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**Investigating the Effects of Intrahippocampal Glucose Administration on Spatial Working
Memory in Rats**

An Honors thesis presented to the
Department of Biological Sciences
University at Albany, State University of New York
in partial fulfillment of the requirements
for graduation with Honors in Biological Sciences
and
graduation from The Honors College

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Second Reader: Paolo E. Forni, Ph.D.

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Abstract

Insulin is a peptide hormone released by beta pancreatic cells. Insulin's best-known function is to regulate absorption of glucose into peripheral tissue: this occurs via activation of the phosphoinositide 3-kinase (PI3K) signaling cascade and subsequent translocation of glucose transporter 4 (GLUT4) to the cell surface. This canonical peripheral insulin signaling pathway appears to exist in essentially identical form within the central nervous system (CNS), so that insulin promotes entry of glucose into neural cells and subsequent increased metabolism. In order to maintain proper function, insulin-responsive hippocampal neurons and glia require glucose metabolism; a catabolic energy-yielding process that requires insulin signaling to provide glucose. *In vivo* microdialysis studies have shown difficulty-correlated depletion of hippocampal extracellular fluid (ECF) glucose concentration during a cognitively-demanding task, indicating that the hippocampus not only depends on glucose metabolism for proper function but regularly functions under conditions of suboptimal supply. We hypothesized that blockade of insulin signaling might attenuate glucose-induced hippocampal cognitive enhancement via disruption of the insulin signaling pathway, preventing the entry of additional glucose via GLUT4. The present study aimed to investigate insulin's role as a mediator of the enhancement of spatial working memory by exogenous glucose *in vivo*. Unexpectedly, unilateral intrahippocampal administration of supraphysiological glucose in a novel, larger microinjection volume significantly impaired spatial working memory. Our data also suggest that within the hippocampus, insulin signaling may possibly work through alternative substrates, such as insulin-like growth factors I or II. Administration of supraphysiological glucose resulted in no significant effect on hippocampal function, suggesting that unilateral administration may be insufficient to enhance hippocampal function. Our results also suggest glucose-induced improvement of hippocampal function may work in a dose-dependent manner in the hippocampus. These unexpected findings contribute to the knowledge of glucose-induced hippocampal enhancement and its relation to proper insulin signaling.

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Introduction

Insulin is a peptide hormone secreted by the pancreas to regulate the absorption of blood glucose into tissue. Insulin is largely known to work in the periphery as a hormonal regulator of glucose uptake in muscle and adipose tissue. Insulin is also known to regulate glucose metabolism through the activation of the phosphoinositide 3-kinase (PI3K) signaling cascade and subsequent translocation of glucose transporter 4 (GLUT4). Insulin receptors are tyrosine receptor kinases. When insulin binds to the insulin receptor, the intracellular domains of the insulin receptor auto-phosphorylate at tyrosine residues, enabling downstream tyrosine phosphorylation of insulin receptor substrates (IRS) 1 and 2, and PI3K. PI3K activates protein kinase B (Akt), leading to the translocation of the insulin-responsive glucose transporter 4 to the cell surface. Importantly, this canonical insulin signaling pathway appears to be similar in both the periphery and the central nervous system, specifically including the hippocampus (McEwen & Reagan, 2004; Reagan, 2005). Studies have shown that the hippocampus expresses a high level of insulin receptors (Dore, Kar, Rowe, & Quirion, 1997), as well as GLUT4 (McEwen & Reagan, 2004; Reagan, 2005; Vannucci et al., 1998) and PI3K (Figlewicz & Szot, 1991), suggesting it is insulin-responsive. Consistent with this, insulin has been shown to enhance hippocampal function evidenced by increased performance in a spontaneous alternation task following intrahippocampal administration (McNay et al., 2010). Our laboratory has found success in using a small antibody-like anti-insulin peptide to impair spatial working memory by inactivating intrahippocampal insulin (McNay et al., 2010), confirming a key role for endogenous intrahippocampal insulin in memory processes.

