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Is the Effect of Glucose on Hippocampal Memory Insulin-Dependent?

Senior honors thesis presented to the
Department of Biological Sciences and
The Honors College of the
University at Albany, SUNY.

Roi Ankawa
May 2016

Abstract

Insulin is now established as a key regulator of brain mechanisms that include both glucose metabolism and synaptic plasticity, especially within the hippocampus. However, the complex set of signaling cascades mediating these effects is not yet understood. Recent studies, many from our lab, have established that insulin plays multiple roles in the brain: in addition to regulation of energy supply, metabolism, and feeding, our work has shown that hippocampal insulin is a key modulator of learning and memory. Exogenous insulin enhances, while pharmacological blockade of intrahippocampal insulin impairs, both metabolism and cognition. Moreover, when systemic insulin signalling is impaired, such as in Type 2 diabetes, hippocampal function and metabolism are again impaired. Memory processes both in the hippocampus and elsewhere (e.g. amygdala) are well established to be sensitive to glucose supply: performance on memory tasks is limited by glucose availability, and provision of additional glucose supports enhanced task performance. Systemically, insulin regulates glucose transport from the blood into cells; conversely, glucose regulates insulin synthesis and release from the pancreas, so that the two molecules mutually regulate. Although this relationship between insulin and glucose has been well studied, there has been little work on their interaction in the brain. For instance, although we have shown that insulin regulates hippocampal glucose metabolism, it is unknown whether glucose acts to enhance memory via stimulation of insulin release within the hippocampus, or whether insulin's procognitive effects are via stimulation of glucose metabolism or a direct modulation of plasticity. In this study, Glut4, an insulin-dependent glucose transporter found on some hippocampal neurons, was directly blocked. Indinavir, a Glut4 inhibitor, was injected directly into the dorsal hippocampus of rats in the presence or absence of a peritoneal glucose injection in order to assess changes in cognition. It was found that indinavir treatment significantly impaired cognition in spontaneous alternation tasks, reduced anxiety, and, surprisingly, and had no effect on cognitive performance in a novel object recognition task. These data support a novel role for GluT4 as a mediator of hippocampal memory processing and suggest that insulin acts to regulate cognitive function at least in part via GluT4-mediated glucose transport into neurons. In the presence of indinavir, glucose was unable to enhance memory, consistent with this interpretation and suggesting that enhancement of hippocampal memory by glucose may require hippocampal insulin signaling. Post-mortem molecular studies of hippocampal protein expression provided further insight into the molecular impact of both glucose treatment and GluT4 blockade.

Introduction:

Glucose is well known as the main fuel source of the brain. Cognitive demand leads to decreased glucose concentrations during spatial memory tasks, with glucose demands directly corresponding to the complexity of the task (McNay et al., 2000). This study indicates that the limitation imposed on memory processing is the concentration of glucose, with a specific focus on the hippocampus. Metabolically, it is widely known that upon increase in systematic glucose concentration, following a meal for example, insulin is released to lower glucose concentration; the aim of the body is to maintain homeostasis of fuel supply.

In addition to studies linking the effect of glucose to cognition, insulin's role on similar tasks has been studied as well. Literature focuses on both the physiological disorders involving impaired insulin signaling, as well as on specific alteration of insulin signaling on the cellular level. For instance, diabetes mellitus type 2 (T2DM) is a metabolic disorder where the lack of insulin sensitivity leads to hyperglycemia. Patients suffering from T2DM have been seen to experience progressive cognitive deficits (Cukierman et al., 2005). Further, it has been shown that hyperinsulinemia associated with T2DM makes patients more susceptible to developing Alzheimer's disease (Kroner, 2009). Excess insulin has been linked to the accumulation of beta amyloid, as the two molecules share a breakdown pathway.

Studies on the cellular and molecular levels make a connection between insulin activity and learning and memory processes. Evaluation of insulin signaling in the central nervous system revealed the presence of insulin receptors distributed throughout the hippocampus (Zhao et al., 1999) alluding to a significant role of insulin receptors on memory processing. Further, direct administration of insulin to the hippocampus was shown to enhance cognitive function in spatial memory tasks. The same study showed that administration of increasing concentrations of insulin versus cognitive function yields a graph shaped like an inverted U, indicating an optimal

level of insulin for cognition. Alteration from this optimal level leads to cognitive impairments (McNay et al., 2010). Furthermore, intranasal insulin administration in humans improved memory performance, suggesting a potential treatment for Alzheimer's disease (Benedic et al., 2004).

