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Insulin modulates hippocampally-mediated spatial working memory via glucose transporter-4

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Abstract

The insulin-regulated glucose transporter, GluT4, is a key molecule in peripheral insulin signaling. Although GluT4 is abundantly expressed in neurons of specific brain regions such as the hippocampus, the functional role of neuronal GluT4 is unclear. Here, we used pharmacological inhibition of GluT4-mediated glucose uptake to determine whether GluT4 mediates insulin-mediated glucose uptake in the hippocampus. Consistent with previous reports, we found that glucose utilization increased in the dorsal hippocampus of male rats during spontaneous alternation (SA), a hippocampally-mediated spatial working memory task. We previously showed that insulin signaling within the hippocampus is required for processing this task, and that administration of exogenous insulin enhances performance. At baseline levels of hippocampal insulin, inhibition of GluT4-mediated glucose uptake did not affect SA performance. However, inhibition of an upstream regulator of GluT4, Akt, did impair SA performance. Conversely, when a memory-enhancing dose of insulin was delivered to the hippocampus prior to SA-testing, inhibition of GluT4-mediated glucose transport prevented cognitive enhancement. These data suggest that baseline hippocampal cognitive processing does not require functional hippocampal GluT4, but that cognitive enhancement by supra-baseline insulin does. Consistent with these findings, we found that in neuronal cell culture, insulin increases glucose utilization in a GluT4-dependent manner. Collectively, these data demonstrate a key role for GluT4 in transducing the procognitive effects of elevated hippocampal insulin.

Keywords

Insulin; Glucose transporter; Hippocampus; Memory

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Conflicts of interest

The authors declare no competing financial interests.

1. Introduction

In peripheral tissues, insulin's canonical effect is to regulate GluT4-mediated glucose uptake and glucose utilization [1]. Although neurons express both GluT4 and insulin receptors (IRs), the role of insulin signaling in controlling neuronal glucose utilization is less clear [2–6]. Because insulin stimulates GluT4 translocation from intracellular storage pools [via GluT4 vesicles (GSVs)] in neuronal cells [4,6], it is possible that insulin can affect glucose transport and hence fuel supply in the brain through GluT4.

Insulin's effects on memory are PI3K-dependent, but downstream mechanisms following PI3K-activation are not clear [7]. While some studies have shown an effect of insulin signaling on glucose utilization in the brain as a whole, others found no effect [7–9]. Because intrahippocampal glucose administration has similar cognitive effects as intrahippocampal insulin [7,10–22], and insulin's well-established action in the periphery is to facilitate glucose utilization, it is possible that insulin's cognitive effects are mediated through enhanced GluT4-mediated glucose uptake and subsequent glucose utilization in the brain.

GluT4 shows primarily neuronal localization in specific brain regions in rat, and its expression is greatest in the perikarya. The hippocampus has high expression of GluT4 in pyramidal cells [23–26]. GluT4 translocation occurs following stimulation by a variety of kinases including PI3 K, several atypical protein kinase c (aPKC) isoforms, and Ca^{2+} /calmodulin kinase II [CaMKII; [27–30]]. Intriguingly, many of these proteins are necessary for memory formation [31–42]. Thus, it is likely that GluT4 activation occurs during hippocampally-mediated memory formation, which is known to be sensitive to glucose supply and metabolism [43,44]. In this study, we used pharmacological inhibition of GluT4-mediated glucose uptake to assess the involvement of hippocampal GluT4 in insulin-mediated memory enhancement and neuronal glucose utilization.

2. Materials and methods

2.1. Animals

All procedures were approved by the University at Albany Institutional Animal Care and Use Committee prior to experimentation.

For *in vivo* experiments, adult male Sprague-Dawley rats (Charles River, Wilmington, MA), approximately 300 g at time of arrival, were housed in pairs on a 12:12 h light:dark schedule with food and water available *ad libitum*. Rats were given at least one week to acclimate prior to any surgery and again after surgery but prior to testing, during which time they were handled extensively. Rats were housed singly following surgery. Each rat was used only once. All testing was done during the light cycle, including generation of hippocampal cell cultures. For generation of hippocampal cell cultures used for *in vitro* experiments, brains were dissected on the day of birth, from postnatal day 1 (P1) rats.

