

Glucose attenuates impairments in memory and CREB activation produced by an $\alpha 4\beta 2$ but not an $\alpha 7$ nicotinic receptor antagonist

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ABSTRACT

Glucose improves memory for a variety of tasks when administered to rats and mice near the time of training. Prior work indicates glucose may enhance memory by increasing the synthesis and release of the neurotransmitter acetylcholine in the brain. To investigate if specific acetylcholine receptor subtypes may mediate some of the memory-enhancing actions of glucose, we examined the effects of subtype-specific nicotinic acetylcholine receptor antagonists on memory in Fischer-344 rats and also examined the ability of glucose to reverse drug-induced impairments. Pre-training peripheral injections of methyllycaconitine (MLA) or dihydro-beta-erythroidine (DH β E), which are specific $\alpha 7$ and $\alpha 4\beta 2$ nicotinic receptor antagonists, respectively, dose-dependently impaired retention latencies in an inhibitory avoidance task when tested 7-days but not 1 h after training. Immediate post-training glucose injections attenuated the impairments, but were more effective in attenuating the DH β E-induced impairments. Likewise, peripheral or direct intrahippocampal injections of MLA or DH β E dose-dependently impaired spatial working memory scores on a spontaneous alternation task. Concurrent administration of glucose reversed DH β E- but not MLA-induced impairments. CREB phosphorylation downstream of cholinergic signaling was assessed 30 min after spontaneous alternation testing and intrahippocampal drug infusions. Both MLA and DH β E impaired hippocampal CREB phosphorylation; glucose reversed DH β E- but not MLA-induced deficits. The effectiveness of glucose in reversing DH β E- but not MLA-induced impairments in behavioral performance and CREB phosphorylation suggests that activation of $\alpha 7$ receptors may play an important role in memory enhancement by glucose.

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1. Introduction

In humans and rodents, rises in blood glucose in response to stressful or emotional stimuli mediate memory improvement for the associated events. Likewise, glucose administration prior to or shortly after an event can increase its salience, converting a relatively unimportant event to a memorable one (Gold and Korol, 2010; Korol and Gold, 2007, 1998; Messier, 2004). In young adult rodents, increases in blood glucose enhance memory in a wide variety of behavioral tasks, ranging from tests of spatial working memory to assessments of memory at long intervals after training (cf. Gold, 2008). Glucose can also reverse age-related memory impairments in these same tasks, improving performance in old rats to the levels seen in young rats (Gold, 2005; McNay and Gold, 2001; Morris et al., 2010; Salinas and Gold, 2005).

Glucose is thought to work directly in the brain to enhance cellular and molecular memory processes, perhaps mediated by enhanced aerobic glycolysis and lactate production in astrocytes (Newman et al., 2011; Suzuki et al., 2011). Previous work suggests that systemically- or centrally-administered glucose may enhance memory by augmenting central release of the neurotransmitter acetylcholine. A number of microdialysis studies show that both peripheral and direct intrahippocampal glucose administration augment training-related acetylcholine release in the hippocampus, concurrent with memory enhancement (Kopf et al., 2001; Morris et al., 2010; Ragozzino and Gold, 1995; Ragozzino et al., 1998, 1996). These effects on acetylcholine release appear to be regionally specific and do not occur in animals at rest (Ragozzino et al., 1998, 1996), suggesting that glucose supports enhanced acetylcholine release in particular brain areas where it is needed to facilitate memory processes.

An open question is whether the memory-improving actions of glucose rely on signaling through some acetylcholine receptor subtypes more than others. Acetylcholine activates both muscarinic

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and nicotinic acetylcholine receptors. The subtypes of these receptors have diverse functions and variable expression patterns throughout the brain. Previous work indicates that peripheral injections of general muscarinic and nicotinic antagonists impair performance in a number of behavioral memory paradigms, including inhibitory avoidance and spontaneous alternation tasks, and that co-administration of glucose attenuates these impairments (Blanchard and Duncan, 1997; Kopf and Baratti, 1994; Ragozzino and Gold, 1991; Ragozzino et al., 1994; Stone et al., 1995, 1991, 1988). These results imply a lack of regional and receptor specificity in glucose's actions, suggesting that glucose could compensate for deficits in muscarinic receptors by promoting signaling through nicotinic receptors, and vice versa. However, the results are difficult to interpret because of the wide-ranging effects of these drugs throughout the body and brain, including effects not specific to memory processes.

