INFLUENCE OF ESTRADIOL ON THE ABILITY OF IGF-I TO IMPACT HIPPOCAMPAL-DEPENDENT MEMORY AND HIPPOCAMPAL SYNAPTIC PROTEINS

AN ABSTRACT
SUBMITTED ON THE SIXTH DAY OF JUNE 2014
TO THE DEPARTMENT OF PSYCHOLOGY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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BY

MELZA R VAN ROIJEN

APPROVED:

JILL DANIEL, Ph.D.
Director

GARY DOHANICH, Ph.D.

BETH WEE, Ph.D.
The ability of insulin-like growth factor-I (IGF-I) to impact the hippocampus and associated behaviors may vary depending upon estrogenic status. Previous work from our lab demonstrated that chronic antagonism of brain IGF-I receptors (IGF-IR) resulted in increased levels of hippocampal synaptic proteins in control-treated ovariectomized (OVX) rats. In contrast, antagonism of brain IGF-IR decreased levels of synaptic proteins in estradiol-treated OVX rats. The goal of the current experiment was to test the hypothesis that effects of chronic agonism of IGF-IR, via peripheral treatment with IGF-I, on hippocampal-dependent memory would also vary with estrogenic status. Furthermore, we assessed the influence of estrogenic status on the ability of IGF-I to impact levels of hippocampal synaptic proteins. OVX rats received chronic peripheral treatment with estradiol or cholesterol control via silastic implants, as well as IGF-I or vehicle via osmotic minipumps. One week after surgeries, place learning and memory on the Morris water maze was assessed via eight place-training trials on the first day and four place-training trials on the second day of testing. Place learning and memory was measured using mean swim path length. Following place training, a probe trial was conducted to assess memory for the location of the hidden platform. Memory on the probe trial was measured via percent time in the target quadrant. Animals were euthanized 24 hours following behavioral testing, and hippocampi were processed for western blotting to determine levels of hippocampal synaptic proteins PSD-95, spinophilin, and synaptophysin. Results revealed no effects of treatment on behavioral measures or on levels of hippocampal synaptic proteins. These data indicate that chronic peripheral administration of IGF-I does not affect hippocampal-dependent memory in a
Morris water maze task and does not impact hippocampal synaptic protein levels in the presence or absence of peripheral estradiol.
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INTRODUCTION

It has been well established that hormones play an important role in mammalian physiology and behavior, but recent advances in research have guided our focus to their effects in the brain and possible implications for learning and memory. Hormones such as insulin-like growth factor-I (IGF-I) and 17β-estradiol (estradiol), the principal estrogen generated by the ovaries, assist in numerous cognitive processes including neurogenesis, neuroprotection, neuroplasticity (Cardona-Gomez, Mendez, DonCarlos, Azcoitia, and Garcia-Segura, 2001) and learning and memory (Daniel, Fader, Spencer, and Dohanich, 1997; Markowska, Mooney, and Sonntag, 1998; Thornton, Ingram, and Sonntag, 2000). Although much of the previous research has been centered around the hypothalamus and sex behaviors, the hippocampus, a brain region important for spatial memory (Rudy, 2008), has also been shown to contain a large number of receptors for these hormones (Araujo, Lapchak, Collier, Chabot, and Quirion, 1989; Bohacek and Daniel, 2009).

Mechanisms of action: IGF-I and estradiol

While the liver is the primary source of peripheral IGF-I synthesis, many tissues throughout the body produce IGF-I in response to localized growth hormone (GH) receptor activation (Liu, Yakar, and LeRoith, 2000). Circulating IGF-I is able to cross the blood-brain barrier (Aberg, Aberg, Hedbacker, Oscarsson, and Eriksson, 2000), and disruption of this transport can impede exercise-induced neurogenesis in the hippocampus (Trejo, Carro, and Torres-Aleman, 2001). IGF-I binding proteins (IGFBPs) bind approximately 99% of the circulating IGF-I, which limits IGF-I receptor (IGF-IR) activity, but provides transport and extends the half-life of the hormone (Janssen and Lamberts, 1999). Free IGF-I, which comprises the remaining percentage of circulating
hormone, binds to IGF-IR causing phosphorylation of the IGF-IR and insulin-receptor substrate-1 (IRS-1) (Sonntag, Ramsey, and Carter, 2005). Phosphorylation of IRS-1 then triggers the IGF-1 signaling cascade that activates extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and phosphotidylinositol-3-kinase/protein kinase B (PI3K/Akt) pathways (Sonntag et al., 2005). These downstream pathways have implications in learning and memory because of their ability to promote protein synthesis, enzyme activation and inactivation, gene expression, and vesicle trafficking (Saltiel and Kahn, 2001).