2.2. Surgeries

Rats were anaesthetized with isoflurane and a single microinjection cannula (Plastics One) was stereotactically implanted in the dorsal hippocampus using aseptic surgical technique. Cannulae coordinates were 5.6 mm posterior to bregma, +4.6 lateral, and 3.3 ventral from dura. The coordinates correspond to the left dorsal hippocampus. Rats received the analgesic acetaminophen in their drinking water following surgery and were then allowed a two-week long recovery period prior to testing.

2.3. Drug treatments and microinjections

Indinavir sulfate (IND) and atazanavir (ATZ) were purchased from Toronto Research Chemicals, Inc. Nelfinavir mesylate (NFV) and insulin were purchased from Sigma, Inc. All drugs and controls were brought to final concentrations in artificial extracellular fluid (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; [45]) and fresh stocks were prepared immediately prior to testing. Microinjections were administered to the dorsal hippocampus 10 min prior to behavioral testing at a flow rate of 1.25 μ l/min over 4 min for a total volume of 0.5 μ l.

2.4. Spontaneous alternation (SA)

To assess spatial working memory we examined spontaneous alternation behavior in a 4-arm plus-maze. Rats tend to visit the least-recently visited arm, using spatial working memory to recall arm-visit history [46,47]; they are allowed to explore the maze freely for 20 min, after which alternation scores are calculated by dividing the percentage of alternations (defined as a visit to each of the 4 arms within each span of 5 consecutive entries), with chance performance being 44%. We and other researchers have used this task extensively over the past decade to examine brain metabolism, insulin signaling, and spatial working memory in rats [7,48–51].

2.5. In vivo [14 C]-2DG injections and analyses

To assess glucose metabolism during SA testing we used a modification of Louis Sokoloff's protocol for assessing glucose phosphorylation using 2DG [52–55]. 2DG is phosphorylated by hexokinase and is functionally “trapped” as the nonmetabolizable product [14 C]-deoxyglucose-6-phosphate, making it an ideal metabolic marker. This method is well validated as a direct measure of brain metabolism [52–55]. The amount of radioactivity (nCi/g) present in [14 C]-2DG-injected tissues is directly proportional to the rate of glucose utilization.

[14 C]-2DG with specific activity of 250–350 mCi/mmol was purchased from PerkinElmer (Cat. No. NEC720A050UC). All radioactive procedures were approved by the University at Albany Radioactive Care Committee. A dose of 16.5 μ Ci/100 g was administered through intraperitoneal (IP) injection into each rat immediately prior to testing. The experimental outline is presented in Fig. 1A. Briefly, immediately following IP injections of 16.5 μ Ci/100 g, pair-housed rats were brought into the testing room. One rat from each pair was randomly picked up and immediately placed back into its home cage while the other rat was tested for 20 min on the SA task. Immediately after testing, both rats were anesthetized with isoflurane, decapitated, and whole brains were rapidly removed and frozen on dry ice. The

entire procedure took less than 30 min, within the range shown to minimize loss of 2-deoxyglucose-6-phosphate from cells [56]. Brains were sliced into 20 μ m sections on a Lieca Cryostat (Sweden), mounted on glass slides (Fischer Scientific), and allowed to dry overnight. The following day slides were appositioned to a storage phosphor screen (GE) next to a set of precalibrated [14 C] standards and sealed for one week in a cassette. After one week of incubation phosphor screens were analyzed on a Typhoon Imager (GE), and images were analyzed by densitometry. Following imaging each section was Nissl stained. Hippocampi from each section were normalized to corpus callosum [14 C]-2DG uptake, a method of relative quantification for [14 C]-2DG [57]. Plasma samples were taken when rats were killed and analyzed in a Beckman Scintillation Counter to ensure there were no significant differences in [14 C]-2DG injection volumes across animals. One rat in the SA tested group had undetectable levels of plasma [14 C], and his data were removed from all analyses.

2.6. Cell fractionation

Plasma membrane (PM) fractions were collected using a commercially available kit (Biovision). Whole hippocampi were homogenized with a Polytron handheld electric homogenizer at 1 mg/3 μ l lysis buffer with lyophilized protease inhibitors (Biovision). Following initial homogenization 30 μ l of the total lysate was added to 200 μ l ice-cold RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-X) containing fresh protease and phosphatase inhibitors (Pierce) and stored at -80°C until quantification. For the remaining lysate, PM separation proceeded according to the kit's instructions.