There is extensive literature regarding the effects of exogenous glucose on cognition. Previous work has shown an enhancement of spatial working memory following intraperitoneal

administration of glucose in rats (McNay, Fries, & Gold 2000). Also, intra-amygdala bilateral administration of a supraphysiological dose of glucose has been shown to reverse memory deficits caused by intra-septal morphine administration (McNay & Gold, 1998). Additionally, bilateral administration of supraphysiological glucose enhances learning acquisition in a T-maze (Canal, 2005). Supraphysiological glucose represents a concentration of glucose larger than that of the concentration of glucose in non-manipulated hippocampal extracellular fluid (ECF). In the periphery, increases in glucose concentration trigger pancreatic insulin release via activation of ATP-gated potassium channels. The same mechanism exists in the hypothalamus to regulate neuronal firing rates, where glucose acts to signal satiety, and this machinery is also present in the hippocampus. Glucose metabolism has been shown to play an important role in hippocampal function, as both a regulatory and limiting factor (McNay et al., 1998; McNay & Recknagel, 2011). *In vivo* microdialysis studies have shown difficulty-correlated depletion of hippocampal extracellular fluid (ECF) concentrations of glucose during a cognitively-demanding task, indicating that the hippocampus depends on glucose metabolism for proper function and that this function may in fact be limited by glucose supply (McNay et al., 2000). Therefore, it is plausible to hypothesize the hippocampal enhancement by glucose is mediated by insulin signaling. In the present study, we hypothesize that intrahippocampal insulin blockade would attenuate or prevent glucose-induced hippocampal enhancement. Based on the hypothesis, animals co-administered the anti-insulin peptide and supraphysiological glucose should demonstrate less enhancement of spatial working memory.

Spatial working memory is mediated mainly by the dorsal hippocampus and can therefore serve as a marker for hippocampal function. Spontaneous alternation testing has been extensively used in our laboratory and others as a method to measure glucose-sensitive hippocampal cognitive

function (McNay et al., 2000; McNay et al., 2010). According to the optimal foraging theory, rats systematically search new places and environments searching for food (Dember, W.N., 1989). Rats use spatial working memory to remember which place was last visited in order to explore a new place. Spatial working memory could be illustrative of hippocampal functioning as a factor of glucose utilization and insulin signaling or availability in the CNS.

In previous work, neuron-specific insulin receptor knockout (NIRKO) mice have been used to probe the importance of neuronal insulin receptors in cognition and glucose metabolism (Schubert et al., 2003). NIRKO mice showed no alteration in memory or basal neuronal glucose metabolism, potentially suggesting that neuronal insulin receptors are of little importance in glucose metabolism and memory performance (Schubert et al., 2003). However, NIRKO mice are generated with a constitutive knockout of the insulin receptor. The brain compensates for the lack of neuronal insulin receptors, and therefore NIRKO mice do not exhibit a decrement in normal memory performance and basal glucose metabolism. Furthermore, astrocytic insulin receptor knockout mice showed alterations in behavior and metabolic processes (Garcia-Caceres et al., 2016). In this study, astrocytic insulin receptors were deleted in mice at 6 weeks old rather than at conception, therefore restricting the brain's ability to compensate. Their alterations in behavior and metabolic processes further show the effect of insulin signaling via control of blood glucose uptake in the central nervous system (Garcia-Caceres et al., 2016). In the absence of astrocytic insulin receptors, the insulin signaling pathway can no longer be activated, and GLUT4 is not translocated to the cell membrane. In turn, there is less glucose uptake by the cell, less glucose metabolism, and improper function of the central nervous system.

The role of insulin in glucose-induced improvement of hippocampal function has not been fully understood. The present study aimed to investigate insulin's role as a potential mediator of

the enhancement of spatial working memory by glucose in an *in vivo* rat model. Intrahippocampal insulin was inactivated to determine whether glucose can act to enhance hippocampal function independent of insulin's action at the insulin receptor. The current experiment was designed to determine whether glucose-induced enhancement of hippocampal function is dependent upon insulin-activated insulin signaling.

Experiment 1

Materials and Methods

Animals

Subjects were male Sprague-Dawley rats, CD sub-strain (Charles River Breeding Laboratories, Wilmington, MA). Rats were received at 10 weeks old. Rats were housed in pairs, with food and water available *ad libitum*, and were maintained on a 12-hour light-dark cycle (on 0700, off 1900). All testing procedures were completed during the light phase. Prior to surgery, rats were handled for a minimum of five minutes daily for a week. Following surgery, rats were handled a minimum of five minutes daily prior to behavioral testing.