Insulin signaling is a complex pathway that has yet to be completely understood. It is known that the activation of insulin receptors via the dimerization of tyrosine residues involves an increase of P13K activity, a kinase involved in cell growth, proliferation, and differentiation. Moreover, it has been recently shown that using insulin-like growth factor affects the phosphorylation of CREB, which is correlated to memory enhancement (Alberini and Chen, 2012). Another key player in insulin signaling is GluT4. In contrast to various other cellular glucose transporters, GluT4 is an insulin-dependent glucose transporter found in the hippocampus. Recently, the activation of GluT4 is thought to be the connection between insulin's effects on cognitive function as described above. GluT4 is the main molecular target in the present study. Using Indinavir, a drug previously used for the treatment of HIV/AIDS, and a GluT4 antagonist, this study directly alters hippocampal insulin signaling and measures its effect on cognitive function.

Glucose and insulin both play significant roles in cognitive processes, as indicated by the memory enhancement produced separately by the administration of each molecule into the hippocampus. Given their interdependent actions systematically, it is imperative then to further our understanding of the way these molecules work together to affect cognition. One may ask two questions; is the effect of glucose on memory a product of insulin signaling? Or, on the contrary, is the effect of insulin on memory mediated by the presence of glucose? This current study tackles the first question: in order to investigate if memory is dependent on insulin

signaling within the brain, subjects were divided into two groups either receiving a microinjection of indinavir or aECF directly into the dorsal hippocampus. Each group was further divided into glucose or saline receiving groups, where the treatment was co-administered by peritoneal microinjection. Using this method, this study indirectly tests the dependency of insulin on the effect of glucose on memory by ceasing the uptake of glucose into the cell.

In order to measure cognition, three behavioral tasks were performed: spontaneous alternation, novel object recognition, and open field task. Administration of indinavir resulted in a significant decrease in percent alternation in a four-armed maze, and a reduction in anxious behavior in comparison to subjects receiving aECF. The administration of glucose did not significantly affect behavior in spontaneous alternation and novel object recognition tasks. However, glucose administration in open field tasks seemed to ameliorate the effect of indinavir. The effects of co-administration of glucose and indinavir were not significantly different from that of glucose and saline co-administration with aECF. Collectively, this data indicates that GluT4, and hence insulin signaling, plays a key role in cognition. In regards to glucose administration, the data presented in this study is inconsistent with previous studies; it was expected that subjects receiving glucose would show enhancement in memory performance in comparison to the aECF/saline-administered groups, and to the two groups receiving the indinavir treatment. The lack of differences between the indinavir-administered groups indicates the need for further studies. Molecular studies of essential hippocampal proteins involved in insulin signaling and learning and memory are currently in progress.

Methods:

Animals:

40 Male Sprague-Daley rats (Charles River) were studied, arriving at 10 weeks of age. Rats were individually housed with food and water available on a 12:12h light:dark schedule. Room/cage temperature was maintained at 25 degrees Celsius. Animals were handled 5 minutes every day a week prior to surgery, which was done 10 days following animal arrival. Rats were handled everyday post-surgery until behavioral testing and animal sacrifice were done a week following surgery. All animal were randomly assigned group during behavioral testing day. All procedures were approved by the University at Albany, SUNY, animal care facility.

Surgery:

Rats were anesthetized with isoflourine (5% by air) before surgery with oxygen was delivered at the same time. Throughout the duration of the procedure isoflourine was lowered to 3% and oxygen was lowered to 2%. All animals received a microinjection guide cannula directly into the left hippocampus with coordinate relative to bregma: 5.2 mm posterior to bregma, +4.8 lateral, and 3.8 ventral from dura. The Nose-bar was set to 5mm above the interaural line. Rats received 4mL injection of sterile saline (1mL before and 3mL after surgery). During surgery a 1:1 epinephrine/marcain was administered drop-wise to prevent the bleeding. Directly following anesthetic removal, rats were placed in a warm incubator until recovery from anesthesia. Animals receive 5mg/kg dose of rimadyl right after surgery and a 2 mg rimidyl tablet for three days post surgery. Animals were recovering for a week post surgery where they are handled for 5 minutes everyday.

Intraperitoneal injection procedure:

Injection was given using a 3mL syringe and administrated to the left abdomen of each rat. Injection was given 10 minutes prior to the start of behavioral testing directly preceding

microinjections. Injections were given according to body weight taken prior to injection 250mg/kg glucose in saline. Saline was given as control.

Microinjection procedure:

Rats were injected with either Indinavir sulfate (Toronto Research Chemicals, Inc) or aECF (aECF; 153.5 mM Na, 4.3 mM K, 0.41 mM Mg, 0.71 mM Ca, 139.4 mM Cl, buffered at pH 7.4; (McNay and Sherwin, 2004)) following an i.p. injection of either glucose or saline. Indinavir was brought to final concentrations of 100nM in artificial extracellular fluid (aECF), HCl was used drop-wise to allow indinavir to go into solution and a pH 6 was measured for all animals including aECF receiving groups. Microinjections were administered to the dorsal hippocampus 10 min prior to behavioral testing at a flow rate of 1.25 μ l/min for a total volume of 0.5 μ l. Solution took 4 minutes to enter the brain area and the probe was left for an additional 2 minutes to make sure no residual solution is left in the probe. Rats enter the maze 10 minutes after the start of injection.