Estradiol is predominantly synthesized in the ovaries, but the adrenal gland, testes, and brain (Prange-Kiel, Wehrenberg, Jarry, and Rune, 2003) are also capable of estradiol production. During classical nuclear receptor-mediated action, estradiol binds nuclear estrogen receptors (ERα or ERβ) and forms dimers (Vasudevan and Pfaff, 2007), which bind to estrogen response elements (EREs) (Klinge, 2000). This newly formed complex prompts gene transcription, which results in enhanced neuroprotection, synaptogenesis, and long-term potentiation (LTP) (Mukai, Kimoto, Hojo, Kawato, Murakami, Higo, Hatanaka, and Ogiue-Ikeda, 2010). Additionally, estradiol can activate non-classical or membrane-mediated pathways. In this case, estradiol binds to membrane-bound ERs that trigger ERK/MAPK (Zhao and Brinton, 2007) and PI3K/Akt (Spencer-Segal, Tsuda, Mattei, Waters, Romeo, Milner, McEwen, and Ogawa, 2012), cellular kinase cascades that exert rapid effects and may also result in gene transcription.

**IGF-I, estradiol, and cognition**

Diminished levels of peripheral IGF-I (Koltai, Zhao, Lacza, Cselenyak, Vacz, Nyakas, Boldogh, Ichinoseki-Sekine, and Radak, 2011;Thornton et al., 2000) and brain
IGF-I (Sonntag, Bennett, Khan, Thornton, Xu, Ingram, and Brunso-Bechtold, 2000) are associated with decreased neurogenesis in the hippocampus, as well as impaired spatial memory (Trejo, Llorens-Martin, and Torres-Aleman, 2008). Furthermore, rats with increased levels of peripheral IGF-I, resulting from growth-hormone-releasing hormone (GHRH) treatment, performed significantly better on a spatial memory task than their control counterparts (Thornton et al., 2000). Aged male rats given intracerebroventricular (ICV) infusions of IGF-I showed increased hippocampal IGF-I concentrations (Sonntag et al., 2000) as well as improved spatial memory during a place discrimination task (Markowska et al., 1998). Additionally, administration of systemic GHRH partially restores IGF-I levels in senescent rats (Thornton et al., 2000). These findings suggest that localized and circulating IGF-I may be critical components in hippocampal-dependent memory.

Localized and circulating estradiol have also been implicated in hippocampal-dependent memory. Diminished serum estradiol, following menopause, is associated with cognitive deficits in women (Sherwin, 1988). Ovariectomy in rats, an animal model of menopause, results in decreased dendritic spine density in the CA1 region of the hippocampus (Woolley and McEwen, 1992). Furthermore, increased spine density in this area has been correlated with the formation and expression of associative memories in a hippocampal-dependent task (Leuner, Falduto, and Shors, 2003). Even natural fluctuations in estrogen throughout the ovarian cycle affect hippocampal-dependent memory. Low-estrogen periods have been linked to diminished cognitive performance on spatial memory tasks compared to high-estrogen periods (Frick and Berger-Sweeney, 2001), and clinical trials revealed a correlation between decreased estrogen and impaired
verbal memory in women (Rosenberg and Park, 2002; Sherwin, 2003). Our lab, however, has demonstrated that estradiol administration shortly after the cessation of ovarian function mitigates this cognitive deficit in rats (Daniel et al., 1997; Daniel, Hulst, and Berbling, 2006; Rodgers, Bohacek, and Daniel, 2010).

