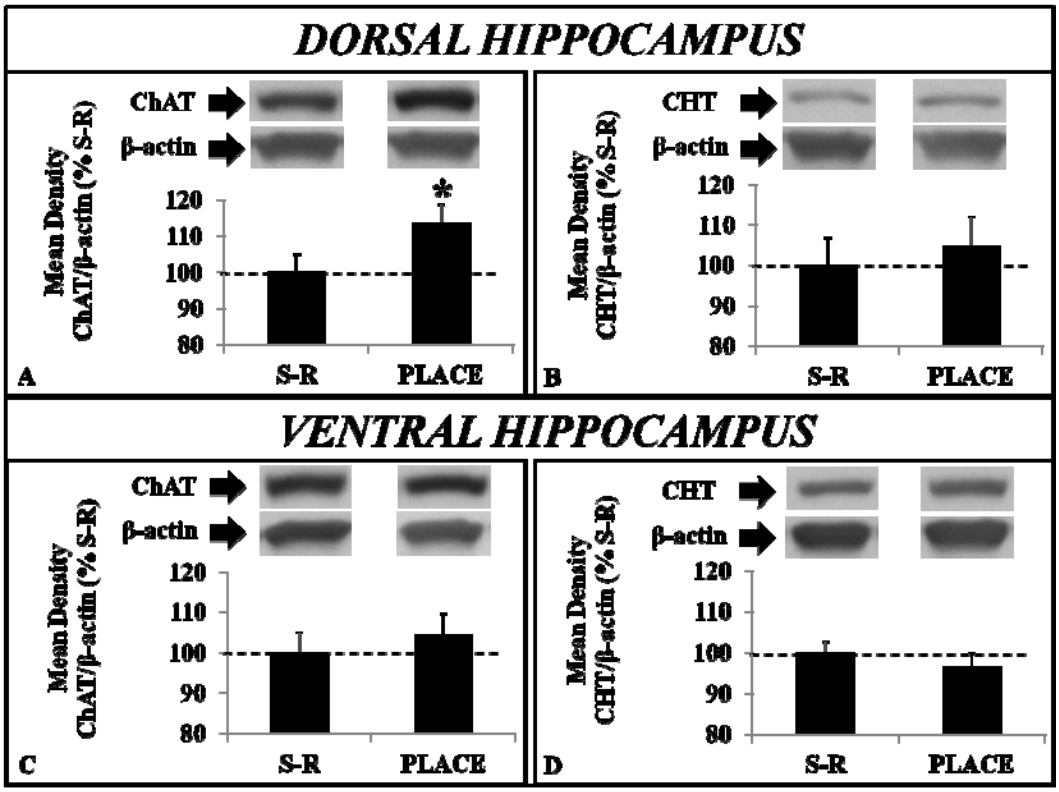

Figure 25. Expression of choline acetyltransferase (ChAT) and the high affinity choline transporter (CHT) in the dorsal (**A,B**) and ventral (**C,D**) hippocampus as a function of learning strategy preference. (**A**) Rats that adopted a place learning strategy had relatively higher levels of ChAT, but not (**B**) CHT in the dorsal hippocampus than rats that adopted a stimulus-response (S-R) learning strategy (*$p < 0.05$ vs. 100%, the mean density of rats that adopted an S-R strategy). No differences were found in protein levels of (**C**) ChAT or (**D**) CHT in the ventral hippocampus as a function of learning strategy preference.

Experiment 9

Primary aim: *To determine if disruption of cholinergic neurotransmission by administration of a muscarinic receptor antagonist directly into the hippocampus impacted learning strategy preference.* Because low doses of either peripheral (von Linstow Roloff et al., 2007) or intrahippocampus (Brito et al., 1983; Carli et al., 1997) administration of the muscarinic receptor antagonist scopolamine has been shown to impair spatial learning, but spare striatum-dependent learning, we predicted that scopolamine administered directly into the hippocampus would bias rats away from a hippocampus-dependent strategy and toward a striatum-dependent learning strategy on a dual-solution learning task. ***Summary of findings:*** *Vehicle-treated rats exhibited a high level of performance early in training on the dual-solution cued-platform water maze task (CPWM), although statistical indicators of learning were not significant. Intrahippocampus administration of scopolamine resulted in poorer performance during training relative to vehicle treated rats, as indicated by longer path lengths during training trials.*

Animals

Male Long-Evans rats (N = 50), purchased from Harlan Laboratories Inc., arrived at the animal care facility at approximately 65 days of age. Rats were housed individually

throughout the experiment and were allowed to acclimate to the facility for approximately 1 week prior to surgery.

Surgery and scopolamine administration

Stereotaxic surgery. At approximately 70 days of age, rats were anesthetized by intraperitoneal injections of ketamine (100mg/kg) and xylazine (7mg/kg) before placement into a stereotaxic frame for bilateral implantation of indwelling cannulae into the hippocampus (3.3 mm posterior to bregma, 2 mm lateral to the mid-line, and 3.0 mm ventral to the skull surface). Bilateral guide cannulae constructed of 23-ga thin-wall stainless-steel tubing were lowered through trephine holes to 1 mm above the target site and anchored to the skull with dental acrylic. Inner cannulae constructed of 26-ga stainless-steel tubing extended 1 mm beyond the tips of the guide cannulae into the target site to maintain patency of the guide cannulae.

Scopolamine administration. Following 7-10 days of recovery, each rat received bilateral infusions of either scopolamine hydrobromide (20 µg per side) or 0.9% saline vehicle (Fader et al., 1998) bilaterally in volumes of 0.5 µl delivered at a flow rate of 1.0 µl /min with a Harvard infusion pump attached to a 10-µl Hamilton microsyringe that was connected to a 26-gauge applicator cannulae by polyethylene tubing (PE10). The applicator cannulae used for infusions extended 1 mm beyond the tips of the guide cannulae into the target sites. At the end of the experiment, cannulae placements were verified upon visual inspection while sectioning with a cryostat and further confirmed by staining sections with cresyl violet (Figure 26).

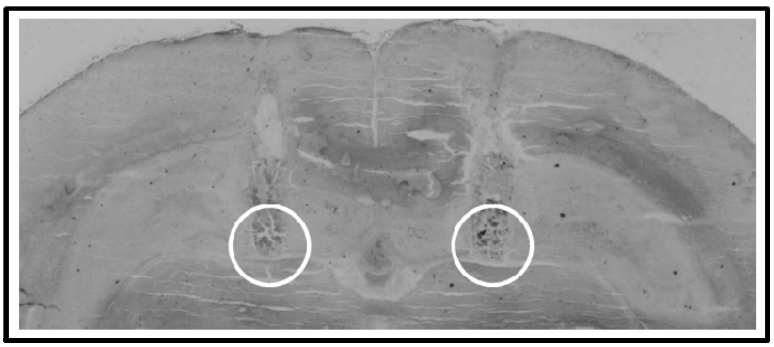

Figure 26. Brain section stained with cresyl violet to confirm cannulae placement into hippocampus. Representation of acceptable placement at most posterior and ventral position.

Behavioral testing procedures

Dual-solution cued-platform water maze (CPWM) task. Rats were handled for 3-5 min each day for 7 consecutive days prior to behavioral testing. Five minutes after infusion, rats were tested on the dual-solution CPWM task (Figure 13). The dimensions of the maze, the platform, the visual cue, the temperature and color of the water, as well as all procedures were conducted in accordance with Experiment 8. However, following training rats received four probe trials, rather than just one.

Statistical analyses

ANOVA with a within-subjects effect of trial and a between-subjects effect of drug (vehicle, scopolamine) were conducted to separately examine learning on training trials (1-8). When warranted, between group differences and within group learning strategy preferences on the first probe trial (place versus response/stimulus-response) and across all 4 probe trials were determined by conducting χ^2 analyses or independent samples *t*-tests, respectively. Statistical significance was indicated by $p < 0.05$.

Behavioral testing results

Dual-solution CPWM task. Of the rats with confirmed bilateral cannulae placement into the hippocampus that were included in behavioral analyses, 13 were administered vehicle and 16 were administered scopolamine. The remaining rats were eliminated due to cannulae misplacement or complications during surgery. When compared to vehicle-treated rats, scopolamine-treated rats exhibited a robust impairment in learning the dual-solution CPWM task [$F(1,27) = 12.20$, $p < .01$], which was indicted by longer path lengths during training trials 1-8 (Figure 27A). There was no effect of trial on learning during training [$F(7,189) = 1.07$, $p = 0.39$], such that path lengths did not decrease as training progressed. However, because there was an indication of an interactive effect of trial as a function of scopolamine treatment during training on path lengths [$F(7,189) = 1.75$, $p = 0.10$], within-condition exploratory analyses were conducted to examine the learning deficit. Strikingly, for saline-treated rats, the path length to locate the platform did not decrease as training progressed [$F(7,84) = 1.39$, $p = 0.22$], which was an effect likely due to the high level of performance that occurred early in training, an effect not observed in any of our other experiments. There was also no effect of training trial on learning in scopolamine-treated rats [$F(7,105) = 1.72$, $p = 0.11$].

On the first probe trial, approximately one-half of the saline-treated rats exhibited a place learning strategy [$\chi^2(1) = 0.08$, $p = .78$, *data not shown*], an effect consistent with the results from Experiment 8. However, the robust learning deficit exhibited by scopolamine-treated rats did not warrant an examination of learning strategy preference. As indicated in Figure 27B, additional exploratory analyses revealed that

scopolamine-treated rats tended to exhibit longer path lengths to locate the platform across all probe trials when compared to vehicle-treated rats [$F(1,27) = 3.63$, $p = .07$].