The emergence of drugs targeting specific acetylcholine receptor subtypes in the brain, as opposed to general muscarinic or nicotinic receptors, makes it possible to begin evaluating which receptor subtypes may be important to the memory-enhancing effects of glucose. Two nicotinic receptor subtypes, $\alpha 4\beta 2$ and $\alpha 7$, possess the functional properties and regional distribution that make them potential mediators of glucose's effects (Cincotta et al., 2008; Graef et al., 2011; Kenney and Gould, 2008; Levin et al., 2006). Neuronal nicotinic receptors are ionotropic receptors composed of various combinations of five membrane-spanning subunits. Nine α ($\alpha 2$ – $\alpha 10$) and three β ($\beta 2$ – $\beta 4$) subunits have been identified, making a large number of receptor subtype combinations possible. However, the heteromeric $\alpha 4\beta 2$ and homomeric $\alpha 7$ receptor subtypes have garnered the most attention due to their high levels of expression in the brain and roles in a variety of cognitive processes. In many situations, $\alpha 4\beta 2$ and $\alpha 7$ receptors appear to function similarly. For example, a number of studies have utilized direct brain infusions of methyllycaconitine (MLA) or dihydro- β -erythroidine (DH β E), which are competitive antagonists for $\alpha 7$ and $\alpha 4\beta 2$ receptors, respectively, to assess working memory performance in the radial arm maze. Infusions of either MLA or DH β E into the ventral or dorsal hippocampus, amygdala, or frontal cortex induced working memory impairments, with only minor dose-related differences between the drugs (Addy et al., 2003; Bettany and Levin, 2001; Chan et al., 2007; Levin et al., 2002; Nott and Levin, 2006). In other cases, there are distinct functional differences between the $\alpha 4\beta 2$ and $\alpha 7$ receptor subtypes. For instance, there are several studies indicating a specific role for $\alpha 4\beta 2$ but not $\alpha 7$ receptors in mediating the effects of nicotine on enhancing memory for contextual fear conditioning (Davis and Gould, 2006, 2007; Davis et al., 2007; Kenney et al., 2012).

Recent evidence indicates that glucose and nicotinic acetylcholine receptors activate a similar downstream molecular signaling pathway related to memory formation. Specifically, cAMP response element binding protein (CREB) is a transcription factor widely implicated in the formation of long-lasting memory and long-term changes in synaptic plasticity (Alberini, 2009; Benito and Barco, 2010; Carlezon et al., 2005; Morris and Gold, 2012; Silva et al., 1998; Yin and Tully, 1996). A number of studies indicate that nicotine administration activates CREB in a variety of neuronal cell types in both *in vitro* and *in vivo* models (Chang and Berg, 2001; Hu et al., 2002; Nakayama et al., 2001; Pascual et al., 2009; Tang et al., 1998). Likewise, glucose administration following inhibitory avoidance training reverses age-related memory impairments, with parallel activation of CREB phosphorylation. Consistent with the view of regional selectivity in the memory-enhancing actions of glucose, there is a high degree of regional variability in glucose-mediated increases in CREB phosphorylation, including within subregions of the hippocampus (Morris and Gold, *in press*).

The following work examines the effects of the $\alpha 7$ antagonist MLA and the $\alpha 4\beta 2$ antagonist DH β E on memory processes in the inhibitory avoidance and spontaneous alternation tasks. Given that the general nicotinic antagonist mecamylamine impairs behavioral performance in both of these tasks, a major prediction was that one or both of these subtype-specific antagonists would impair performance similar to mecamylamine, perhaps distinguishing which nicotinic receptor subtype mediates the effects of mecamylamine. We hypothesized that any differences in the effects of MLA and DH β E may help determine which nicotinic receptor signaling processes are important in facilitating memory formation in these tasks.