Mechanisms through which IGF-I and estradiol affect cognition

One possible mechanism through which IGF-I (Sonntag et al., 2000) and estradiol (Woolley et al., 1992) affect cognition is through their ability to induce structural changes in hippocampal synapses by increasing dendritic spine density. This increase enhances synaptic efficacy, an integral part of memory formation, by permitting new and strengthened contacts between cells (Leuner et al., 2003). Parallel increases in molecular components of the synapse, including pre- and postsynaptic proteins, have been implicated in many processes that influence cognition such as signal transduction (Migaud, Charlesworth, Dempster, Webster, Watabe, Makhinson, He, Ramsay, Morris, Morrison, O'Dell, and Grant, 1998), receptor organization (Kim and Sheng, 2004), and regulation of dendritic spine formation and function (Feng, Yan, Ferreira, Tomizawa, Liao, Zhuo, Allen, Ouimet, and Greengard, 2000). For example, transgenic mice that are genetically manipulated to have a mutant form of postsynaptic density-95 (PSD-95), a support protein involved in the maturation of excitatory synapses (El-Husseini, Schnell, Chetkovich, Nicoll, and Bredt, 2000), show a dramatic decline in spatial learning (Migaud et al., 1998). Postsynaptic proteins PSD-95 and spinophilin, a protein responsible for the regulation of dendritic spine development, maintenance, and morphology (Feng et al., 2000), have been correlated with synapse and spine density proliferation, and are therefore used as indicators of synaptic formation (Spencer, Waters,
Romeo, Wood, Milner, and McEwen, 2008). Synaptophysin, a presynaptic vesicle protein, is also considered a marker of synaptic formation (Spencer et al., 2008).

Hormone-induced increases in hippocampal neurogenesis are also believed to enhance hippocampal-dependent memory. IGF-I (Aberg et al., 2000; Lichtenwalner, Forbes, Bennett, Lynch, Sonntag, and Riddle, 2001; Trejo et al., 2001) and estradiol (Ormerod, Lee, and Galea, 2003) both regulate neurogenesis in the dentate gyrus, which is noteworthy because cognitive impairments have been associated with deficits in this area (Lichtenwalner et al., 2001). Intriguingly, the two hormones given in unison have a synergistic effect on neurogenesis (Perez-Martin, Azcoitia, Trejo, Sierra, and Garcia-Segura, 2003), which suggests that their mechanisms of action may be related.

**Interaction of IGF-I and estradiol in the brain**

The interaction of IGF-I and estradiol is believed to impact cognition by regulating hippocampal synaptic plasticity. One mechanism by which this may be accomplished is through the cross-regulation of IGF-I and estrogen receptor expression. In rat brains, estradiol and ER activity can modulate IGF-IR expression, and IGF-IR activity can control ER expression (Cardona-Gomez et al., 2001). Furthermore, IGF-I is able to regulate ER-mediated transcription by triggering intracellular kinase signaling pathways. For instance, IGF-I utilizes the ERK/MAPK pathway to enhance the activity of ERα in the absence of estradiol (Patrone, Gianazza, Santagati, Agrati, and Maggi, 1998), and uses the PI3K/Akt pathway to diminish the activity of ERα in the presence of estradiol (Mendez and Garcia-Segura, 2006). Additionally, estradiol is able to control IGF-IR signaling by activating ERK/MAPK (Cardona-Gomez et al., 2001) and PI3K/Akt (Cardona-Gomez, Mendez, and Garcia-Segura, 2002) pathways.
Further evidence that IGF-I and estradiol share a common mechanism emerged with the administration of specific antagonists, which create a pharmacological blockade of IGF-I and estradiol receptors. The neuroprotective effects of estradiol were impeded when the IGF-IR antagonist JB1 was administered, and the neuroprotective effects of IGF-I were prevented when the ER antagonist ICI 182,780 was administered (Azcoitia, Sierra, and Garcia-Segura, 1999).

Previous work in our lab demonstrated that IGF-I infused ICV increased levels of PSD-95 and IGF-I receptor (IGF-IR) in the hippocampus of young adult ovariectomized (OVX) rats chronically treated with estradiol (Mainguy M., 2012). In another experiment, IGF-I infused ICV decreased levels of PSD-95 and IGF-IR in the hippocampus of young adult OVX rats not treated with estradiol (Mainguy M., 2012). In a separate set of experiments, in which ICV administration of JB1 was used to block brain IGF-IRs, estradiol-induced increases in PSD-95, spinophilin, and synaptophysin were blocked in the hippocampus, and estradiol-induced improvements in spatial working memory were attenuated (Nelson, Springer, and Daniel, 2014). Intriguingly, in the absence of estradiol, JB1 actually increased hippocampal levels of PSD-95, spinophilin, and synaptophysin. Taken together, these studies suggest that IGF-I and JB1 influence levels of synaptic hippocampal proteins differently in the presence or absence of estradiol. The effects of IGF-I may therefore be contingent on estradiol exposure, which would suggest that crosstalk between IGF-I, estradiol, and their associated receptors has important implications in hippocampal-dependent learning and memory.
**Goal of experiment and hypothesis**

The goal of the current study was to determine the influence of estradiol on the ability of IGF-I to impact the hippocampus and hippocampal-dependent memory. At this time no study has investigated how peripheral administration of IGF-I affects place learning and levels of hippocampal synaptic proteins in the presence or absence of estradiol. To achieve this, young adult ovariectomized rats received estradiol or cholesterol vehicle capsules as well as osmotic minipumps that delivered IGF-I or saline to the periphery. The Morris water maze was employed to test differences in place learning. Additionally, tissue was collected to assess independent and interactive effects of exposure to estradiol and treatment with IGF-I on hippocampal levels of the postsynaptic proteins PSD-95 and spinophilin as well as the presynaptic protein synaptophysin.