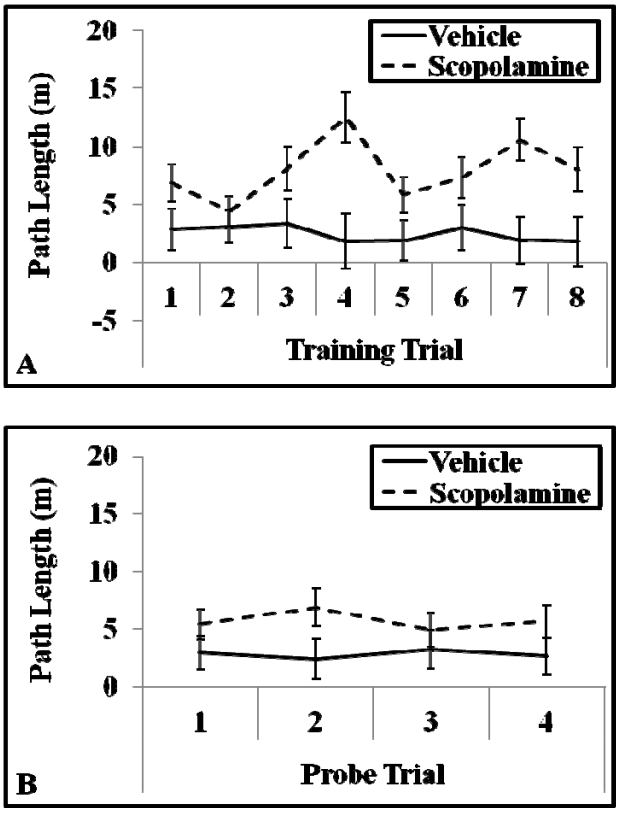

Figure 27. Path length to locate the platform during training and probe trials in rats administered either intrahippocampus scopolamine or saline 5 min prior to training. (**A**) Path lengths for rats administered scopolamine were significantly greater compared to rats treated with saline across all training trials ($p < 0.01$). (**B**) Path lengths for scopolamine-treated rats also tended to be longer than saline-treated rats across all probe trials ($p = 0.07$).

Discussion

As summarized in Table 1, the current set of experiments indicate that spatial cognition is impacted by biological sex and gonadal hormones, affective outcomes, and cholinergic neurotransmission in the hippocampus.

	SPATIAL RECOGNITION MEMORY	***LEARNING STRATEGY PREFERENCE***
Biological Sex and Gonadal Hormones		
Biological Sex	-	Males = Better learning/place bias
Estrous Cycle	Proestrus = Enhanced	No effect
Estradiol	-	Enhanced place bias
G-1	Enhanced	-
ORX	Impaired	Attenuated learning
Testosterone	Enhanced	-
Affective Outcomes		
Trait Anxiety	Low = Enhanced	Low = Place bias
Stress/Reminders	No effect	Striatum-based strategy bias
Cholinergic Neurotransmission in Hippocampus		
ChAT/CHT	-	Elevated ChAT = Place bias
mAchR	-	Antagonism impaired learning

Table 1. The effects of biological sex, gonadal hormones, affective outcomes, and cholinergic neurotransmission in the hippocampus on spatial recognition memory and learning strategy preference in adult rats. OVX = ovariectomy; ORX = orchidectomy; ChAT = choline acetyltransferase; CHT = choline uptake transported; mAchR = muscarinic receptors.

Male rats exhibited better learning and a greater preference for a place learning strategy than female rats. In female rats, gonadal hormones modulated spatial learning and memory, as well as learning strategy preference, albeit in a task-specific manner. However, in male rats, spatial learning and memory, but not the preference for a place

learning strategy on the dual-solution water T-maze task was modulated by gonadal hormones. Although gondal hormones did not impact learning strategy preference in male rats, higher levels of trait anxiety were associated with both poorer spatial recognition memory and a greater preference for a stimulus-response learning strategy in male rats. Further, for male rats, exposure to a single episode of stress resulted in a preference for a stimulus-response learning strategy, an effect which was maintained by exposure to additional reminders of the stressor after the effects of the initial stressor dissipated. With regard to cholinergic neurotransmission in the hippocampus, the expression of proteins that regulate acetylcholine synthesis were elevated in male rats that adopted a place learning strategy relative to males rats that adopted a stimulus-response strategy. Finally, disruption of muscarinic receptor function in the hippocampus impaired learning on a dual-solution task, which could be solved by executing either a place or a stimulus-response strategy.

Sex Differences and Learning Strategy Preference in Adult Rodents

In **Experiment 1**, male rats were more accurate than female rats in their initial arm choices during the training phase of a dual-solution water T-maze task. Subsequently, the results of a probe trial revealed that only male rats exhibited a bias toward a place strategy. Female rats did not display a preference for either a place or a response strategy, and, unexpectedly, their performance during both training trials and the probe trial was unaffected by the stage of the estrous cycle in which they were tested.

The results from Experiment 1 confirm previous studies that have investigated sex differences in learning strategy (Kanit et al., 1998; Kanit et al., 2000a; Jonasson et al.,

2004). Taken together, the results indicate that female rats are not as reliant on place learning strategies as male rats on some tasks. In accordance with previous research (Packard, 1999; Chang and Gold, 2003b; Jonasson et al., 2004; Elliott and Packard, 2008), male rats in both the first and second experiments exhibited a preference for a place learning strategy on a dual-solution T-maze task. Therefore, the previously reported male advantage on hippocampus-dependent tasks (Jonasson, 2005; Luine and Dohanich, 2008) corresponds with a greater reliance on a place learning strategy on tasks that can solved by relying on either a hippocampus-based place learning strategy or a striatum-based response learning strategy (Kanit et al., 1998; Kanit et al., 2000a; Jonasson et al., 2004). Given the poorer performance of female rats on spatial tasks (Jonasson, 2005; Luine and Dohanich, 2008), in addition to their greater reliance on response learning strategies (Kanit et al., 1998; Kanit et al., 2000a; Jonasson et al., 2004), the hippocampus-based memory system of female rats may not control learning to the extent that it does males.

In addition to exhibiting a preference for a place learning strategy (Packard and McGaugh, 1996; Packard, 1999; Cahill and Baxter, 2001; Jonasson et al., 2004; Packard and Wingard, 2004), adult male rats also performed better than females on single-solution learning tasks that required execution of either a place or a response strategy (Blokland et al., 2006; Schmidt et al., 2009). However, the ability of male rats to rapidly adapt to a specific strategy in order to solve a particular task also corresponds with a more rigid style of navigation (Williams et al., 1990). Specifically, the performance of male rats on a hippocampus-based radial-arm maze task suffered only when the geometry of the room changed, not when the location of landmarks were altered. On the other hand, compared

to male rats, female rats exhibited more flexibility in the way they navigated toward a goal on the same radial-arm maze task, such that their performance was unaffected when either the geometry of the room or landmarks within the room were changed. With this in mind, an alternative approach to interpreting the findings from the dual-solution water T-maze task in Experiment 1 is that the superior performance of male rats exhibited during training, in conjunction with their propensity to adopt a place learning strategy, is a manifestation of a rigid navigational style (Williams et al., 1990).

Gonadal Hormones and Learning Strategy Preference in Adult Rats

Unlike the effects of ovarian hormones on learning strategy preference documented elsewhere, female rats in Experiment 1 failed to exhibit a learning strategy bias on a dual-solution water T-maze task regardless of the stage of the estrous cycle in which they were tested (Korol et al., 2004; McElroy and Korol, 2005; Sava and Markus, 2005; Pleil and Williams, 2010). The discrepancy between results may be due to several factors. Elevated levels of estradiol typically result in a preference for a place strategy when testing occurs on dry-land tasks motivated by food-reward (Korol and Kolo, 2002; Korol et al., 2004; Quinlan et al., 2008). Alternatively, the effects of estradiol on water-based tasks have been less consistent (Kanit et al., 2000a; Sava and Markus, 2005; Pleil and Williams, 2010; Rummel et al., 2010; Tunur et al., 2010). In fact, on some water-based tasks, elevated levels of estradiol were associated with a preference for a stimulus-response learning strategy (Kanit et al., 2000a). Therefore, on some water-based tasks, the effects of ovarian hormones may have been obscured by heightened levels of stress and anxiety, which consequently increased the likelihood of adopting a response learning

strategy (Elliott and Packard, 2008; Ferragud et al., 2010). Interestingly, on a dual-solution water T-maze task, which was similar to the task employed in Experiment 1, pre-exposure to the context of the testing environment without the presence of water enhanced preference for a place learning strategy in mice that were in proestrus (Tunur et al., 2010). Therefore, pre-exposure to the context of the testing environment may have been necessary to bias rats in proestrus toward a place learning strategy on the dual-solution water T-maze task in Experiment 1.

In addition to the nature of the testing environment, and in contrast to the majority of water-based learning strategy tasks (Kanit et al., 2000a; Pleil and Williams, 2010; Rummel et al., 2010), testing on dry-land mazes requires satisfaction of a strict criterion during training before a probe trial is administered (Korol et al., 2004; McElroy and Korol, 2005; Quinlan et al., 2008). As could be the case for Experiment 1, either insufficient (Pleil and Williams, 2010) or excessive (Rummel et al., 2010) training on water-based tasks may have obscured the effects of gonadal hormones on learning strategy in female rats. Historically, attempts to correlate the stage of the estrous cycle with spatial ability have yielded contrasting results (Dohanich, 2002). Therefore, the most parsimonious explanation for the discrepant findings between Experiment 1 and others is that the relatively small sample size (Korol et al., 2004), in conjunction with the demands of the task (Gibbs, 2007; Rummel et al., 2010) and nature of the testing environment, were not conducive to detecting the effects of ovarian hormones on learning strategy preference.