Surgery

Standard sterile stereotaxic procedures were used to unilaterally implant a 6 mm microinjection guide cannula (Plastics One, Torrington, CT) into the left dorsal hippocampus at 11 weeks old as described previously (McNay et al., 2000). Rats were anesthetized by inhalation of 5%:2% isoflurane:oxygen. The rat was weighed to determine injection dosages. The anesthetization was maintained at 3%:2% isoflurane:oxygen inhalation via nosecone for the duration of the surgery. Saline was administered subcutaneously (s.c) (1 mL before incision and 3 mL upon completion) to maintain hydration. A 5 mg/kg dose of carprofen (s.c) and a 10 mg/kg dose of enrofloxacin (s.c) were administered prior to the incision. The coordinates used for targeting the dorsal hippocampus were +4.6 mm lateral from the midline, -5.6 mm posterior to bregma, and 3.3 mm ventral from the dura based on Paxinos and Watson (1997). After non-specific placement of two anchor screws on the skull, the microinjection guide cannulae were implanted and secured with dental cement. Immediately following surgery, rats were placed in an incubated

recovery cage until they recovered from anesthesia. Rats received doses of carprofen (s.c., 5 kg/mg) and enrofloxacin (s.c., 10 kg/mg) daily for three days post-surgery.

Reagents

The vehicle for all groups was artificial ECF (aECF; 153.5mM Na, 4.3mMK, 0.41mMMg, 0.71mM Ca, 139.4mM Cl, 1.25mM glucose, buffered at pH 7.4 (McNay & Sherwin, 2004b)) including the anti-insulin peptide (Abcam, ab31906, Cambridge, United Kingdom) and anti-Erb2, the anti-insulin peptide control (Abcam, ab31889, Cambridge, United Kingdom). Supraphysiological glucose concentration was determined to be 33.4 mM according to McNay and Gold, (1998).

Microinjection

Microinjections were given to maze-tested animals over eight minutes in a total volume of 1 μ L (0.13 μ L/min) into the left dorsal hippocampus twenty minutes prior to testing. Injections were administered through a 33 ga injection cannula that extended 1.0 mm below the guide cannula. The injection cannula was connected to a 25 μ L Hamilton syringe via fluorinated ethylene propylene (FEP) tubing (Harvard Apparatus, Holliston, MA), which was locked into an infusion pump (Harvard Apparatus, Holliston, MA). Injection cannula remained in place for two minutes following the completion of injection to ensure full diffusion of the drug.

Experimental Groups

Subjects were randomly assigned to four groups in a 2 (glucose) x 2 (anti-insulin peptide) factorial design: (1) supraphysiological glucose in aECF and anti-insulin peptide ($N=6$); (2) supraphysiological glucose in aECF and anti-insulin peptide control ($N=6$); (3) basal glucose in aECF and anti-insulin peptide ($N=6$); and (4) basal glucose in aECF and anti-insulin peptide

control ($N=7$). The experimenter was blinded to the drug conditions until the completion of testing to eliminate potential for experimenter-bias.

Table 1. The experimental design. SP denotes supraphysiological concentration of glucose (33.4 mM).

Experimental Groups

		Glucose Conditions	
		+Glucose	-Glucose
Affibody Conditions	+Anti-insulin peptide (ab31906)	+Glucose (SP) +Anti-insulin peptide	-Glucose (Basal) +Anti-insulin peptide
	+Affibody Control (anti ErbB2)	+Glucose (SP) +Affibody Control	-Glucose (Basal) +Affibody Control

Spontaneous Alternation Testing

Ten minutes after the initiation of microinjection, rats were placed into the center of a four-arm elevated plus-maze facing the same direction each time and were allowed to explore for twenty minutes. The plus-maze was cleaned with 70% ethanol both before testing and in between subjects. Spontaneous alternation has been extensively used to test spatial working memory as a measure of hippocampal function (McNay et al., 2000). A successful entry/exit is defined by all four limbs of the rat crossing the threshold of the arm. The measure of spatial working memory performance used was percent 4/5 alternation. An alternation is counted when the rat visits all four arms within any span of five consecutive arm choices. The maximum number of alternations is $N-4$, where N

is the total number of arms entered. The actual number of alternations made is expressed as a percentage of this number (McNay et al., 2010). Chance level on this measure is 44%.