Based on previous work in our lab that showed antagonism of brain IGF-IR attenuated estradiol-induced improvements in spatial working memory (Nelson et al., 2014), we hypothesized that agonism of IGF-IR, via peripheral administration of IGF-I, would enhance spatial memory on the Morris water maze in young ovariectomized rats in the presence of estradiol; however, IGF-I would not have an effect in the absence of estradiol. Furthermore, we anticipated estradiol-controls would have impaired performance compared to cholesterol-controls, as has been previously reported in a study of spatial memory (Daniel, Roberts, and Dohanich, 1999). Additionally, because antagonism of brain IGF-IR decreased levels of hippocampal synaptic proteins in the presence of estradiol and increased levels in the absence of estradiol (Nelson et al., 2014), we hypothesized that agonism of IGF-IR would increase levels of hippocampal
PSD-95, spinophilin, and synaptophysin in the presence of estradiol and decrease levels in the absence of estradiol. Moreover, we predicted estradiol-controls would have higher levels of hippocampal synaptic proteins compared to cholesterol-controls, as has been previously reported (Nelson et al., 2014).

MATERIALS AND METHODS

Subjects

Forty-eight female Long-Evans hooded rats (approximately 2 months of age) were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). Rats were housed individually in a temperature-controlled vivarium under a 12-hour light, 12-hour dark cycle (lights on at 7:00 A.M.) and had unrestricted access to food and water. Surgeries and subsequent behavior testing and killing of the animals were conducted in two cohorts of 24 rats each to facilitate procedures. All groups were equally represented in all cohorts. Animal care was in accordance with guidelines set by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996), and all procedures were approved by the Institutional Animal Care and Use Committee of Tulane University.

Ovariectomy, estradiol, and IGF-I treatment

One week after arrival, rats were ovariectomized while under anesthesia induced by injection of ketamine (100 mg/kg, ip; Bristol Laboratories, Syracuse, New York) and xylazine (7 mg/kg, ip; Miles Laboratories, Shawnee, Kansas) and implanted with 5-mm SILASTIC brand capsules (0.058 in. inner diameter and 0.077 in. outer diameter; Dow Corning, Midland, Michigan) on the dorsal side of their necks. The capsules contained
either 25% 17β-estradiol (E) (Sigma-Aldrich, St Louis, Missouri) diluted with cholesterol or 100% cholesterol (Ch) vehicle. We reported previously that implants of these dimensions and estradiol concentrations maintain blood plasma estradiol levels of 26-47 pg/ml, which fall within the physiological range of cycling female rats (Bohacek and Daniel, 2007; Bohacek and Daniel, 2010). Along with the capsule, an osmotic minipump (model 2004, ALZET) was inserted subcutaneously on the dorsal side of the neck. Pumps delivered either IGF-I (5 µg/kg per day; GroPep Pty., Adelaide, Australia) or diluted 0.9% saline vehicle (Veh), at a flow rate of 0.25 µL/h. In combination with exercise, peripheral chronic administration of IGF-I at this dose and regime has been shown to increase hippocampal neurogenesis in aged rats (Koltai et al., 2011). Hormone treatments were designed such that half of the rats given estradiol received IGF-I (E + IGF-I, n = 12) and half received vehicle (E + Veh, n = 12). Similarly, half of the rats given cholesterol received IGF-I (Ch + IGF-I, n = 12) and half received vehicle (Ch + Veh, n = 12). Following surgeries, animals were given one week to recover before behavioral testing.