Behavioral testing:

Spontaneous Alternation (SA): the animals were placed in the middle of a four-arm maze, facing the same direction each time. It was noted every time an animal moved to a new arm or showed interest by placing more than half of their body in the arm. The spontaneous alternations were recorded for 15 minutes. An alternation is counted when the subject visited all 4 arms within a span of 5 consecutive arm choices. The actual number of alternation made is expressed as a percentage to the total numbers of arms entered. The animals were allowed 5 minutes to rest post task where they were placed back in their cage. The maze was cleaned with 70% ethanol between each animal and at the start of testing day.

Novel Object Recognition:

training took place 5 minutes after SA. For the training phase the animals were placed in a clear box with two of the same objects orientated and placed in the same way. Time was collected every time the animal showed interest in the object by investigating/sniffing it. Animal interactions with both the left and right objects were recorded for 5 minutes. The animals were allowed 30 minutes of rest back in their home cages. After 30 minutes the animals were placed in the same box with a novel object and one of the familiar object. The same measurements were collected as in training. The novel object was randomized between placements. The box was cleaned with 70% ethanol between each animal.

Open field: the animals were placed in a box that has 16 squares drawn on it. Every time the animals were in the middle 4 boxes the time was collected. Animals were in the box for five minutes. The box was cleaned with 70% ethanol prior to testing and between animals. Rats were sacrificed following the task.

Sacrifice and Tissue collection:

animals were anesthetized using isoflourine and after ensuring unconsciousness rats were guillotined. Blood samples were collected as well as the left and right hippocampus and prefrontal cortex.

Tissue preparation:

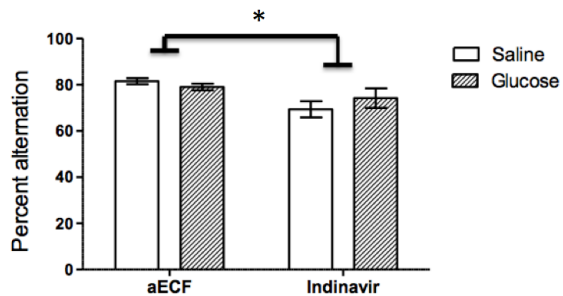
Following collection hippocampus tissues were immediately transferred to 150uL of homogenization buffer and were treated with handheld tissue grinder. Samples were divided into total and plasma membrane. 30uL of the homogenized tissue were placed in 100uL RIPA buffer to prepare the total sample. Plasma membrane samples that were only placed in homogenized buffer were further processed using membrane extraction protocol.

Statistical Analysis:

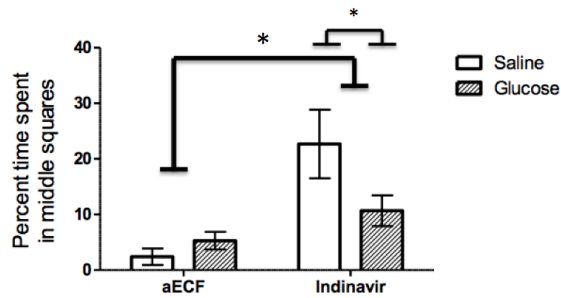
All tests were conducted using two-tailed, unpaired t-tests or one-way analysis of variance (ANOVA) with individual group differences versus control determined by Dunnett's Multiple Comparison post hoc tests. Significance was taken >0.05 . N of each behavioral group was between 8-10.

Results:

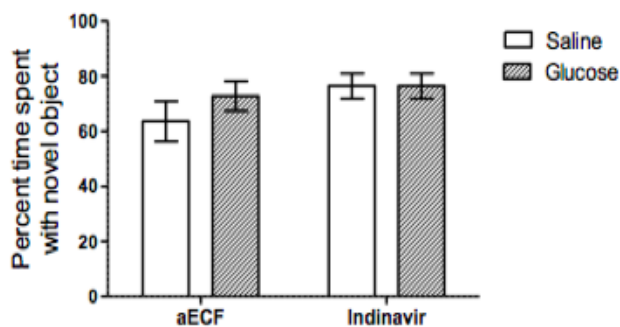
Indinavir administration impaired spatial memory performance: Through trials of spontaneous alternation tasks, it was shown that the two groups administered indinavir scored significantly lower than those administered aECF ($p=0.0049$).