Euthanasia and Molecular Testing

Rats were euthanized via isoflurane and decapitation ten minutes after completion of spontaneous alternation testing. Brains were rapidly extracted, the left and right hippocampi were dissected, and blood plasma was collected. All samples were immediately placed on dry ice for subsequent protein analysis.

Results

Five subjects receiving supraphysiological glucose scored below chance level (44%) during spontaneous alternation testing. A chi-square analysis was conducted to determine whether there was arm bias. Two subjects exhibited arm bias and therefore were excluded from further analysis. In order to assess whether supraphysiological glucose affected spatial working memory performance, a 2 (basal glucose, supraphysiological glucose) X 2 (anti-insulin peptide, anti-insulin peptide control) ANOVA was conducted. Contrary to the hypothesized outcome, there was a main effect of supraphysiological glucose ($F(1,19)=7.313, p<.05$) collapsed across peptide conditions, indicating that supraphysiological glucose significantly impaired spatial working memory ($M=47.9, SD=11.7$) compared to basal glucose ($M=60.4, SD=9.1$). There was not a main effect of type of peptide used ($F(1,19)=0.229, p>.05$) collapsed across glucose conditions, indicating that the anti-insulin peptide did not impair spatial working memory ($M=55.7, SD=11.9$) compared to anti-insulin peptide control ($M=54, SD=12.5$). There was no significant interaction between anti-insulin peptide conditions and glucose conditions ($F(1,19)=0.179, p>.05$).