While stage of the estrous cycle did not impact the performance of female rats in Experiment 1, orchidectomy significantly impacted different facets of performance in

male rats on the dual-solution water T-maze task employed in **Experiment 2**. Removal of testicular hormones decreased arm choice accuracy during the training phase of a dual-solution water T-maze task. However, on the probe trial, orchidectomy did not significantly reduce the preference for a place learning strategy. Therefore, the results of Experiments 1 and 2 indicate that the previously reported male advantage on hippocampus-dependent learning and memory tasks (Jonasson, 2005; Luine and Dohanich, 2008) corresponds with a greater reliance on a place learning strategy and that removal of testicular hormones impacts learning, but only minimally affects the preference of male rats for a place learning strategy. Importantly, the results from the dual-solution water T-maze task in Experiment 2 align well with previous findings, which have demonstrated that testicular hormones modulate spatial learning in male rodents (Harrell et al., 1990; Daniel et al., 2003; Edinger et al., 2004; Gibbs, 2005; Sandstrom et al., 2006; Spritzer et al., 2008; Benice and Raber, 2009; Hasegawa and Mochizuki, 2009; Spritzer et al., 2011b). In addition, that orchidectomy did not affect learning strategy preference on the dual-solution T-maze task in Experiment 2 is also consistent with a previous study, which indicated that testosterone did not impact the learning strategy used to solve a matching-to-position T-maze task (Gibbs, 2005). Taken together, the results of Experiments 1 and 2, in conjunction with those reported elsewhere, indicate that the presence of testicular hormones are important for spatial learning and memory, but that gonadal hormones differentially impact learning strategy preference in male and female rats (Korol, 2004; Gibbs, 2005).

The Effects of Testosterone on Spatial Cognition in Adult Male Rats

In Experiment 3, orchidectomy-induced deficits in spatial cognition on a hippocampus-dependent version of a Y-maze task were rescued by testosterone treatment. The deficits emerged in a delay-dependent fashion, such that orchidectomy impaired spatial recognition memory, and treatments that produced higher levels of testosterone restored cognition when a longer delay of 48 h was employed between the information and retention trials. In orchidectomized rats, the deficit in spatial cognition was associated with both reduced body weight gain and atrophy of the androgen-sensitive ischiocavernosus muscle, effects that were abrogated by testosterone treatment. Correlational analyses confirmed that higher levels of testosterone were predictive of both heavier ischiocavernosus muscle weights and enhanced spatial recognition memory, indicated by a greater preference for the novel arm of the Y-maze on the retention trial following the 48-h delay. The results indicate that testosterone modulates spatial cognition on a task that is not motivated by either food reward or water escape, but instead takes advantage of the propensity of rodents to prefer novel spatial environments.

Results from the Y-maze tasks employed in Experiment 3 are consistent with a growing body of research conducted over the last decade, which has indicated a beneficial role for testosterone on a variety of spatial tasks (Daniel et al., 2003; Khalil et al., 2005; Sandstrom et al., 2006; Spritzer et al., 2011b; McConnell et al., 2012). However, the results of earlier studies that examined the effects of testosterone and other androgens on spatial cognition in male rodents were largely inconsistent, such that androgen treatment either had no effect (Smith et al., 1996) or impaired the performance (Goudsmit et al., 1990) of male rats. Arguably, the cognitive demands of the tasks are

one of several factors that that may have contributed to the discrepancies between earlier and more recent studies. Spatial working memory, which categorizes information specific for a particular sequence of trials, was more sensitive to the effects of testosterone than reference memory, which categorizes information that remains the same from trial to trial (Olton and Papas, 1979), on tasks motivated by either food reward (Spritzer et al., 2008) or water escape (Sandstrom et al., 2006).

In Experiment 3, spatial recognition memory on the Y-maze task was sensitive to the effects of testosterone, but only when the delay between the information and retention trials was relatively longer. Interestingly, recent evidence indicates that testicular hormones influence memory retention on object location tasks (McConnell et al., 2012), a relatively non-aversive task in which optimal performance is contingent upon detecting changes in the spatial configuration of two identical objects (Ennaceur et al., 1997). However, in the study by McConnell and colleagues, performance was assessed using a fixed delay of 30 min between all information and retention trials. The delay-dependent results from the Y-maze tasks employed in Experiment 3 that featured a 24-h delay and a 48-h delay align well with the delay-dependent effects observed on water based spatial tasks (Sandstrom et al., 2006). The results from Experiment 3 extend previous findings (McConnell et al., 2012) and indicate that testosterone modulates spatial performance on tasks that take advantage of the tendency of rodents to seek out novel spatial environments, but only when the demands of the task becoming sufficiently challenging. With this in mind, it also is important to note the poorer performance of orchidectomized rats during the training phase of the dual-solution water T-maze task in Experiment 2, an effect which corresponds with the results of previous reports that have documented

slower rates of spatial learning following removal of testicular hormones (Harrell et al., 1990; Daniel et al., 2003; Edinger et al., 2004; Gibbs and Johnson, 2008; Spritzer et al., 2008; Spritzer et al., 2011b). Importantly, although learning on spatial tasks is often slower in orchidectomized rats, removal of testicular hormones does not necessarily cause learning impairments. After sufficient training, orchidectomized rats eventually reach the level of performance exhibited by rats with testosterone (Harrell et al., 1990; Daniel et al., 2003; Spritzer et al., 2008; Spritzer et al., 2011b).

The Non-Mnemonic Effects of Testosterone

Because the effects of testosterone in Experiment 3 emerged in a delay-dependent fashion, and because there were no differences between hormone conditions in general activity on the Y-maze task, it is reasonable to conclude that orchidectomy-induced deficits in spatial recognition memory were not due to non-mnemonic factors. However, testosterone has been shown to reduce both depressive-like and anxiety-like behavior on a variety of tasks that categorize affective outcomes (Frye and Seliga, 2001; Carrier and Kabbaj, 2012). In addition, following orchidectomy, treatments that produce higher levels of testosterone have been shown to attenuate cellular activation in brain areas that regulate the stress response (Viau et al., 2003; Lund et al., 2006; Goel et al., 2011), as well as attenuate the stress-induced release of adrenocorticotrophin hormone (ACTH) and corticosterone (Handa et al., 1994; Viau and Meaney, 1996; Seale et al., 2004; Goel et al., 2011). Interestingly, exogenous administration of corticosterone has been shown to disrupt the retrieval of a recently formed spatial memory (de Quervain et al., 1998; Ferguson and Sapolsky, 2008; Goel et al., 2011). Further, attenuation of corticosterone

synthesis prevented stress-induced deficits in spatial memory on both a water maze task (de Quervain et al., 1998) and a Y-maze task (Wright et al., 2006). Therefore, we cannot entirely rule out the possibility that testosterone modulated activation of the stress response, and the subsequent release of corticosterone, which ultimately impacted spatial recognition memory in Experiment 3, as well as the rate of learning on the dual-solution water T-maze in Experiment 2. Following orchidectomy, treatments that produce higher levels of testosterone may be necessary to overcome cognitive impairments that result from dysregulation of the hypothalamic-pituitary-adrenal axis and the removal of other testicular hormones and synthesizing enzymes that play a role in spatial cognition (Sandstrom et al., 2006; Spritzer et al., 2011b; McConnell et al., 2012).

In accordance with previous findings, both the reduction in body weight gain (Gentry and Wade, 1976) and the atrophy of the ischiocavernosus muscle that resulted from orchidectomy in Experiment 3 were abrogated by testosterone treatment (Collins et al., 1992). Previous studies indicated that orchidectomy-induced deficits in spatial learning were not associated with alterations in activity on a food-rewarded version of the radial-arm maze task, (Daniel et al., 2003). However, it is conceivable that because testosterone modulates appetite (Gentry and Wade, 1976), the reinforcing properties of food reward may have been attenuated by orchidectomy. This possibility is especially intriguing considering the rewarding properties of testosterone (Packard et al., 1998; Frye et al., 2002), as well as the modulatory role that the hormone plays on other reward related outcomes, which occur in response to sexual experience and drugs of abuse (Hughes et al., 1990; Minerly et al., 2008). That the orchidectomy-induced spatial recognition memory impairment in Experiment 3 was associated with a robust reduction

in body weight gain, but no alteration in activity level, supports the contention that spatial tasks that are not motivated by food reward may be more appropriate for examining the effects of long-term orchidectomy and testosterone treatment in male rats.

For rats in Experiment 3 that received treatments producing higher levels of testosterone, the divergent effects of testosterone treatment on body weight and the relative weight of the ischiocavernosus muscle likely resulted from elevated levels of the testosterone metabolites dihydrotestsoterone (DHT) and estradiol. Hypertrophy of the ischiocavernosus muscle results from over activation of androgen receptors by testosterone or DHT (Collins et al., 1992). Given that estradiol treatment attenuates food intake and weight gain that occurs in female rats following ovariectomy (Wade, 1972; Beatty et al., 1975), the slight attenuation in weight gain that occurred in orchidectomized rats with higher levels of testosterone likely resulted from elevated levels of estradiol. Although elevated, it is also important to note that plasma testosterone levels found in orchidectomized rats implanted with two testosterone-filled capsules in Experiment 3 mimicked the levels that occur following sexual activity (Bonilla-Jaime et al., 2006), and therefore, were no greater than levels that occur naturally. When considered in light of previous reports (Gibbs, 2005; Spritzer et al., 2011b), these findings together indicate that higher levels of testosterone, and in all likelihood DHT and estradiol (McConnell et al., 2012), enhance spatial cognition and differentially impact body weight gain and the weight of the ischiocavernosus muscle.