2.7. Western blotting

Protein concentration was determined using a commercially available kit for the bicinchoninic assay (Pierce). 10–20 μ g of protein was placed in sample buffer in denaturing and reducing conditions. Samples tested for total protein content were heated at 95°C for 5 min prior to sample loading. Samples analyzed for plasma membrane associated proteins were not heated prior to gel loading. Samples were resolved on 8% or 12% SDS polyacrylamide mini-gels (Pierce or Bio-Rad) and transferred onto PVDF membranes (Bio-Rad) followed by detection with appropriate primary antibodies. anti-rabbit directed against GluT4 (Millipore Cat. No. 07-1404), GluT1 (Abcam Cat. No. ab652), GluT3 (Abcam Cat. No. ab41525). β -actin (Sigma Cat. No. A2228) was used as a loading control. Following wet-transfer at 350 mA in the cold for 60 min, PVDF membranes were blocked for one hour with 5% milk or 5% BSA in tris-buffered saline containing 0.1% tween-20. Membranes were incubated with primary antibodies in blocking solution overnight at 4°C in conditions optimized for immunodetection by each antibody. Secondary incubation in biotin-conjugated goat anti-mouse (Pierce) or goat anti-rabbit (Vector) IgG was followed by incubation in HRP streptavidin (Pierce) for enhanced immunoreactivity, both for one hr at room temperature. Immunoblots were developed with chemiluminescent detection using the SuperSignal West Pico Chemiluminescent Kit (Pierce). Images were taken in a Bio-Rad ChemiDoc XRS Image Analyzer. Membranes were then stripped with Restore Plus Stripping Buffer (Pierce) and reprobed for loading controls or total protein content. Band intensity was measured by densitometry in ImageQuant software and normalized to loading controls or total protein

content. To control for between gel variations in band intensity, vehicle control values were normalized to 100% and treatment groups were analyzed as percents of control values.

2.8. Primary hippocampal neuronal cell culture

At postnatal day 1, rat pup hippocampi were rapidly extracted and placed in ice-cold Hibernate-A (HBA; Brain Bits) plus B-27 vitamin supplement and GlutaMAX mixture (Invitrogen). Hippocampi were then preheated and transferred to HBA (minus calcium) and Papain (Worthington) for digestion. The cells were further dissociated manually and evenly suspended in solution. The cells were pelleted and resuspended in a Neurobasal-A (NBA) media containing 2% B27 vitamin supplement, 0.5% fibroblast growth factor (FGF), 0.2% GlutaMAX growth supplement, and 0.05% gentamycin antibiotic (Invitrogen). Cell densities were measured using trypan blue exclusion, and plated at 800 cells/mm² on 65 mm poly-D-lysine precoated plates. Cells were cultured at 37 °C and 5% CO₂ in the NBA mixture listed above for one week before testing. Fresh media were added every 3 days. Each experimental manipulation was performed in 3 separate replications, and data were averaged.

2.9. In vitro [¹⁴C] 2-deoxy-D-glucose uptake ([¹⁴C]-2DG)

This protocol was obtained from Dr. Kyriaki Bakirtzi [58], optimized for similar experimental conditions in cerebellar neurons. Primary hippocampal neuronal cells were transferred to fresh serum-free medium 4 h before each experiment. Plates were washed with glucose-free Krebs-Ringer HEPES (KRH) medium and treated with 100 nM insulin or vehicle control (KRH medium) for 15 min. Treatments (IND or vehicle [KRH medium] control) were then added to the medium for 15 min. A mixture of [¹⁴C]-2DG (PerkinElmer Cat. No. NEC720A050UC specific activity 250–350 mCi/mmol) and unlabeled 2-deoxyglucose was added for 3.5 min at 37 °C. Glucose uptake was stopped by washing plates several times with ice-cold KRH with 25 mM glucose and 10 μM cytochalasin b to stop further glucose uptake. Cells were collected in glucose-free KRH containing 0.1% sodium dodecyl sulfate (SDS), and radioactivity was determined by liquid scintillation counting in a Beckman Scintillation Counter.