**Morris water maze**

One week after surgeries, rats were habituated to the Morris water maze before being tested on a spatial learning and memory task. Habituation consisted of a 60 second, individual free swim in a white circular pool (1.8 m in diameter and 0.6 m in height). The pool was filled with water (to a depth of approximately 35 cm), and made opaque by adding 150 ml of non-toxic white tempera paint (Crayola, Easton, PA). The water temperature was maintained at approximately 25°C. Extramaze cues surrounding the pool were fixed at specific locations and were visible to the rats while in the maze. One day after habituation, a transparent Plexiglass escape platform (12 cm in diameter) was placed
roughly 2 cm below the water surface, in the southwest quadrant of the pool. Maze performance was recorded by a video camera suspended above the maze and interfaced with a video tracking system (HVS Imaging, Hampton, UK). Place learning was assessed on eight place-training trials on the first day and four place-training trials on the second day. Each female was placed into the pool from a quasirandom start point and allowed a maximum of 60 seconds to escape to the platform where it remained for 15 seconds. Rats that failed to escape were guided to the platform by the experimenter. The position of the platform remained constant across trials. Trials were separated by two minutes and thirty seconds. Performance was assessed using mean swim path length. After the final place-training trial on the second training day, the platform was removed for a 60 second probe trial. Percent time spent in each of the four quadrants of the pool was recorded.

**Western blotting**

Western blotting procedures were used to determine the independent and interactive effects of exposure to estradiol and treatment with IGF-1 on hippocampal levels of the postsynaptic proteins PSD-95 and spinophilin and the presynaptic protein synaptophysin.

**Tissue dissection and processing**

One day after behavioral testing, the second cohort of rats were killed by decapitation under anesthesia induced by ketamine and xylazine. Hippocampi from the left and right hemisphere were dissected on ice, quick-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, 0.1μM microcystin, and 1%
protease inhibitor cocktail (Sigma-Aldrich). Samples were centrifuged for 15 min at 1000 x g at 4 C, protein concentration of supernatants were 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 each sample, 15 μg of total protein was loaded and separated at 200 V on 10% SDS-PAGE gels (Bio-Rad) for 60 minutes (PSD-95, spinophilin/neurabin-II, synaptophysin). Molecular weight markers (Kaleidoscope; Bio-Rad) were included with each run. Proteins were transferred to nitrocellulose membranes at 100 V for 60 minutes. The membranes were cut to allow for simultaneous development of the protein of interest with the loading control, β-actin. The membranes were blocked with 5% nonfat dry milk in 0.1% Tween/1 x Tris-buffered saline (TTBS) at room temperature for 1 h. This was followed by incubation with primary antibody for PSD-95 (rabbit monoclonal, 1:15,000; Millipore, Billerica, MA), spinophilin or neurabin II (mouse monoclonal, 1:1,125; Santa Cruz; Santa Cruz, CA), synaptophysin (mouse monoclonal, 1:10,000; Sigma, St. Louis, MO) and β-actin (mouse monoclonal, 1:15,000; Santa Cruz; Santa Cruz, CA) overnight at 4 C in 5% nonfat dry milk-TTBS (PSD-95), 1% nonfat dry milk-TTBS (spinophilin and synaptophysin), or TTBS (β-actin). Blots were washed three times for 15 min each with TTBS and incubated with 5% nonfat dry milk containing goat anti-rabbit IgG (Santa Cruz, Santa Cruz, CA; PSD-95, 1:10,000) and goat anti-mouse IgG (Santa Cruz, Santa Cruz, CA; spinophilin, 1:4,000; synaptophysin, 1:10,000; β-actin, 1:10,000). Blots were washed again for 15 min each and incubated for 1 min with the chemiluminescent
substrate SuperSignal West Femto (Pierce) for PSD-95 or with the chemiluminescent substrate ECL (Pierce) for spinophilin, synaptophysin, and β-actin. They were then exposed to film (Kodak Biomax MR) for varying durations to capture optimal signal intensity. Films were imaged using MCID 7.0 imaging software (Interfocus Imaging Ltd., Cambridge, England), and a single userdefined template was established for each blot to measure optical density x area (DxA) for bands of interest. Mean values were expressed as a percentage relative to control β-actin for each blot. Samples from each group were equally represented on each blot.

**Estradiol treatment efficacy**

At the time the rats were killed, uteri were extracted and 1-cm-long sections of the right uterine horns (cut at the base) were weighed to verify efficacy of estradiol treatment. Additionally, proper removal of the ovaries and integrity of the implanted capsules were verified. One animal from the E + Veh group was excluded (from all analyses) after uterine weight indicated ineffective estradiol treatment.