The Effects of Androgens on the Hippocampus

From a neurobiological standpoint, both the previously reported male spatial advantage and the differential navigational style of male and female rats exhibited in Experiment 1 are modulated by the presence of early hormones (Dawson et al., 1975; Williams et al., 1990; Roof, 1993b; Isgor and Sengelaub, 1998, 2003). Female rats treated with either estradiol or testosterone in early life performed similarly to their male counterparts on hippocampus-dependent tasks in adulthood (Williams et al., 1990; Roof, 1993b). Likewise, the removal of testosterone in male rats during early life abolished the male spatial advantage in adulthood (Williams et al., 1990). In addition to behavioral differences in spatial ability, gonadal hormone manipulations in early life also altered the morphology and function of the adult hippocampus. When compared to adult female rats, adult male rats exhibited a wider dentate gyrus (Roof, 1993a), an increased number of dendritic branches in CA3 (Isgor and Sengelaub, 2003), and increased acetylcholine release within the hippocampus (Mitsushima et al., 2009). However, exposing females to testosterone or estradiol in early life abolished the sexual dimorphisms that presented in the hippocampus during adulthood (Roof, 1993a; Isgor and Sengelaub, 2003; Mitsushima et al., 2009). Much like the male spatial advantage and the sexually-dimorphic navigational style, the male preference for a place learning strategy exhibited in Experiment 1 and 2 was likely contingent upon the presence and organizational effects of gonadal hormones in early life.

Although the organizing effects of gonadal hormones impact spatial cognition and the corresponding neurobiological endpoints in the hippocampus, disruptions to the structure and function of the hippocampus after removal of testicular hormones may have

moderated the poorer performance exhibited by rats orchidectomized as adults during the training phase of the dual-solution water T-maze task in Experiment 2 and on the Y-maze task in Experiment 3 that featured a 48-h delay between the information and retention trials. Although estradiol treatment has been shown to enhance spatial learning (Gibbs, 2005) and memory (Luine and Rodriguez, 1994; Packard and Teather, 1997b; McConnell et al., 2012) in adult male rats, androgens, more so than estradiol, modulate the expression of a variety of morphological and neurochemical endpoints in the hippocampus believed to underlie spatial cognition. In the hippocampus, androgens have been shown to affect synaptic plasticity (Pouliot et al., 1996; Harley et al., 2000; Sakata et al., 2000; Smith et al., 2002; Ziehn et al., 2012), acetylcholine release (Mitsushima et al., 2009; Ziehn et al., 2012), NMDA receptor binding (Kus et al., 1995; Romeo et al., 2005b) phosphorylation of protein kinases (Nguyen et al., 2005; Rossbach et al., 2007; Hatanaka et al., 2009; Carrier and Kabbaj, 2012), the expression of transcription and neurotrophic factors (Nguyen et al., 2009; Li et al., 2012a), neurogenesis (Spritzer and Galea, 2007; Benice and Raber, 2010), the density of synaptic spines in area CA1 (Leranth et al., 2003), and the morphology of dendrites in areas CA1 and CA3 (Hatanaka et al., 2009; Li et al., 2012a). As would be expected, many of these cellular responses are dependent upon androgen receptors (Nguyen et al., 2005; Hatanaka et al., 2009; Okamoto et al., 2012). Accordingly, impaired spatial learning and memory on water maze tasks result from administration of an androgen receptor antagonist directly into the hippocampus (Naghdi et al., 2001; Edinger and Frye, 2007) or genetic mutations of the receptor, which render it fully insensitive to androgens (Jones and Watson, 2005). Because androgen receptors also play a large role in mediating the stress response

(McCormick et al., 2002; Zuloaga et al., 2011), it is important to characterize the contributions of the receptors on spatial tasks that are inherently less stressful than water based tasks.

It has become increasingly apparent that the hippocampus contains the necessary enzymatic machinery required for the *de novo* synthesis of both estrogens and androgens (Hojo et al., 2004; Konkle and McCarthy, 2011). Although orchidectomy does not cause a reduction in levels of androgens in the hippocampus (Okamoto et al., 2012), the expression of androgen receptors in the hippocampus, particularly in area CA1, is significantly reduced following the removal of testicular hormones (Kerr et al., 1995; Xiao and Jordan, 2002). Therefore, it is tempting to consider that orchidectomy-induced impairments in spatial memory result from a reduction in the expression of androgen receptors in the hippocampus. Further support for this notion comes from findings in female rats in which estradiol treatment of ovariectomized rats resulted in both an enhancement in spatial cognition and higher levels of estrogen receptor α in the hippocampus (Rodgers et al., 2010).

The Role of Estrogen Receptors in Spatial Cognition

Together, the results of **Experiments 4a and 4b** indicate that activation of the putative estrogen membrane receptor GPR30 in ovariectomized rats mimics the enhancement in spatial recognition memory on a Y-maze task that occurs when levels of ovarian hormones are naturally elevated during proestrus. Consistent with the results of Experiment 3, no differences in activity were found during the retention trial as a function of stage of the estrous cycle or drug treatment, which indicates that memory

enhancements were not influenced by non-mnemonic factors. The enhancement in spatial recognition memory exhibited by rats that were in proestrus during the information trial of the Y-maze task in Experiment 4a is consistent with the results of previous studies (Conrad et al., 2004). Elevated levels of ovarian hormones that occur during proestrus also have been associated with enhanced spatial cognition on water maze tasks designed to characterize spatial reference memory (Frick and Berger-Sweeney, 2001; Rubinow et al., 2004; Sava and Markus, 2005), and on a variety of other tasks designed to assess spatial working memory, such as the object location task (Frye et al., 2007; Paris and Frye, 2008; Walf et al., 2009), the radial-arm maze task (Pompili et al., 2010) and the spontaneous alternation task (Walf et al., 2009).

To date, only two other studies, both from the same laboratory, have examined the relationship between the GPR30 receptor and cognition. In line with the results of Experiment 4b, chronic daily administration of 5 µg of G-1 to ovariectomized rats for over 1 month enhanced spatial learning on a delayed matching-to-position T-maze task and mimicked the beneficial effects of chronic estradiol treatment (Hammond et al., 2009). In a follow-up study, chronic daily administration of 40 µg of G-15, a GPR30 antagonist, for over 1 month abrogated the learning enhancing effects of simultaneous estradiol treatment on the same spatial task (Hammond et al., 2012). When considered in light of the results of the Y-maze task employed in Experiment 4b, activation of the GPR30 receptor is sufficient to enhance cognition in ovariectomized rats on a variety of spatial tasks.

Although the results of Experiment 4b illustrate a role for the putative estrogen membrane receptor GPR30 in spatial recognition memory, the contributions of estrogen

receptors α and β to spatial recognition memory remains to be determined. However, administration of agonists that selectively activate either estrogen receptor α or β have been show to enhance performance on a variety of other spatial tasks, which include object placement tasks (Frye et al., 2007; Walf et al., 2008; Jacome et al., 2010), a delayed matching-to-position T-maze task (Hammond et al., 2009), a radial-arm maze task (Liu et al., 2008), and reference memory versions of the water maze task (Rhodes and Frye, 2006; Qu et al., 2012). In line with these findings, the beneficial effects of estradiol in ovariectomized rats on spatial cognition are contingent upon activation of estrogen receptors, such that administration of antiestrogens abrogated the enhancing effects of estradiol on place learning (Zurkovsky et al., 2006) and spatial cognition (Chen et al., 2002). Likewise, when levels of estradiol are elevated, expression of estrogen receptor β was necessary for enhancements in spatial cognition to occur on an object location task (Walf et al., 2008), a water version of a delayed nonmatching-to-position Y-maze task (Liu et al., 2008), and a spontaneous spatial alternation task (Walf et al., 2009). Further, delivery of viral vectors that resulted in the overexpression of estrogen receptor α in the hippocampus was sufficient to enhance spatial learning and memory in aged ovariectomized rats (Witty et al., 2012) and ovariectomized mice with genetic deletion of estrogen receptor α (Foster et al., 2008). Importantly, activation of either the estrogen receptor α or β isoform does not enhance all aspects of spatial cognition. For instance, chronic administration of the estrogen receptor α agonist propyl pyrazole triol (PPT) or the estrogen receptor β agonist diarylpropionitrile DPN (Neese et al., 2010), mimicked the effects of chronic estradiol treatment (Wang et al., 2009b; Neese et al., 2010) and impaired performance on an operant delayed spatial alternation task. Examining the

contributions of the GPR30 receptor on spatial tasks that are adversely affected by estradiol would further elucidate the mechanisms by which estradiol influences different aspects of spatial cognition.