Indinavir administration decrease anxious behavior: Through trials of open field tasks, general locomotive activity, exploratory behavior, and anxiety were measured. By comparing the percentage of time that the rats spent in the middle four squares in comparison to the edges, it was found that indinavir administered rats were significantly less anxious than rats receiving aECF, evidenced by the significantly higher percentage spent in the middle squares ($p=0.029$). Additionally, within the group of the rats administered indinavir, co-administration of glucose significantly decreased the effect observed in the absence of glucose ($p=0.0244$). No significant differences were found between the indinavir/glucose group and the aECF/glucose group.



Indinavir did not significantly affect recognition memory: In order to test memory recognition, rats were presented with a two-object recognition task. It was found that upon administration of indinavir, the subject's ability to recognize objects significantly improved.



Discussion

Previous studies in the field of learning and memory directly correlate the effect of both insulin and glucose to cognitive enhancement. It is known that in the periphery, these molecules directly affect one other, but little work has been done focusing on their interaction in the brain. The present study suggests that the effects of these molecules on cognition are not independent of each other; rather, the presence of one causes the observed cognitive effect upon sole administration of the other. The question arises as to which one is responsible. Here, we begin by looking at insulin as the main modulator and ask whether enhancement of glucose on cognition

is a result of insulin signaling. In order to alter insulin signaling, we focused specifically on GluT4, an insulin dependent glucose transporter. GluT4 is known to be present in the hippocampus and causes upregulation of memory formation upon administration. We used the GluT4 antagonist, indinavir.

Due to previous studies on GluT4, it was expected that administration of indinavir would cause impairment in cognitive performance. We also expected that glucose administration alone would enhance cognition, as previously shown. We used spontaneous alternation tasks as a measure of spatial memory processing, novel object recognition as a cognitive task for recognition memory, and open field tasks as a measure for anxious behavior.

As expected, indinavir administration significantly decreased cognitive performance in comparison to a control group in spatial working memory tasks. Co-administration of glucose and indinavir yields the same results as administration of indinavir with saline. This provides the possibility that glucose effect on memory is indeed insulin dependent, since indinavir treatment was not affected by glucose treatment. However, the administration of glucose alone (aECF microinjection) did not affect memory as expected if the cognitively ameliorative effect of glucose is dependent on insulin signaling, because proper insulin signaling is still intact. This raises the question of whether the lack of effects seen in the indinavir group is actually due to the dependency of glucose on indinavir, or rather, a lack of effect of glucose in this study.

Since it was previously shown that glucose levels significantly decrease during memory tasks, and administration of glucose significantly improves cognitive tasks, we question the effect of glucose in this study. One possibility for our lack of effect could be that simply not enough glucose was given to cause improvement of performance, and perhaps an additional study should be carried out with administration of higher glucose concentrations. Another

possibility is that since glucose decline is associated with cognitive demands, it is possible that the four-armed maze was not complex enough to result in a significant decrease in glucose. The latter explanation is the most probable one; by observing the result of the SA task one can see that the control animals reached nearly 80% alternation. In the previously reported studies the controls reached about 50% alternation. This comparison suggests that the maze setup in the facility is not challenging enough for the animals. These results are rather inconclusive because the controls do not show consistent results as seen in previous studies. We are interested in investigating this odd occurrence by carrying out a simple study of administering glucose or insulin to subjects to obtain a better understanding of these inconsistencies concerning the effect of glucose.

Open field tasks also showed significant differences between the indinavir and aECF groups. The indinavir/saline treated group showed significantly less anxiety by spending more time in the center of the field in comparison to the control. Furthermore, groups administered indinavir/glucose were significantly more anxious than those that received indinavir in the absence of glucose. Statistically, this group was comparable to both groups that did not receive indinavir. This might suggest a potential role of glucose in balancing or protecting anxious behavior; it can be seen that a significant decrease in anxious behavior was brought back to baseline upon administration of glucose along with the agent that lowered anxiety. This hypothesis may also explain the lack of effects seen in the administration of glucose alone in comparison to the control. Open field task was the only task in which the administration of glucose showed a significant effect.

Taken together, the presented behavioral testing data demonstrate the importance of GluT4 in cognition, further showing the importance of downstream hippocampal insulin

signaling in such processes. However, we cannot conclude whether the known effect of glucose on cognition is insulin dependent due to the inconsistent results obtained from our controls.

In order to further investigate the effect of indinavir and glucose on cellular and molecular processes, we are currently working on western blotting for protein analysis in the hippocampus. The target proteins are those directly participating in insulin signaling, including Akt, p-Akt, P13K, and Glut4, as well as proteins involved in learning and memory, including NMDA-R, AMPA-R, CREB, and their phosphorylated counterparts.

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