**Statistical analyses**

Data were analyzed using SPSS Statistics 19.0 (IBM Corp., Armonk, NY). Place learning data across all 12 trials (two-trial blocks), as measured by mean swim path length, were analyzed using repeated measures ANOVA (estradiol treatment x IGF-I treatment x two-trial block). Memory over 24 hours (day one trial eight and day two trial one) was evaluated using repeated measures ANOVA (estradiol treatment x IGF-I treatment x trial). Percent time spent in the target quadrant during the probe trial was analyzed using a two-way ANOVA (estradiol treatment x IGF-I treatment) with a between-subjects design. Mean swim speed was also assessed for place learning across
all 12 trials (two-trial blocks) using a repeated measures ANOVA (estradiol treatment x IGF-I treatment x two-trial block). Additionally, a repeated measures ANOVA (estradiol treatment x IGF-I treatment x trial) was used to analyze mean swim speed for day one, trial eight and day two, trial one. Western blotting data were analyzed with a two-way ANOVA (estradiol treatment x IGF-I treatment). A one-way ANOVA, with estradiol treatment as the factor, was used to test for differences in uterine weight.

RESULTS

Water maze

As illustrated in Fig. 1, there were no significant main or interactive effects of estradiol treatment and/or IGF-I treatment on place learning and memory in the Morris water maze. Analyses revealed no effects of treatments on place learning over 12 trials (two-trial blocks) (See Fig. 1A). However, there was a significant main effect of block, $F(5,215) = 26.841, p < .001$, indicating all groups learned the task over the two day period. Similarly, there were no effects of treatments on memory for the location of the hidden platform over 24 hours (See Fig. 1B). There was, however, a significant main effect of trial, $F(1,43) = 8.648, p < .05$, indicating an effect of the 24 hour delay on memory. Finally, there were no effects of treatments on memory for the location of the hidden platform during the probe trial (See Fig. 1C). Results suggest that chronic peripheral administration of IGF-I does not influence hippocampal-dependent memory, as measured in the Morris water maze, in the presence or absence of estradiol.
Additionally, there were no main or interactive effects of estradiol treatment and/or IGF-I treatment on mean swim speed over 12 trials (two-trial blocks) (See Fig. 2A). Analyses revealed a significant main effect of block, $F (5,215) = 3.989, p < .05$, (See Fig. 2B). Contrasts suggest all rats swam more slowly during earlier trials compared to later trials. Furthermore, although there was no interactive effect of IGF-I treatment by block on mean swim speed over 12 trials (two-trial blocks) (See Fig. 2A), there was a significant interaction of estradiol treatment by block, $F (5,215) = 2.538, p < .05$, indicating mean swim speed differed by block depending upon estradiol treatment (See Fig. 2B). Analyses revealed no effects of treatments or trial on mean swim speed over 24 hours.

**Western blots**

*PSD-95*

Western blots revealed a single band of PSD-95-like immunoreactivity at approximately 95 kilodaltons (kDa). Analyses revealed no significant main or interactive effects of estradiol treatment and/or IGF-I treatment on protein levels of PSD-95 in the hippocampus (See Fig. 3). Additionally, there were no effects of treatments on levels of β-actin, the loading control. Furthermore, estradiol-controls did not have significantly higher levels of hippocampal PSD-95 compared to cholesterol-controls. Results indicate that chronic peripheral administration of IGF-I does not affect levels of hippocampal PSD-95 in the presence or absence of estradiol.

*Spinophilin*

Western blots revealed a single band of spinophilin/neurabin-II-like immunoreactivity at approximately 132 kDa. Analyses revealed no significant main or
interactive effects of estradiol treatment and/or IGF-I treatment on protein levels of spinophilin in the hippocampus (See Fig. 4). Additionally, there were no effects of treatments on levels of β-actin, the loading control. Furthermore, estradiol-controls did not have significantly higher levels of hippocampal spinophilin compared to cholesterol-controls. Results indicate that chronic peripheral administration of IGF-I does not affect levels of hippocampal spinophilin in the presence or absence of estradiol.

**Synaptophysin**

Western blots revealed a single band of synaptophysin-like immunoreactivity at approximately 37 kDa. Analyses revealed no significant main or interactive effects of estradiol treatment and/or IGF-I treatment on protein levels of synaptophysin in the hippocampus (See Fig. 5). Additionally, there were no effects of treatments on levels of β-actin, the loading control. Furthermore, estradiol-controls did not have significantly higher levels of hippocampal synaptophysin compared to cholesterol-controls. Results indicate that chronic peripheral administration of IGF-I does not affect levels of hippocampal synaptophysin in the presence or absence of estradiol.