The Effects of Estradiol on Spatial Memory Consolidation

In Experiment 5, posttraining administration of estradiol enhanced memory for a place learning strategy on a series of probe trials conducted 24 h following completion of training on a dual-solution cued-platform water maze (CPWM) task. Ovariectomized rats treated with estradiol immediately following training exhibited a greater percentage of place learning responses on the probe trials than rats treated with vehicle. The results from Experiment 5 are consistent with a growing body of research that has demonstrated a role for estrogen during the period of memory consolidation, which occurs shortly after learning (Luine and Frankfurt, 2012). Notably, both estradiol (Packard et al., 1996; Packard and Teather, 1997b; Luine et al., 2003; Gresack and Frick, 2006; Frye et al., 2007; Walf et al., 2008; Inagaki et al., 2010; Jacome et al., 2010; Inagaki et al., 2012) and testosterone (Edinger et al., 2004) treatments enhanced hippocampus-based learning and memory in memory in gonadectomized rodents when administered immediately following training. For instance, administration of estradiol immediately following training on a single-solution version of the hidden-platform water maze task resulted in shorter escape latencies to reach the platform across a series of probe trials, which indicated an enhancement in spatial memory (Packard and Teather, 1997b). Results from Experiment 5, which featured similar training and testing demands to the paradigm employed by Packard and colleagues, extend these findings and demonstrate that the

enhancement in spatial cognition in ovariectomized rodents following posttraining administration of estradiol biases rats toward a place learning strategy on a task in which reliance upon a stimulus-response learning strategy also provides solution.

When considering the results from Experiment 4b, in which administration of the GPR30 agonist G-1 enhanced spatial recognition memory in ovariectomized rats, it is tempting to consider the role that estrogen receptors played in the preference for a place learning strategy exhibited by ovariectomized rats in Experiment 5. Posttraining administration of selective agonists for either estrogen receptors α (Frye et al., 2007) or β (Rhodes and Frye, 2006; Inagaki et al., 2010; Jacome et al., 2010) mimicked the spatial memory enhancing effects of estradiol on both object placement and water maze tasks. As is the case with Experiment 5, delivery of estradiol conjugated to cyclodextrin or suspended in ethanol enhances absorption and facilitates clearance of sex steroids (Pitha et al., 1986; Taylor et al., 1989), which accelerates the onset but limits the duration of estradiol action, thereby minimizing the contributions of the intracellular estrogen receptors α and β. Considering the limited window of action induced by delivery of estradiol in ethanol, and that GPR30 in the hippocampus (Brailoiu et al., 2007; Hammond et al., 2011) is likely involved in the rapid induction of intracellular processes that underlie cognition (Fan et al., 2010), it is conceivable that the greater preference for a place strategy in estradiol-treated rats was mediated by activation of GPR30 in the hippocampus. Alternatively, estradiol administered into the striatum also impaired response learning within 2 h of treatment (Zurkovsky et al., 2011). Therefore, we cannot rule out the possibility that the estradiol-induced place strategy preference on the dual-solution CPWM task was a manifestation of an impaired memory for use of a stimulus-

response learning strategy induced by the activation of GPR30 located in the striatum (Hammond et al., 2011).

In ovariectomized rodents, estradiol treatment has been shown to alter levels of general activity (Morgan and Pfaff, 2001), attentional processes (McGaughy and Sarter, 1999), affective outcomes (Bowman et al., 2002; Walf and Frye, 2005; Charoenphandhu et al., 2011), and the responsiveness of the HPA axis (Viau and Meaney, 1991), confounding effects that may impact the influence of estradiol on cognition when levels are elevated prior to and during training. Importantly, delayed administration of estradiol 2 h following completion of training does not influence cognition, which indicates that the memory enhancing effects on spatial memory (Packard and Teather, 1997b; Luine et al., 2003), and in all likelihood the place strategy bias observed in Experiment 5, occurred independent of non-mnemonic influences. Therefore, when considered in light of the results from previous research (Packard and Teather, 1997b), the greater preference for a place learning strategy on the dual-solution CPWM task following estradiol treatment emerged a function of enhanced mnemonic processing. With this in mind, the non-mnemonic influences of estradiol, in addition to the demands of the task, may explain the discrepancies in learning strategy preference exhibited by female rats in Experiments 1 and 5. Because elevated levels of estradiol potentiate the stress response (Viau and Meaney, 1991), the preference for a place learning strategy that typically emerges during proestrus (Korol et al., 2004) may have been abrogated by the stress associated with water maze testing on the dual-solution T-maze task in Experiment 1.

Anxiety, Spatial Learning and Memory, and Learning Strategy

Two important conclusions can be drawn from the results of **Experiment 6**. First, there was a high degree of concordance in spatial cognition across the tasks that characterized distinctly different types of spatial learning and memory. Performance on a land-based Y-maze task was correlated with learning strategy preference on a dual-solution visible-platform water maze (VPWM) task. Specifically, better spatial recognition of the novel arm of the Y-maze, indicated by a greater percentage of entries into the novel arm during the retention trial (Conrad et al., 1996), predicted a bias toward the use of a place learning strategy on the dual-solution VPWM task, which was indicated by longer path lengths on the probe trial (Kim et al., 2001). Second, the level of inherent, or trait anxiety, as indicated by behavior in an open field, was correlated with performance on both learning and memory tasks. Lower levels of anxiety, indicated by a greater percentage of time in the center of the open field, were associated with better place recognition memory on the Y-maze task and a bias toward the use of a place learning strategy on the dual-solution VPWM task.

Previous studies indicated that higher levels of anxiety expressed in an open field and on an elevated plus-maze were related to poorer performance on a hidden platform version of the water maze, an aversive spatial learning and memory task motivated by the stress associated with water escape (Herrero et al., 2006). Extending these findings, the results of Experiment 6 indicated that higher levels of inherent anxiety also are associated with poorer performance on a Y-maze task, a relatively non-aversive spatial learning and memory task (Conrad et al., 1996). Therefore, the results from the Y-maze task in Experiment 6, in conjunction with the results from the reference memory version of a

water maze task reported elsewhere (Herrero et al., 2006), indicate that natural variations in anxiety, which exist independent of stress exposure, modulate hippocampus-based spatial learning and memory.

More entries into the novel arm on retention trials of the Y-maze task has been interpreted as evidence of better spatial recognition memory (Conrad et al., 1996). However, it should be noted that novel arm entries could be influenced by other variables. For instance, rats with higher levels of trait anxiety might enter the novel arm of the Y-maze less frequently because of their fear of novelty rather than poorer place recognition memory. If this was the case, and performance on the Y-maze task characterized anxiety, rather than spatial cognition, then the alternative interpretation is still consistent with the notion that higher levels of anxiety are associated with a bias toward a striatum-based learning strategy (Packard and Wingard, 2004). However, Conrad and colleagues, who routinely employ the Y-maze task, demonstrated that rats exposed to chronic stressors exhibited an impairment in spatial recognition memory on the Y-maze task but displayed normal novelty-seeking behavior (Wright and Conrad, 2005). Therefore, although fear of the novel arm cannot be completely ruled out as a contributing factor in the results of Experiment 6, this interpretation seems unlikely.

In Experiment 6, the subject sample was not dichotomized into place learners and stimulus-response learners. Performance was evaluated as continuous variables reflected by behaviors in the open field and the dual-solution VPWM task (Kim et al., 2001). Rats that expressed lower levels of anxiety in the open field exhibited the hallmark behaviors indicative of place learning on the dual-solution VPWM task, longer path lengths to reach the escape platform. Conversely, rats that expressed higher levels of anxiety in the open

field demonstrated behaviors on the VPWM task that were indicative of stimulus-response learning, shorter path lengths to reach the escape platform. When considered in light of the notion that the hippocampus and the striatum interact to control learning (Packard and McGaugh, 1996; Gold, 2004), higher levels of trait anxiety negatively impact performance on hippocampus-based tasks (Herrero et al., 2006). Further, as is the case with the results of Experiment 6, higher levels of trait anxiety subsequently redirect control over learning toward the striatum on tasks that can be solved by relying on either brain structure.

The results from Experiment 6, in which heightened levels of trait anxiety were associated with both impaired spatial memory on the Y-maze task and a bias toward a stimulus-response learning strategy on the dual-solution VPWM task, are consistent with an increasingly growing body of literature that has examined the effects of anxiety on learning strategy. Administration of anxiogenics, which decreased the amount of time rats spent in the open arms of the elevated plus-maze, also impaired acquisition of a hippocampus-dependent task (Wingard and Packard, 2008), facilitated performance on a striatum-dependent task (Wingard and Packard, 2008), and resulted in a bias toward a response learning strategy on a dual-solution task in which reliance upon either memory system could be used to solve the task (Packard and Wingard, 2004). As would be expected, the memory-enhancing effects on the striatum-based memory system induced by anxiogenic agents are prevented by co-administration of an anxiolytic (Leong et al., 2012). Conceivably, administration of pharmacological agents that alleviate anxiety would attenuate impairments in spatial learning and memory (Herrero et al., 2006) and circumvent the bias toward striatum-based learning strategies exhibited by rats that

express naturally higher levels of anxiety, levels which may predispose rats to cognitive impairments following exposure to subsequent stressors (Bellani et al., 2006).

Stress, Reactivation of an Aversive Memory, and Learning Strategy

Collectively, the results from **Experiment 7** indicate that (1) exposure to a remote acute stressor biases rats toward a striatum-dependent learning strategy and (2) after the effects of an acute stressor subside, reactivation of the aversive memory maintains the preference for striatum-dependent learning strategies and heightened levels of emotionality. One week after exposure to an inescapable footshock, shocked rats exhibited lower levels of activity on the retention trial of a Y-maze task that was administered 30 min following the first reminder of the stressor. However, there was no effect of the initial stressor, or an additional reminder of the stressor, on spatial recognition memory on the Y-maze task. One month after exposure to footshock, rats that were shocked, but not reminded of the stressor, exhibited a bias toward a response learning strategy on a dual-solution water T-maze task. Likewise, shocked rats that were reminded of the stressor 30 min prior to the probe trial of the water T-maze task also tended to exhibit a preference for a response learning strategy. Two months after exposure to footshock, shocked rats that were reminded of the stressor 30 min prior to the probe trial of a dual-solution VPWM task exhibited a preference for a stimulus-response learning strategy. At this time point, rats exposed to the footshock, but never reminded of the stressor, as well as rats that were not shocked, did not demonstrate a bias for a stimulus-response learning strategy on the VPWM task. Four days following the probe trial of the VPWM, rats repeatedly reminded of the stressor exhibited significantly lower

levels of activity than both the group of rats that were not shocked, as well as the group that were shocked but never reminded of the stressor. Collectively, the results from Experiment 7 indicate that exposure to a single episode of stress induces long-lasting effects on learning strategy preference, effects that can be maintained by exposure to reminders of the stressor.