2.10. Statistical analysis

For comparisons of two groups we used Student's *t* test; for more than two groups we used analysis of variance (ANOVA) followed by Bonferroni *post hoc* tests to compare individual groups. An α level of 0.05 (two-tailed) was set for significance. All statistical analyses used SPSS version 17 or Prism 5.

3. Results

3.1. Increased glucose uptake in the dorsal hippocampus during SA testing

Several previous studies have shown a transient, task-associated decrease in hippocampal extracellular glucose during spontaneous alternation testing (e.g. [43,59,60]), and similar results have been obtained in other brain areas during tasks that require those areas [61]. This finding has been interpreted as being due to increased local glucose utilization during SA testing. However, other mechanisms could explain the decrease, such as decreased peripheral glucose supply. Therefore, the purpose of Experiment 1 was to confirm whether

SA testing does indeed increase glucose utilization in the hippocampus. The experimental outline is shown in Fig. 1A. A $2 \times 2 \times 2$ factorial ANOVA with region (dorsal and ventral), side (left and right) and condition (control and SA) variables revealed significant main effects of region ($F(1, 46) = 3.95, p < 0.05$) and condition ($F(1, 46) = 30.04, p < 0.05$), as expected, with highest glucose utilization values in the dorsal hippocampus of SA-tested rats (Fig. 1D). A potential three-way interaction effect was not observed, $F(1, 46) = 1.07, p = 0.31$, suggesting that side of hippocampus was not an important factor in predicting glucose utilization during SA testing. Comparison of the dorsal and ventral hippocampus of SA tested rats shows significantly increased glucose utilization in the dorsal hippocampus relative to the ventral hippocampus (Bonferroni *post hoc* test, $p < 0.05$). These data are consistent with findings linking the dorsal hippocampus to spatial memory and the ventral hippocampus to memories with aversive emotional context. Additionally, it also lends support to the well-established idea that SA is a non-aversive task [51,62].

3.2. SA testing increased glucose transporter trafficking in the hippocampus

To determine whether SA testing increased plasma membrane expression of GluT4 relative to other glucose transporters, pair-housed rats were brought into the testing room and tested for SA behavior for 20 min, or left in their home cage for 20 min. Immediately after testing rats were killed, hippocampi were dissected, and protein levels were measured. The experimental outline is summarized in Fig. 2A.

We measured trafficking of 3 glucose transporters: GluT1 (expressed both on astrocytes [MW 45 kDa; GluT1–45] and endothelial cells [MW 55 kDa; GluT1–55]), GluT3 and GluT4 (both primarily neuronal in rat). Based on the literature discussed above, showing that learning activates many upstream modulators of GluT4 trafficking, we hypothesized that GluT4 trafficking to the plasma membrane would occur as a result of SA testing. Results of a 2×4 ANOVA, which examined condition ('Control' and 'Maze-tested') and the ratio of plasma membrane to total glucose transporter expression (PM:Total; GluT1–45, GluT1–55, GluT3, and GluT4) showed a significant interaction effect $F(3, 66) = 5.46, p < 0.05$ (Fig. 2B and C). Bonferroni *post hoc* tests showed that GluT4 was increased in the Maze-tested condition relative to control ($p < 0.05$). None of the other GluTs were significantly increased relative to their respective controls. These data demonstrate that amongst the glucose transporters assessed, only GluT4 trafficking was increased following 20 min of SA testing, suggesting that GluT4 is specifically activated by memory training.

3.3. GluT4 inhibition alone did not impair SA performance

To determine whether the increased trafficking of GluT4 to the plasma membrane that occurred during SA testing was necessary for performance on SA, we used the drugs NFV and IND to assess the role of insulin signaling and GluT4 on SA performance, respectively. NFV and IND both impair glucose translocation through GluT4. However, IND is a selective inhibitor of GluT4-mediated glucose uptake, and blocks GluT4's intracellular binding domain. NFV inhibits Akt/PI3 K activity as well as GluT4 activity. Consistent with previous work showing that Akt/PI3 K was critical for normal SA performance [7], NFV impaired SA behavior (Student's *t* test, $p < 0.05$; Fig. 3A and B). A separate experiment

showed that neither 40 ng nor 200 ng IND significantly impaired SA performance (Fig. 3B and C).