**Hormone treatment efficacy**

As illustrated in Fig. 6, there was a significant difference between groups in uterine weight, $F (1,45) = 67.23, p < .001$, indicating that estradiol treatments were effective. Estradiol-treated rats had larger uteri (mean ± SEM, 77.96 ± 4.95 mg) than cholesterol-treated rats (30.75 ± 3.05 mg).
DISCUSSION

The results of the present study indicate that agonism of IGF-IR, via chronic peripheral administration of IGF-I, does not affect place learning and memory on the Morris water maze in young ovariectomized rats in the presence or absence of estradiol as originally hypothesized. These findings conflict with previous work that showed antagonism of brain IGF-IR attenuated estradiol-induced improvements in spatial working memory (Nelson et al., 2014). Furthermore, performance on the place learning and memory task is not impaired in estradiol-controls vs. cholesterol-controls, as has been previously reported (Daniel et al., 1999). Additionally, chronic peripheral administration of IGF-I does not differentially impact levels of hippocampal PSD-95, spinophilin, or synaptophysin depending upon estrogenic status. These results contrast with former findings that showed antagonism of brain IGF-IR decreased hippocampal synaptic protein levels in the presence of estradiol and increased hippocampal synaptic protein levels in the absence of estradiol (Nelson et al., 2014). Moreover, the current study did not show an estradiol-induced increase in hippocampal synaptic protein levels vs. cholesterol-controls, as has been formerly shown (Nelson et al., 2014).

Inconsistencies between the behavioral tasks employed in the current and former study may have attributed to our conflicting results. Supplemental aerobic exercise and stress, that are associated with the Morris water maze but not the radial-arm maze, may have interactive effects with IGF-I and/or estradiol. For instance, it has been established that both systemic IGF-I injection and aerobic exercise facilitate the uptake of blood IGF-I by neurons in the brain (Carro, Nunez, Busiguina, and Torres-Aleman, 2000). Exercise also significantly enhances spatial memory and increases blood and hippocampal IGF-I
levels in young male rats (Cetinkaya, Sisman, Kiray, Camsari, Gencoglu, Baykara, Aksu, and Uysal, 2013). Furthermore, testing on the Morris water maze has been shown to prevent spine synapse density increases in the CA1 of OVX rats that typically occur with estradiol administration in behaviorally naïve animals (Frick, Fernandez, Bennett, Prange-Kiel, MacLusky, and Leranth, 2004). In addition to exercise, stress associated with the current behavioral task may have been an important factor. Previous work has shown that acute stress leads to a decrease in plasma IGF-I (Davis and Peterson, 2006) and an increase in serum estradiol (Shors, Pickett, Wood, and Paczynski, 1999), in sunshine bass and rats respectively. Finally, it has been demonstrated that IGF-I infusions significantly improve place learning on the Morris water maze in old male rats (Markowska et al., 1998). The aforementioned findings suggest IGF-I and estradiol may have interactive effects with exercise and/or stress that influenced our results. The Morris water maze was chosen for the current study because previous research has shown IGF-I to effectively attenuate spatial memory deficits on this task (Lupien, Bluhm, and Ishii, 2003; Trejo et al., 2008).

Additionally, although it has been established that IGF-I can cross the blood-brain barrier, it is conceivable that peripheral administration of the hormone, used in the present study, influenced the body and brain differently than ICV infusions, employed in the former study (Nelson et al., 2014). For instance, in the current study, the IGF-IR agonist was administered via a subcutaneous mini-pump implant, which delivered the hormone throughout the entire periphery. Thus, effects of IGF-I were not restricted to the brain. In the former study, a mini-pump was attached to a brain cannula that delivered the IGF-IR antagonist directly into the lateral ventricle (Nelson et al., 2014), which at least
partially limited the area influenced by the drug. Thus, disparate routes of administration may have led to different non-mnemonic effects that altered results. Our current study used peripheral administration, instead of ICV infusions, because we aimed to more closely mimic the beneficial influences of circulating IGF-I on the hippocampus (Trejo et al., 2001) and hippocampal-dependent memory (Lupien et al., 2003). Additionally, peripheral administration has more applications for human use than ICV infusions.