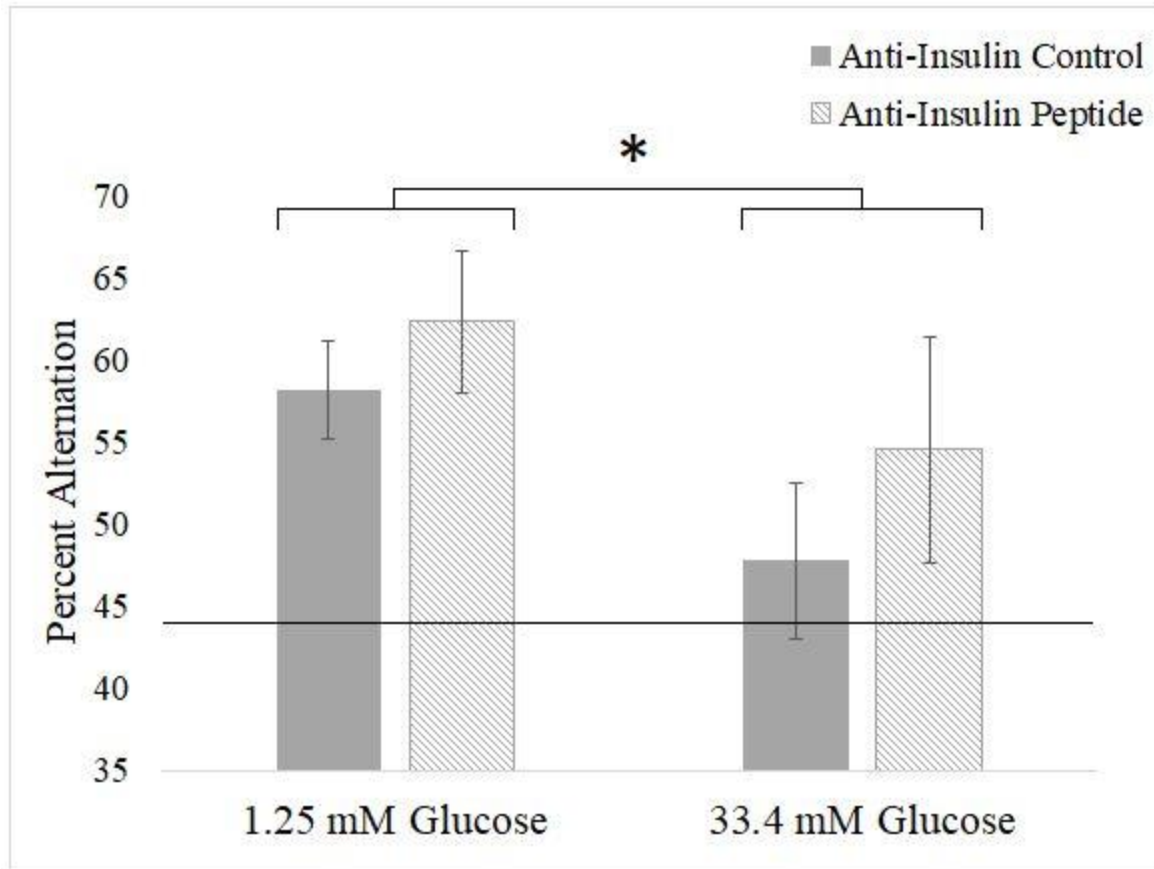


Figure 1. Mean 4/5 percent alternation performance on a +-maze, expressed as a percentage of possible alternations. A horizontal line is included to display chance level of performance (44%). Error bars represent +/-1 SEM. ANOVA: $F(1,19)=7.313$. * $p<.05$.

Experiment 2

In Experiment 1, we found discrepant results after attempting to enhance hippocampal function as measured by spontaneous alternation testing via intrahippocampal administration of 1.0 μL glucose (33.4 mM) and anti-insulin peptide control in a 1:1 ratio. Here, we adjusted our methods to replicate past literature by administering glucose to the left hippocampus in a standard volume (0.5 μL).

Materials and Methods

Subjects were male Sprague-Dawley rats, CD sub-strain (Envigo, Huntingdon, United Kingdom). Subjects were housed, handled, and surgicized as described in Experiment 1. aECF containing supraphysiological glucose (33.4 mM glucose) and normal aECF (1.25 mM glucose) solutions were created as described in Experiment 1. Microinjection, behavioral testing, and euthanasia were performed as described in Experiment 1 with an exception: while the microinjection rate remained 0.13 $\mu\text{L}/\text{min}$, the volume decreased from 1 μL to 0.5 μL .

Experimental Groups

Subjects were randomly assigned to two groups: (1) supraphysiological glucose in aECF ($N=7$) and (2) basal glucose in aECF ($N=8$). The experimenter was blinded to the drug conditions until the completion of testing to eliminate the potential of experimenter-bias.

Results

In order to assess the effects of supraphysiological glucose on spatial working memory, an independent group *t*-test was conducted. There was no significant effect of supraphysiological glucose ($t(13)=0.412, p>.05$) indicating that supraphysiological glucose ($M=55.4, SD=7.9$) did not affect spatial working memory compared to basal glucose ($M=57.8, SD=12.9$).