3.4. GluT4 inhibition prevented insulin's cognition enhancing effects

Previous research showed that acute administration of intrahippocampal insulin enhances SA performance [7]. Here, we tested the hypothesis that GluT4 is necessary for the cognitive enhancing effects of insulin (Fig. 4). ATZ, which is a PI that has little to no effect on GluT4 activity [63], was used to control for potential non-GluT4 dependent cognitive effects of the drugs. The one-way ANOVA was significant $F(3, 37) = 6.28, p = 0.001$. A Bonferroni *post hoc* comparison revealed that 100 μ U insulin enhanced SA performance relative to control, ($p < 0.01$), and also relative to animals treated with both insulin and ATZ ($p < 0.05$). However, when GluT4 was blocked with IND, insulin failed to enhance SA performance. These data show that GluT4 activity is necessary for the memory enhancing effects of insulin on SA behavior.

3.5. GluT4 is critical for insulin-mediated glucose uptake in hippocampal neurons

One of the mechanisms by which insulin has been proposed to exert cognitive effects on hippocampal-dependent memory is by increasing neuronal glucose metabolism. Such a hypothesis is consistent with research showing that intrahippocampal glucose administration can enhance learning [64]. Our data show that insulin's memory enhancing effects in the hippocampus depend on functional hippocampal GluT4. Therefore, it is likely that a key mechanism of action for insulin's effects on hippocampal memory are indeed by enhancing neuronal glucose utilization, since GluT4 is primarily expressed in neurons in the rat CNS [5].

The effects of insulin and glucose transporter inhibitors on glucose utilization were tested in neuronal cell culture; neurons are the predominant or only cell-type in the rat CNS known to express GluT4 [5]. Results of a one-way ANOVA showed a significant main effect of treatment, $F(3, 19) = 5.57, p = 0.007$ (Fig. 5). Bonferroni *post hoc* analyses revealed that insulin enhanced glucose utilization relative to control ($p < 0.05$), and that indinavir prevented insulin's effect on glucose utilization ($p < 0.05$). Indinavir by itself had no effect, which is consistent with our behavioral data that demonstrated indinavir alone had no significant effects. These data are consistent with other data from cerebellar neurons showing that neuronal GluT4 is necessary for insulin-mediated glucose uptake enhancement in the brain [58].

4. Discussion

In this study, we used selective inhibition of hippocampal GluT4 to probe the involvement of GluT4 in hippocampally-mediated memory. Our findings suggest that the acute effects of GluT4 inhibition on spatial working memory, at least in the SA task used here, are only detectable following insulin-stimulated GluT4 translocation. Blocking GluT4 alone did not impair glucose utilization in hippocampal neurons, but blocking GluT4 impaired insulin-stimulated glucose utilization suggesting that baseline levels of GluT4 do not have prominent effects on mediating neuronal glucose utilization. The implications of this study

are that in the brain, the acute effects of supra-baseline insulin to enhance spatial working memory may require functional GluT4. We make specific note of “supra-baseline” insulin’s effects here, because a previous study found that inhibition of endogenous insulin (i.e. baseline insulin) in the hippocampus impaired SA performance [7]. This suggests, that suprabaseline insulin affects memory via distinct pathways (i.e. mediated through GluT4) from baseline insulin (i.e. not mediated through GluT4). Increased PM expression of GluT4 observed following SA testing as observed in Experiment 2 is unnecessary for SA performance. These data suggest that increased hippocampal GluT4 PM expression observed during the SA testing may be involved in memory processes different from spatial working memory, such as perhaps memory consolidation. In a previous study where we varied the retention interval between learning and testing, and used the inhibitory avoidance paradigm, we found that intrahippocampal administration of IND to the hippocampus did indeed impair memory when give 10 min prior to the acquisition phase of testing [65]. Alternatively, increased PM GluT4 expression may be a response to increased metabolic load (including drainage of extracellular glucose) during memory, and might perhaps play a greater role during more prolonged cognitive demand.

A main finding of this study was that the impact of exogenous insulin on hippocampal memory and glucose utilization was GluT4-dependent. Although insulin was administered directly to the hippocampus, it is important to point out that such administration does indeed have physiological relevance; that is, physiologically-relevant conditions such as those following a meal, or in response to elevated blood glucose, would lead to enhanced insulin production, which may affect the hippocampus directly. Moreover, insulin may be locally produced in the brain [66], a possibility that remains controversial and requires further investigation. Current clinical trials are underway that are assessing the impact of intranasal insulin on memory in individuals with mild cognitive impairment and Alzheimer’s disease ([Clinical-Trials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01767909) identifier NCT01767909), thus our results may suggest GluT4 as a novel mechanism for insulin’s precognitive effects.