A large body of literature has documented the detrimental effects of acute stress on spatial learning and memory in rodents (Cazakoff et al., 2010). Of particular relevance to the findings from Experiment 7, exposure to a reminder of an acute stressor interferes with the retrieval of spatial memories (Zoladz et al., 2010). Specifically, exposure to a reminder of a footshock immediately prior to a retention trial on a water-based version of the radial-arm maze task effectively disrupted retrieval of a recently-formed spatial memory. The discrepancy between results from the study by Zoladz et al., (2010) and results from the Y-maze task in Experiment 7 are likely due to a combination of factors. First, the delay interval between the learning and the retrieval phase was 24 h in the study by Zoladz et al., (2010), whereas in the current study the delay was only 4 hours. A significantly longer delay on the Y-maze (McLaughlin et al., 2008) may have been necessary to detect differences in memory retrieval between stress conditions. An additional difference between the paradigms to take into consideration is the nature of the environment in which memory was assessed. Conceivably, in the study by Zoladz et al., (2010), the aversive nature of the water maze interacted with the reminder of the stressor to impact memory retrieval, an effect which was obscured by the relatively non-aversive nature of the Y-maze task in the current study.

On the retention trial of the Y-maze task in Experiment 7, all rats exposed to the initial footshock one week earlier, regardless of exposure to an additional reminder of the stressor, exhibited lower levels of activity as indicated by a reduced number of total arm entries. Consistent with this result, previous research has established that exposure to inescapable footshock often results in a generalized state of anxiety, which is indicated by lower levels of activity in novel environments (van Dijken et al., 1992; Baldi et al., 2004; Diehl et al., 2007; Daviu et al., 2010; Hawley et al., 2011a). As indicated by the total number of entries on the retention trial of the Y-maze task, exposure to a reminder of the stressor did not increase levels of hypoactivity induced by the footshock beyond that displayed by rats that were only exposed to footshock. However, it is interesting to note that three of the rats in the *shocked/reminded* stress condition failed to move from the start arm on the retention trial of the Y-maze. Therefore, although spatial recognition memory on the Y-maze task was not affected by reminders of the aversive event, exposure to a reminder of the stressor may have impacted anxiety and fear-like behavior (Korte et al., 1999), which may have contributed to the deficits in spatial cognition reported elsewhere (Zoladz et al., 2010). This interpretation is consistent with findings from Experiment 6 and those reported elsewhere, which indicate that higher levels of anxiety-like behavior are associated with poorer spatial recognition memory on the Y-maze task, as well as poorer reference memory on a water maze task (Herrero et al., 2006), and poorer place learning on single-solution learning strategy tasks (Wingard and Packard, 2008; Packard and Gabriele, 2009).

In Experiment 7, rats subjected to footshock 1 month earlier exhibited a propensity to adopt a response learning strategy on a dual-solution water T-maze task

regardless of additional exposure to a reminder of the stressor. Although not necessarily consistent with our original prediction, the preference for a response learning strategy exhibited by rats exposed to inescapable stress is a finding that dovetails with previous research. Specifically, when administered either immediately prior to training trials (Kim et al., 2005) or immediately prior to a retrieval trial (de Quervain et al., 1998), exposure to inescapable shock resulted in memory impairments on the probe trial of hippocampus-dependent versions of the water maze. In accordance with the current findings, exposure to shock prior to training on a water-based version of a dual-solution learning strategy task shifted control over learning away from the hippocampus and toward the striatum-dependent memory system (Kim et al., 2001). Rats exposed to inescapable shock exhibited a greater preference for a stimulus-response learning strategy on a probe trial conducted 24 h after training when compared to rats that were not subjected to inescapable shock. Similar to the findings from the dual-solution water T-maze task in Experiment 7, exposure to inescapable shock did not impact learning during training trials, rather, the deleterious effects on cognition manifested as an inability to retrieve a particular spatial memory (de Quervain et al., 1998; Kim et al., 2005), which subsequently biased rats toward using the striatum-dependent memory system (Kim et al., 2001). Therefore, the results from the dual-solution water T-maze task in Experiment 7 extend earlier findings, which indicated that repeated exposure to stressors (Schwabe et al., 2008; Ferragud et al., 2010), or to an acute stressor that terminated just prior to testing (Kim et al., 2001), biased rodents toward striatum-based learning strategies. Accordingly, exposure to inescapable shock prior to training on a dual-solution learning task resulted in a preference for a response learning strategy that extended well beyond 24 h.

Exposure to a single episode of inescapable stress exerts rather long-lasting effects on affective behaviors (Adamec et al., 2007; Belda et al., 2008; Mikics et al., 2008a; Mikics et al., 2008b). Furthermore, memory for footshock strengthens with the passage of time (McGaugh, 1966). Consistent with these findings, in Experiment 7, memory for the footshock strengthened from the reminder that took place just prior to the retrieval trial on the Y-maze task (Reminder I), which occurred 1 week after footshock, relative to the reminders that took place prior to the probe trials on the learning strategy tasks (Reminders II and III), which occurred 1 and 2 months after footshock. Of particular importance to the findings from the dual-solution water T-maze task employed in Experiment 7, in rodents, the deleterious effects of footshock on emotionality were most pronounced 28 days after exposure to footshock (Siegmund and Wotjak, 2007; Mikics et al., 2008a; Mikics et al., 2008b). For instance, mice subjected to a single episode of inescapable footshock exhibited maximal signs of generalized fear 28 days later, which was characterized by higher levels of freezing to a neutral tone that did not predict footshock, when compared to mice tested for generalized fear 1 day after exposure to the footshock stressor (Siegmund and Wotjak, 2007). Given the relationship between emotionality and learning strategy (Packard, 2009), the bias for a response learning strategy on the dual-solution water T-maze task in Experiment 7 expressed by rats exposed to footshock, but not subjected to subsequent reminders of the stressor, may have resulted from an extended period of fear incubation in which memory for footshoock strengthens as a function of time (Houston et al., 1999; Golub et al., 2009; Pickens et al., 2009; Pamplona et al., 2010).

It is worth noting, that in Experiment 7, the intensity (0.6 mA) and duration (3 s) of the footshock was considerably mild relative to those employed in previous studies, which demonstrated a long-lasting effect of footshock on emotionality (Siegmund and Wotjak, 2007; Mikics et al., 2008a; Mikics et al., 2008b). Perhaps, as the results of Experiment 7 and others indicate (de Quervain et al., 1998), when cognitive testing occurs in an aversive environment, such as a water maze, exposure to relatively milder forms of shock may be sufficient to impact spatial cognition, even when testing occurs at a much later time point. As would be expected, footshock stressors of greater intensity (Baldi et al., 2004; Mikics et al., 2008b) and longer duration (Pickens et al., 2009; Pickens et al., 2010) maintain the expression of fear-like behavior, which can last for upwards of 2 months after cessation of stress exposure (Pickens et al., 2009; Pickens et al., 2010). In Experiment 7, rats exposed to footshock, but not subjected to subsequent reminders of the stressor, may have exhibited a preference for a striatum-dependent learning strategy on the dual-solution VPWM task, which took place 2 months after exposure to footshock, had the intensity of the stressor been greater or of longer duration.

Two months after exposure to the initial stressor, the preference for a striatum-based learning strategy on the dual-solution VPWM task dissipated in shocked rats that were never reminded of the stressor in Experiment 7. In contrast, only shocked rats that were reminded of the aversive event exhibited a preference for a stimulus-response learning strategy on the dual-solution VPWM task. When considered within the context of previous studies (Zoladz et al., 2010), poorer performance on hippocampus-dependent tasks, which occurs following reactivation of an aversive memory, corresponds with the expression of a stimulus-response learning strategy. Admittedly, because rats in

Experiment 7 were subjected to three reminders of the stressor it is still uncertain if the stimulus-response learning strategy exhibited by rats on the dual-solution VPWM task was due entirely to the reminder that preceded the probe trial or to a cumulative effect of multiple reminders of the footshock stressor.

On the open field test in Experiment 7, the lower levels of activity exhibited by the *shocked/reminded* group relative to both the *not shocked/reminded* and the *shocked/not reminded* group indicates that repeated reactivation of the aversive memory heightened emotionality (van Dijken et al., 1992). Rodents reminded of an aversive event exhibited heightened levels of emotionality as indicated by less time spent on the open arms of an elevated plus-maze (Korte et al., 1999; Louvart et al., 2005), heightened startle reflexes (Pynoos et al., 1996), prolonged latencies to begin feeding on a novelty-suppressed feeding task (Hawley et al., 2011a; Hawley et al., 2012b), lower levels of social interaction (Louvart et al., 2006; Siegmund and Wotjak, 2007) and reduced sexual motivation (Hawley et al., 2011a; Hawley et al., 2012b). Exposure to reminders of the footshock may have been necessary to maintain the heightened levels of emotionality (Korte et al., 1999), which occurred in response to the initial stressor (van Dijken et al., 1992), required to rats toward use of the striatum-dependent memory system on the dual-solution VPWM learning strategy task that was conducted 2 months after the stressor. Interestingly, heightened levels of emotionality, which impair learning, can be maintained for an indefinite period of time by exposure to reminders of the initial footshock stressor (Maier, 2001). Given that retrieval of a spatial memory is vulnerable to disruption by reactivation of a remote aversive memory (Zoladz et al., 2010), it is likely

that a striatum-dependent learning strategy would dictate learning in rats reminded of the initial stressor for as long as the memory for the footshock persisted.