Finally, there may have been complications with the estradiol capsules used in the current study that would have resulted in our rats having unintentionally high mean blood plasma estradiol levels. Because OVX significantly reduces circulating estradiol, capsules were implanted to maintain estradiol levels in OVX rats at approximately the mid-physiological range of gonadally intact females (Bohacek et al., 2007; Bohacek et al., 2010). Recent work in our lab, however, has indicated that capsules made from the same 25% 17β-estradiol used in the current study yielded mean blood plasma estradiol levels in the high-physiological range (~51 pg/ml) (Svorinic, 2014), instead of the intended mid-physiological range (26-47 pg/ml) used in prior studies (Bohacek et al., 2007; Bohacek et al., 2010; Mainguy M., 2012; Nelson et al., 2014). Researcher variability and/or human error may have attributed to these results. It should be noted, however, that the mean blood plasma estradiol level for OVX cholesterol-treated rats was higher (~21 pg/ml) (Svorinic, 2014) than the typical physiological range of OVX control animals. We have previously reported in OVX rats that cholesterol-control implants produce estradiol levels below detection limit of the assay (8.70 ± 2.77 pg/ml) (Bohacek et al., 2010). These results suggest that the assay may have been inaccurate. If the assay was in fact correct, however, these findings imply that our rats may have received an unintentionally high
dose of estradiol, which would explain why our current results do not replicate former
findings.

In summary, the results of the present study indicate that agonism of IGF-IR, via
chronic peripheral administration of IGF-I, does not affect place learning and memory on
the Morris water maze in young ovariectomized rats in the presence or absence or
estradiol as originally hypothesized. Performance on the place learning and memory task
is also not impaired in estradiol-controls vs. cholesterol-controls. Additionally, chronic
peripheral administration of IGF-I does not differentially impact levels of hippocampal
PSD-95, spinophilin, or synaptophysin depending upon estrogenic status. Moreover,
estradiol treatment does not increase levels of hippocampal synaptic proteins compared to
cholesterol-controls. For future experiments, further investigation into the interactive
effects of IGF-I, estradiol, exercise and stress; routes of administration; and effects of
estradiol-dose are needed.
REFERENCES


FIGURE CAPTIONS

Fig. 1. Effects of estradiol treatment and/or IGF-I treatment on spatial memory during the Morris water maze task. Place learning over 12 trials as measured by mean swim path length. Performance displayed in two-trial blocks. Learning was exhibited by a decrease in mean swim path length over 12 trials, indicating all groups learned the task (Fig. 1A). Memory for the location of the hidden platform over 24 hours as measured by mean swim path length. Change in performance from day one trial eight to day two trial one (Fig. 1B). Memory for the location of the hidden platform as measured by percent time spent in the target quadrant during the probe trial (Fig. 1C). There were no effects of estradiol treatment and/or IGF-I treatment on spatial memory during the place discrimination task.

Fig. 2. Effects of estradiol treatment and/or IGF-I treatment on mean swim speed during the Morris water maze task. Overall motor abilities as measured by mean swim speed over 12 trials (Fig. 2A). Mean swim speed over 12 trials, displayed in two-trial blocks (Fig. 2B). There were no main or interactive effects of estradiol treatment and/or IGF-I treatment on mean swim speed over 12 trials (two-trial blocks). There was a main effect of block, \( p < .05 \). Rats swam more slowly during block one vs. all other blocks, \( *p < .05 \). Additionally, rats swam more slowly during block two vs. all subsequent blocks, \( **p < .05 \).
Fig. 3. Effects of estradiol treatment and/or IGF-I treatment on protein levels of PSD-95 in the hippocampus. There were no effects of estradiol treatment and/or IGF-I treatment on protein levels of PSD-95 in the hippocampus.

Fig. 4. Effects of estradiol treatment and/or IGF-I treatment on protein levels of spinophilin in the hippocampus. There were no effects of estradiol treatment and/or IGF-I treatment on protein levels of spinophilin in the hippocampus.

Fig. 5. Effects of estradiol treatment and/or IGF-I treatment on protein levels of synaptophysin in the hippocampus. There were no effects of estradiol treatment and/or IGF-I treatment on protein levels of synaptophysin in the hippocampus.

Fig. 6. Effects of estradiol treatment on uterine weight. There was a significant difference between estradiol-treated and control-treated groups in uterine weight, **p < .001, indicating that estradiol treatments were effective.
Figure 1.

A

Day 1

Day 2

2-Trial Blocks

B

Day 1 Trial 8 & Day 2 Trial 1

C

Probe Trial

Path Length (m)

% Time in Target Quadrant

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Day 1 Trial 8 & Day 2 Trial 1

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Figure 2.

A

Swim Speed (meters/sec)

Ch + Veh  Ch + IGF-I  E + Veh  E + IGF-I

B

Day 1  Day 2

Swim Speed (meters/sec)

1  2  3  4

2-Trial Blocks
Figure 3.
Figure 4.
Figure 5.

![Graph showing DxA % Beta-Actin for different groups: Ch + Veh, Ch + IGF-I, E + Veh, E + IGF-I. The graph compares Synaptophysin and β-Actin levels across these groups.]
Figure 6.