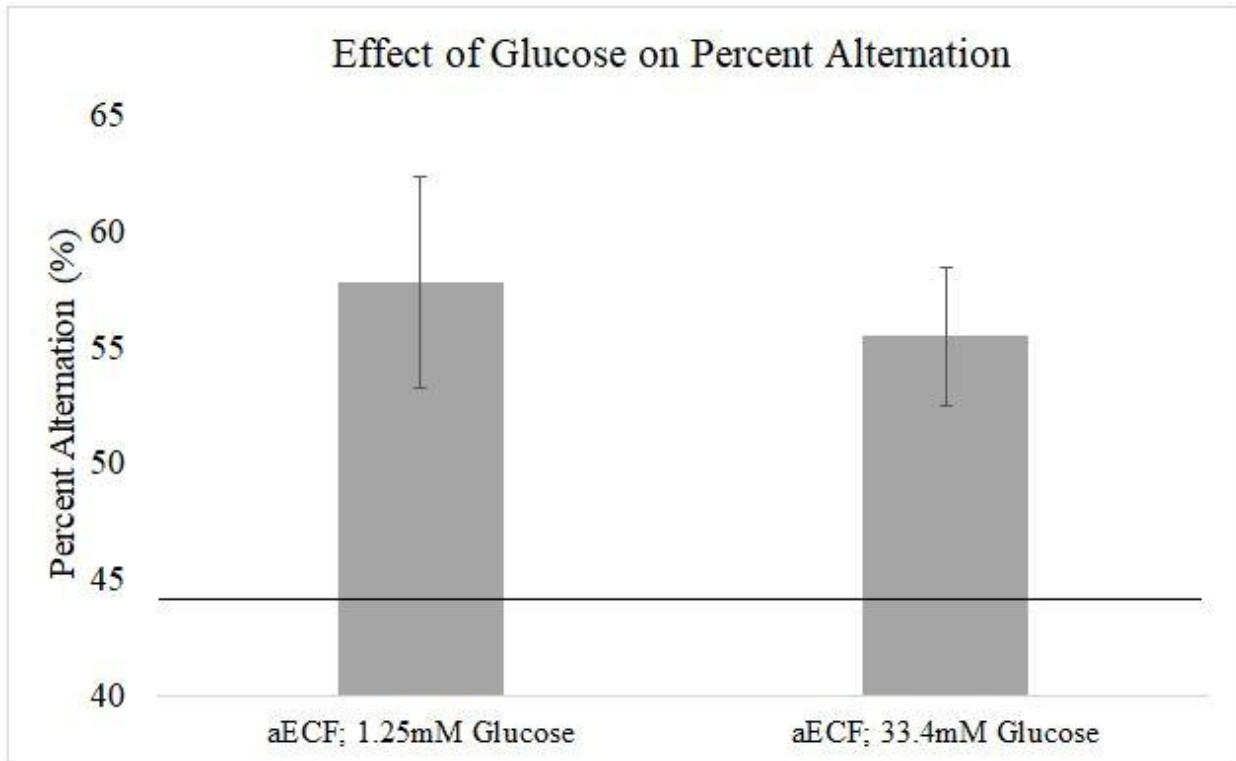


Figure 2. Mean 4/5 percent alternation performance on a +-maze, expressed as a percentage of possible alternations. A horizontal line is included to display chance level of performance (44%). Error bars represent +/-1 SEM. $t(13)=0.412, p>.05$.

Discussion

The aim of this experiment was to determine whether the blockade of intrahippocampal insulin can attenuate or prevent the enhancement of spatial working memory typically observed after systemic or bilateral glucose administration. The prediction was that insulin signaling would be required for glucose-induced improvement in hippocampal function. Given previous results (Canal, 2005; McNay et al., 2000; McNay et al., 2010; Ragozzino et al., 1998), if supraphysiological glucose is co-administered with the anti-insulin peptide, we expected that spatial working memory would be less enhanced than if supraphysiological glucose was administered without the anti-insulin peptide. Moreover, this supports the idea that insulin, rather than insulin signaling, is needed for hippocampal enhancement by glucose. Alternatively, if the intrahippocampal blockade of insulin fails to attenuate or prevent the enhancement of spatial working memory by glucose, then insulin signaling in the absence of insulin may be mediating the effect, since the insulin signaling cascade can effectively work through substrates such as IGFs I and II. IGFs I and II are effective substrates for insulin receptor, and in turn can activate the insulin signaling cascade in the absence of insulin.

Our lab has recently found trouble replicating past results using spontaneous alternation testing as a method of evaluating spatial working memory. After some debate on whether the rat breeder has any effect on spatial working memory outcome, we decided to investigate the differences. Spontaneous alternation results did not significantly differ among the control groups from each breeder (Carter, Fitzgerald & McNay, *unpublished data*). After using Sprague-Dawley rats from two different vendors and observing no significant difference, we do not have data to suggest that rat breeder has an effect on the outcomes of spatial working memory.

Our lab has successfully impaired spatial working memory by blocking intrahippocampal insulin via the delivery of an anti-insulin peptide (McNay et al., 2010). In the present study, anti-insulin peptide had no significant effect on spatial working memory, independent of the glucose conditions (Figure 1). This study was novel in combining an anti-insulin peptide and supraphysiological glucose into one microinjection, rather than separating the injections. Combining the anti-insulin peptide and supraphysiological glucose into one injection provides a method of directly administering both groups into fresh tissue simultaneously. The efficacy of the anti-insulin peptide in impairing spatial working memory may have been affected when mixed in a 1:1 ratio with either glucose condition. Alternatively, insulin blockage may be insufficient to impair spatial working memory, because insulin signaling can work through other substrates, such as IGFs I and II. Although intrahippocampal administration of IGF I did not significantly improve hippocampal function (McNay et al., 2010), this may have been because insulin was still present in the brain. Insulin's higher affinity for insulin receptor may have out-competed IGF I. To assess whether IGF I or II can activate insulin signaling and maintain hippocampal function in the absence of insulin, further investigation should include the blockage of insulin using the anti-insulin peptide followed by the administration of IGF I or II. If IGF I or II successfully maintains hippocampal function, future studies should investigate the efficacy of blocking insulin receptor in impairing spatial working memory. Blockage of the insulin receptor will eliminate the possibility of insulin signaling being activated through alternative substrates such as IGFs I and II. Thus, blocking insulin receptor should effectively inhibit the insulin signaling cascade and the translocation of GLUT4, which allows for the proper investigation of the role of insulin signaling in mediating glucose-induced improvement of hippocampal function.