From a clinical standpoint, the results of Experiment 7 have important implications for our understanding of how stress-induced pathologies like posttraumatic stress disorder (PTSD) impact memory systems. PTSD is an anxiety disorder characterized by the unwanted re-experiencing of thoughts related to the initial trauma and by profound alterations in emotionality, which include a perpetual state of hyperarousal, hypervigilance, and heightened levels of anxiety (Association, 2000). In some instances, the hallmark re-experiencing of intrusive thoughts have been directly linked to both general measures of distress (Dougall et al., 1999; Schooler et al., 1999) and poorer cognitive functioning (Wessel et al., 2002). Together, with the results of Experiment 7 in mind, these findings suggest that intrusive thoughts are associated with a heightened state of emotionality, which can strengthen with time to adversely impact cognitive processes. Whether the re-experiencing of intrusive thoughts is sufficient to redirect control over learning toward the striatum-dependent memory system remains to be determined.

Neurobiological Modulators of Emotionality on Learning Strategy

From a neurobiological perspective, it is particularly intriguing to note that the amygdala is positioned to regulate both the consolidation of an aversive memory as well as the subsequent learning and memory processes that are impacted by stress exposure. Specifically, lesions of the amygdala impaired the recall of fear-associated stimuli (Maren et al., 1996; Wilensky et al., 1999, 2000) and eliminated stress-induced

impairments in spatial cognition (de Quervain et al., 1998; Kim et al., 2001; Kim et al., 2005). With regard to emotionality and learning strategy, the amygdala modulates the shift from the hippocampus to the striatum-based memory system when anxiety is elevated (Packard, 2009). Administration of an anxiogenic drug directly within the amygdala either prior to training (Packard and Wingard, 2004) or prior to a probe trial (Elliott and Packard, 2008) resulted in a preference for a response learning strategy. Furthermore, amygdala inactivation neutralized both the impairing and enhancing effects of anxiogenic drugs on hippocampus-dependent and striatum-dependent learning tasks, respectively (Packard and Gabriele, 2009). Therefore, impairments in spatial learning and memory induced by stress and anxiety (de Quervain et al., 1998; Kim et al., 2005; Sadowski et al., 2009; Schwabe et al., 2010a), which are modulated by the amygdala (Kim et al., 2001; Packard and Wingard, 2004; Packard and Gabriele, 2009), essentially redirect control over learning to the striatum-based memory system (Kim et al., 2001; Packard and Wingard, 2004; Elliott and Packard, 2008). This hypothesis is especially intriguing considering that electrical activation of the amygdala has been shown to suppress long-term potentiation (Vouimba and Richter-Levin, 2005) and dysregualte place cell firing in the CA1 field of the hippocampus (Kim et al., 2012), two phenomena believed to underlie spatial cognition. Conceivably, on the dual-solution VPWM task in Experiment 7, amygdala activation brought about by exposure to a reminder of the stressor resulted in a dysregulation in the functioning of the hippocampus that subsequently allowed the striatum-dependent memory system to emerge and assume control over learning.

In addition to the role of the amygdala, impairments in spatial memory retrieval following exposure to acute stressors are associated with elevated levels of corticosterone (Woodson et al., 2003; Park et al., 2008; Tronche et al., 2010; Li et al., 2012b). Moreover, while systemic administration of corticosterone is sufficient to block the retrieval of a recently formed spatial memory (Coburn-Litvak et al., 2003; de Quervain et al., 2003; Roozendaal et al., 2004; Khaksari et al., 2007; Ferguson and Sapolsky, 2008; Dorey et al., 2011), attenuation of corticosterone synthesis following exposures to either acute (de Quervain et al., 1998; Dorey et al., 2011) or chronic stressors (Wright et al., 2006) rescues stress-induced deficits in spatial cognition. Importantly, the stress-induced rise in corticosterone subsequently results in altered synaptic plasticity in the hippocampus (Diamond et al., 1996; Mesches et al., 1999; Kim et al., 2006; Wong et al., 2007; Cazakoff and Howland, 2010), an effect that is likely modulated by the actions of corticosterone and other neurotransmitters within the amygdala (Akirav and Richter-Levin, 2002; Roozendaal et al., 2004; Vouimba et al., 2006; Vouimba et al., 2007).

Much like the effects of acute stressors, exposure to a reminder of a footshock induces a rise in corticosterone (Hagewoud et al., 2011) and alters synaptic plasticity in the hippocampus (Li et al., 2005). Arguably, the actions of corticosterone may have contributed to the spatial memory impairments observed in previous studies (Zoladz et al., 2010) and the greater reliance on striatum-dependent learning strategies exhibited by rats on the dual-solution VPWM task in Experiment 7 that were reminded of an aversive event. In accordance with the idea that corticosterone release mediates stress-induced alterations in spatial cognition (de Quervain et al., 1998) and learning strategy preference (Schwabe et al., 2010b), deficits in spatial learning and memory exhibited by rats

classified as higher in trait anxiety were associated with reduced mineralocorticoid receptor binding, but not glucocorticoid receptor binding, in the hippocampus (Herrero et al., 2006). Individual differences in the neurochemical profiles of the hippocampus, as well as the amygdala, which impact stress reactivity and the detrimental effects of corticosterone, could account for the differences in spatial recognition memory and learning strategy exhibited in by rats in Experiment 6 that expressed high or low levels of trait anxiety.

Cholinergic Neurotransmission and Learning Strategy

The results from the dual-solution cued-platform water maze (CPWM) task in **Experiment 8** indicate that protein levels of choline acetyltransferase (ChAT), but not choline transporter (CHT), in the dorsal hippocampus, but not ventral hippocampus, were elevated in rats that adopted a place learning strategy relative to rats that adopted a stimulus-response learning strategy. Previous findings demonstrated that extracellular levels of acetylcholine in the hippocampus are indicative of learning strategy preference, such that rats that adopted a place learning strategy on the probe trial of a dual-solution T-maze task exhibited higher levels of acetylcholine release in the hippocampus both prior to and during training compared to rats that adopted a response learning strategy (McIntyre et al., 2003). In accordance with these findings, the results from Experiment 8 indicate that levels of the enzyme that synthesizes acetylcholine (ChAT) also are greater in hippocampus of rats that adopted a place learning strategy relative to rats that adopted a stimulus-response learning strategy, which supports the contention that cholinergic neurotransmission in the hippocampus is involved in learning strategy preference (Gold,

2003; Parent and Baxter, 2004). Taken together, natural variations in cholinergic neurotransmission in the hippocampus predispose rats toward adopting one learning strategy over the other.

Importantly, in Experiment 8, the relationship between learning strategy preference and protein levels of ChAT emerged in only the dorsal hippocampus, not the ventral hippocampus, an effect consistent with the results of lesion studies, which have demonstrated a greater role for the dorsal hippocampus in spatial cognition (Moser et al., 1995; Pothuizen et al., 2004; Zhang et al., 2004; Potvin et al., 2006). In addition, the results from Experiment 8 also align with previous studies that have examined the relationship between learning strategy preference and neurochemical transmission in the hippocampus. Temporary inactivation of sodium channels in the dorsal hippocampus results in a preference for a response learning strategy at a time point when a place learning strategy is typically expressed (Packard and McGaugh, 1996). Alternatively, posttraining administration of glutamate into the dorsal hippocampus maintains preference for a place learning strategy under conditions in which rats normally exhibit a preference for a response learning strategy (Packard, 1999). Despite these findings, the results from the microdialysis study conducted by McIntyre and colleagues (2003) were conducted by placing sampling probes into the ventral hippocampus to detect extracellular levels of acetylcholine, which indicates that neurotransmission in both subdivisions of the hippocampus are involved in learning strategy preference (McIntyre et al., 2003). However, up to this point, the relationship between acetylcholine release in the dorsal hippocampus and learning strategy preference remain to be determined. Given that the dorsal region of the hippocampus is more important for spatial cognition than the

ventral region (Moser et al., 1995), extracellular levels of acetylcholine in the dorsal hippocampus may serve as a better predictor of learning strategy preference that the ventral hippocampus.

In Experiment 8, comparable protein levels of CHT, the sodium-dependent presynaptic transporter that regulates choline uptake, were found in the hippocampus of rats that adopted a place learning strategy relative to rats that adopted a stimulus-response learning strategy. Although protein levels of CHT were not indicative of learning strategy preference on the dual-solution CPWM task in Experiment 8, elevated levels of ChAT and CHT activity in the hippocampus have both been positively correlated with better spatial and learning and memory (Tarricone et al., 1991; Tarricone et al., 1993). However, in light of the results of Experiment 8, it is important to note that ChAT activity in the hippocampus served as a better neurochemical marker of spatial learning and memory on a water maze task than CHT activity (Dunbar et al., 1993). Interestingly, with regard to CHT activity, elevations in choline uptake in the hippocampus were detectable only within a few minutes following completion of training on a spatial leaning task (Marighetto et al., 1993). With this in mind, it is reasonable to believe that levels of CHT activity in the hippocampus immediately after completion of a probe trial on a dual-solution learning task may serve as an indicator of learning strategy preference, despite the fact that there were no difference in protein levels of CHT between rats that adopted a place and rats that adopted a stimulus-response learning strategy in Experiment 8. In addition to examining CHT activity, differentiating between protein levels of CHT located at the presynaptic membrane, which regulate choline uptake, and those located within the cytoplasm, which have been shown to translocate to the membrane in response

to neural activity (Ferguson et al., 2003), would further elucidate the role of cholinergic neurotransmission in learning strategy preference.