The effects of physiological concentrations of insulin on neuronal glucose utilization are unclear. Some studies have shown that insulin enhances neuronal glucose utilization, while others have shown no effect [7–9,58,67–70]. An important consideration in interpretation of our data is that the dose of insulin used to show enhancement of glucose utilization in the cell culture experiment might not reflect physiological levels. This possibility is difficult to assess for two primary reasons; first, normal concentrations of intrahippocampal insulin in rats (and mammals in general) are not clear. One of the earliest studies documenting insulin levels in the brain identified 12 ng insulin/g wet brain tissue, whereas blood had 2 ng/ml [71,72]. Determining how the concentration used in our current study (100 nM) compares to that in wet tissue is not directly possible. However, the dose we used has previously been shown to increase hippocampal glucose utilization *in vivo* [58]. Secondly, we assessed effects on neuronal cell culture, and the conditions may not reflect what actually occurs in an intact brain in response to insulin signaling. In the human brain, there is some evidence that GluT4 is also expressed (albeit to lower extent) on non-neuronal cells, such as microglia and endothelial cells [73], so insulin’s effects in human brain may be more diverse than those observed in rodent studies. Thus, more research is necessary to determine the physiological effects of insulin on neuronal glucose utilization.

One implication of this work is that GluT4 may be an appropriate target for therapeutic intervention. We recently showed that chronic inhibition of brain GluT4 caused marked alterations in hippocampal metabolism and cognitive performance. Conversely, enhancing GluT4 function in the brain of cognitively impaired patients may mimic the procognitive effects of insulin without inducing insulin resistance, which is a key concern associated with chronic hyperinsulinemia [74], and is possibly a limitation of intranasal insulin, which is a treatment being researched to treat Alzheimer's disease [75–77]. Indeed, several treatments that increase the activity of GluT4 such as alpha lipoic acid, AICAR, insulin sensitizing drugs, and histone deacetylase inhibitors also increase memory [19–22]. Continued investigation of treatments that enhance GluT4 translocation or intrinsic activity in the hippocampus may provide novel therapeutic opportunities to remedy memory loss without the ill-effects of a generalized increase in insulin.

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Fig. 1. Regional distribution of glucose utilization in the hippocampus during SA testing. A, Experimental outline for injections of [^{14}C]-2DG. B, Heat maps from representative phosphorimaging of [^{14}C]-2DG phosphorylation. C, [^{14}C] Standards showing standard curve used. D, Regional analysis of [^{14}C]-2DG phosphorylation during SA testing confirming role of dorsal hippocampus in SA behavior ($n = 4$ per group) * $p < 0.05$, two-tailed.

Fig. 2.

Glucose transporter trafficking in the hippocampus during SA testing. A, Experimental outline used to determine the effects of SA testing on glucose transporter trafficking. B, Representative western blots used to determine ratio of plasma membrane to total levels of glucose transporter expression following SA testing. C, Ratio of plasma membrane to membrane glucose transporter expression following SA testing or control ($n = 7-12$ per group). $p < 0.05$, two-tailed.

Fig. 3. Pharmacological inhibition of hippocampal insulin signaling during SA testing. A, Inhibition of PI3 K signaling with NFV reduced SA behavior as measured by decreased alternation scores. B, Inhibition of GluT4-mediated glucose transport with IND had no effects on alternation scores ($n = 5-9$ per group). $p < 0.05$, two-tailed.

Fig. 4.

Insulin-enhancement of SA memory and effects of GluT4-inhibition. A, Intrahippocampal insulin increased SA performance, and this effect was blocked by GluT4-mediated glucose transport inhibition ($n = 10-11$ per group). $p < 0.05$, two-tailed.

Fig. 5. Effects of GluT4-mediated glucose transport inhibition on glucose utilization in primary hippocampal neuronal cell culture. Insulin increased hippocampal glucose utilization, and this effect was blocked by inhibition of GluT4 ($n = 4-5$ hippocampi per group; 3 repetitions). $p < 0.05$, two-tailed.