Unilateral intrahippocampal glucose (6.6 mM) administration has been shown to significantly enhance spatial working memory in rats (Ragozzino et al., 1998). Unexpectedly, we found that unilateral intrahippocampal microinjection of supraphysiological glucose (33.4 mM) significantly impaired spatial working memory compared to basal glucose in a novel, larger microinjection volume (1 μ L) (Figure 1). Following this result, we then attempted to replicate spatial working memory enhancement by microinjecting glucose in a standard volume (0.5 μ L) compared to the microinjection volume in Experiment 1 (1 μ L). Surprisingly, supraphysiological glucose did not significantly affect spatial working memory when administered in standard volume (0.5 μ L).

We found that when delivered in a volume of 1 μ L, intrahippocampal administration of supraphysiological glucose significantly impaired spatial working memory compared to basal glucose (Figure 1). However, when delivered in a volume of 0.5 μ L, intrahippocampal administration of supraphysiological glucose did not significantly affect spatial working memory compared to basal glucose. This finding suggests that microinjection volumes of 1 μ L may damage hippocampal tissue or induce hippocampal hyperglycemia. Induced hyperglycemia has been shown to increase brain ischemia in rats (Gisselsson, L., Smith, M., & Siesjö, B. K., 1999; Nedergaard M., 1987). Thus, if the tissue is damaged, then supraphysiological glucose administration may augment the impairment of hippocampal function.

Although there has been success in significantly enhancing spatial working memory by unilateral intrahippocampal supraphysiological glucose administration (Ragozzino et al., 1998), our data show this method may be insufficient to significantly affect spatial working memory (Figure 2). Systemic and bilateral administration are effective in delivering glucose to both the left and right hippocampi (Canal, 2005; McNay et al., 2000), and in turn are effective in enhancing

spatial working memory. The results of this study suggest systemic and bilateral administration may be more effective than unilateral administration in enhancing hippocampal function.

The concentration of supraphysiological glucose administered in this study follows McNay and Gold (1998), when they performed bilateral intra-amygdala glucose (33.4 mM) administration and reversed deficits caused by intra-septal morphine microinjections. This concentration of glucose has not been shown to enhance hippocampal function when administered unilaterally to the hippocampus. Here, in fact, we show that unilateral intrahippocampal administration of 33.4 mM glucose has no significant effect on spatial working memory (Figure 2). However, there has been success in enhancing spatial working memory by supraphysiological glucose at a lower concentration of 6.6 mM (Ragozzino et al., 1998), which is approximately five times less than the concentration used in this study. These results suggest that glucose-induced enhancement may work in a dose-dependent manner in the hippocampus, similar to insulin. Insulin has been shown to enhance hippocampal function at moderate doses, however the enhancement effect is abolished at lower and higher doses (McNay et al., 2010). Given the results of the current study, glucose may work in a similar fashion in the hippocampus.

Conclusion

The results of this study are inconsistent with past literature regarding the role of exogenous glucose in facilitating hippocampal function as measured by performance on spatial working memory tasks, such as spontaneous alteration testing, that are presumed to be mediated by hippocampal functioning and adequate neuronal metabolism. Unilateral intrahippocampal administration of glucose may not be the most efficient way to affect the hippocampus. Instead, systemic or bilateral administration of glucose should be utilized to enhance spatial working memory as described in previous work (Canal, 2005; McNay et al., 2000). Further investigation

of the role of intrahippocampal insulin as a mediator of the effect of exogenous glucose administration on hippocampal function should include systemic administration of glucose, followed by bilateral intrahippocampal administration of indinavir, a GLUT-4 inhibitor. Based on the outcome of the present study, alternative experimental methods may prove to be better suited to test the *a priori* hypothesis.

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