The results from Experiment 8, in which levels of ChAT were elevated in rats that adopted a place learning strategy, provide further support the hypotheses that the hippocampus and striatum operate in parallel, and at times, compete for control over learning (Gold, 2004). In accordance with the results of Experiment 8, the expression of transcription factors, immediate early genes, and neuronal markers that indicate cell proliferation also were differentially expressed in the hippocampus as a function of learning strategy preference. For example, on a dual-solution learning task, levels of phosphorylated cAMP response element binding protein (CREB), a transcription factor that regulates gene expression, and *c-Fos*, an immediate early gene that dictates protein translation, were elevated in the hippocampus of rats that adopted a place strategy relative to rats that adopted a response strategy (Colombo et al., 2003). Consistent with these findings, and the idea that the hippocampus dictates preference for a place learning strategy, the expression of Ki67, an endogenous marker that signals cell proliferation, also was greater in the hippocampus of female rats that adopted a place learning strategy (Rummel et al., 2010). Interestingly, the relationship between cell proliferation and learning strategy preference appears to be quite different in male and female rats. Following training on a dual-solution task, lower levels of Ki67 were found in the hippocampus of male rats that adopted a place learning strategy relative to those that adopted a response learning strategy (Epp and Galea, 2009). When considered in light of the results from Experiment 8, these findings indicate that the expression of neurochemical endpoints believed to underlie spatial cognition are differentially

expressed in the hippocampus of rodents that exhibit a preference for a place learning strategy. Ostensibly, the nature of these relationships are contingent upon a variety of factors, including biological sex (Epp and Galea, 2009; Rummel et al., 2010) and the timing of brain tissue collection relative to completion of behavioral testing (Colombo et al., 2003).

The results of Experiment 8, in conjunction with those reposted elsewhere (McIntyre et al., 2003), indicate that cholinergic neurotransmission in the hippocampus is involved in learning strategy preference. Alternatively, previous research regarding the modulatory role that the neurotransmitter system in the hippocampus plays in dictating learning strategy preference has been disputed (Janis et al., 1998; Cahill and Baxter, 2001; Bizon et al., 2003; Jonasson et al., 2004; Fitz et al., 2008). With this in mind, the purpose of **Experiment 9** was to determine if disruption of cholinergic neurotransmission in the hippocampus was sufficient to bias rats toward a stimulus-response learning strategy. The results from Experiment 9 indicated that saline-treated rats exhibited a high-level of performance throughout the training phase of the dual-solution CPWM task, an effect which was not observed in other experiments. Conversely, intrahippocampus administration of scopolamine disrupted learning during the training phase of the task. The learning impairment exhibited by rats treated with scopolamine indicates that disruption of cholinergic neurotransmission in the hippocampus impairs learning on a task that can be solved by relying on either a place or stimulus-response learning strategy. Although incongruent with the idea that the hippocampus and striatum operate in an independent fashion to control different types of cognitive processes (Packard and McGaugh, 1992), as well as the idea that disruption of cholinergic neurotransmission in

the hippocampus spares striatum-based stimulus-response learning (Brito et al., 1983; Carli et al., 1997), the findings are likely the result of a combination of factors.

Prior to training on a dual-solution CPWM task, rats in Experiment 9 received 20 µg of scopolamine bilaterally into the hippocampus prior to learning. When administered into the hippocampus, lower doses than that used in Experiment 9 impaired performance on hippocampus-based navigational tasks (Carli et al., 1997; Riekkinen and Riekkinen, 1997). However, somewhat higher doses (35 µg) than the dose used in Experiment 9 also disrupted performance on some non-spatial navigational tasks (Brito et al., 1983). In addition, it also is important to note that acetylcholine release in the hippocampus increases during training on cognitive tasks that are typically considered to be striatum based (Pych et al., 2005b). Accordingly, extracellular levels of acetylcholine in the hippocampus increased well above baseline levels during training on a single-solution version of a response learning task (Pych et al., 2005b). Interestingly, levels of acetylcholine release in the hippocampus of rats during training on a response learning task were identical to levels of acetylcholine release in the hippocampus of rats during training on a place learning task (Pych et al., 2005b). Furthermore, regardless of the demands of the task, levels of acetylcholine in the hippocampus remained elevated above baseline levels throughout the duration of behavioral testing. Therefore, cholinergic neurotransmission in the hippocampus is involved in the initial stages of learning, regardless of the demands of the task, and disruption of cholinergic neurotransmission in the hippocampus with somewhat higher doses of scopolamine can compromise execution of both spatial and non-spatial learning strategies.

The preference for a place learning strategy early in training is contingent upon the demands of the task (Martel et al., 2007). Results from studies that used food-rewarded versions of a dual-solution T-maze task to investigate learning strategy preference found that rats typically prefer a place learning strategy early in training and switch to a response learning strategy as training progresses, which indicates that the two brain systems interact to control learning (Packard and McGaugh, 1996; Cahill and Baxter, 2001; McIntyre et al., 2003; Espina-Marchant et al., 2009). However, rats expressed a preference for a stimulus-response learning strategy following limited training on a dual-solution CPWM task, which was similar to the task employed in Experiment 9. These findings raise the possibility that the hippocampus processes both spatial information (Packard and McGaugh, 1996) and stimulus-response associations (Martel et al., 2007) early in training. This interpretation is especially tempting to consider given the bi-directional interactive nature of the hippocampus and striatum-based memory systems (Lee et al., 2008), and that acetylcholine release is elevated in the hippocampus independent of the cognitive demands of the task (Pych et al., 2005b). Administration of scopolamine following completion of training (Packard and Teather, 1997b) would minimize the potential influence of non-mnemonic effects during training to elucidate the role of cholinergic neurotransmission in the hippocampus on learning strategy preference.

With regard to modulators of cholinergic transmission in the hippocampus, previous research indicates that the combination of ovariectomy and exposure to chronic stress resulted in both a reduction in levels of choline acetyltransferase in the hippocampus and deficits in hippocampus-based learning and memory (Takuma et al.,

2012). In accordance with the working model depicted in Figure 1, deficits in hippocampus-dependent learning and memory as a function of stress exposure (Srikumar et al., 2006), loss of ovarian hormones (Packard and Teather, 1997b), or a combination of both factors (Takuma et al., 2012) were abrogated by drugs that enhance cholinergic neurotransmission. Therefore, it is tempting to consider that in the current set of experiments impairments and enhancements in spatial recognition memory, as well as biases in learning strategy preference, which emerged as a function of gonadal hormones or alterations in affective states, were mediated by cholinergic neurotransmission in the hippocampus.

Summary and Conclusions

Collectively, the results from Experiments 1-5 indicated that male rats exhibited a greater preference for a place learning strategy than female rats on certain learning tasks, an effect that was independent of the activational effects of testosterone. Alternatively, in female rats, higher levels of estradiol immediately following training, but not during training, resulted in a greater preference for a place learning strategy. More importantly, in both sexes, higher levels of gondal hormones were associated with enhanced spatial learning and memory. Further, in female rats, activation of the estrogen membrane receptor GPR30 was sufficient to enhance spatial cognition. The results from Experiments 1-5 contribute to growing body of literature, which indicates that gonadal hormones impact not only *how much* information is learned, but also, *how* information is learned.

Experiments 6 and 7 indicate that subtle alterations in emotionality impact spatial cognition and learning strategy preference. Inherent levels of anxiety, which exist independent of stress exposure or elevations in state anxiety, were associated with both poorer spatial learning and memory and a bias toward a striatum-based learning strategy. In accordance with these findings, exposure to a brief footshock induced long-lasting changes in learning strategy preference, which was indicated by a greater preference for a striatum-based learning strategy 1 month after stress exposure. However, after the effects of the initial stressor subside, additional reminders of the stressor, which heightened emotionality, maintained the preference for striatum-based learning strategies. The results from Experiments 6 and 7 have important implications for furthering our understanding of the affective factors that contribute to the relative use of multiple memory systems.

The results from Experiments 8 and 9 align well with previous findings and support the hypotheses that cholinergic neurotransmission in the hippocampus is involved in learning strategy preference. Specifically, in the hippocampus, preference for a place learning strategy on a dual-solution task was associated with higher levels of a specific protein that regulates cholinergic neurotransmission. However, disruption of cholinergic neurotransmission in the hippocampus disrupted learning on a task that could be solved by executing place or stimulus-response learning strategies. When considered together, the results of Experiments 8 and 9 indicate that although cholinergic neurotransmission is involved in learning strategy preference, learning on certain dual-solution tasks is contingent upon optimal cholinergic neurotransmission in the hippocampus.

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Biography

Wayne Hawley was born June 10th, 1975 in Brockport, New York. He obtained a Bachelor of Science degree in Psychology from The State University of New York at Brockport in 2005 and a Master of Arts degree in Experimental Psychology from Indiana State University in 2008. Shortly thereafter, his interest in the effects of stress and sex-steroids on learning and memory led him to the graduate program in Psychology at Tulane University, where he worked under the mentorship of Gary Dohanich to pursue a doctoral degree in Psychology with a specialization in Behavioral Neuroscience.