We also tested the ability of glucose to attenuate any drug-induced impairments. Based on the convergent behavioral, neurochemical, and molecular evidence of regional selectivity in the memory-enhancing actions of glucose, a major hypothesis was that glucose may enhance memory by facilitating signaling through specific cholinergic receptor subtypes. Thus, examining interactions between the effects of glucose and subtype-specific antagonists may help identify certain nicotinic receptor subtypes that are important to the memory-enhancing effects of glucose.

These experiments utilize both peripheral and direct intrahippocampal injections and include correlations with the expression of phosphorylated CREB (pCREB) in the hippocampus. Although the focus here was on nicotinic receptors, some muscarinic receptor subtypes (especially M_1 receptors) share similar distributions, behavioral effects, molecular signaling pathways, etc. to $\alpha 7$ and $\alpha 4\beta 2$ receptors, and thus may also be important in these contexts. However, muscarinic antagonists tend to have more peripheral side effects compared to MLA and DH β E, which are centrally-acting drugs. In addition, the effects of MLA and DH β E have been examined together in a variety of behavioral studies, including aversive and spatial working memory tasks, allowing for direct comparisons with the present experiments and supporting this investigation of the effects of nicotinic receptor antagonists on memory and on glucose enhancement of memory.

2. Methods

2.1. Subjects

Young adult (3–4 months) male Fischer-344 rats ordered from Taconic Farms (Germantown, NY) were individually housed in translucent cages with a 12-h light/dark cycle (lights on at 07:00 h) and *ad libitum* access to food and water. Animal pain and discomfort were minimized, and all experiments were conducted in accordance with animal care guidelines established by the National Institute of Health and the University of Illinois, which is fully accredited (AAALAC). Rats were handled for 2–3 min each day on 5 consecutive days prior to behavioral training.

2.2. Preparation of drugs

Methyllycaconitine citrate salt hydrate (MLA) was obtained from Sigma–Aldrich (St. Louis, MO). Dihydro- β -erythroidine hydrobromide (DH β E) was obtained from Tocris Bioscience (Minneapolis, MN). MLA, DH β E, and glucose were dissolved in saline for intraperitoneal (i.p.) administration or in artificial cerebral spinal fluid (aCSF; 128 mM NaCl, 2.5 mM KCl, 1.3 mM CaCl₂, 2.1 mM MgCl₂, 0.9 mM NaH₂PO₄, 2.0 mM Na₂HPO₄, 1 mM glucose, pH 7.4) for intrahippocampal administration. The 1.0 mM glucose concentration in the aCSF is based on evidence that this level is seen at baseline in hippocampal extracellular fluid (McNay and Gold, 1999).

2.3. Surgery and intrahippocampal drug infusions

Rats were deeply anesthetized with isoflurane and placed in a stereotaxic apparatus. Stainless steel guide cannulae (Plastics One, Roanoke, VA) were implanted bilaterally into the dorsal hippocampus [coordinates: -3.2 mm from bregma; ± 2.5 mm lateral; -1.4 mm deep from dura], according to the atlas of Paxinos and Watson (2003). Rats were monitored and allowed to recover for 7 days after surgery. Prior to training, microinfusion probes (Plastics One) were inserted through the guide cannulae to a point 1 mm below the guide cannulae tips. Compounds were infused bilaterally into the dorsal hippocampus at a rate of 0.25 μ l/min for 2 min. Infusion probes were left in place for an additional 1 min to allow solutions to diffuse away from the probe tips.

2.4. Inhibitory avoidance training

All training and testing took place between 12:00 and 16:00 h. The inhibitory avoidance apparatus was a trough-shaped alleyway (91 cm long, 22.9 cm wide at the top, 7.6 cm wide at the bottom, and 15.2 cm deep) divided into lit (31 cm) and dark (60 cm) compartments by a sliding door that could be lowered through the floor. Each rat was placed in the lit chamber facing the door. When the rat turned completely around, the door was lowered to a height 2 cm above the floor. When the rat again turned toward the door, a timer was started to record the latency to enter the dark chamber (i.e. the training latency). Upon entering the dark chamber, the rat received a brief foot shock (0.2 mA, 0.4 s) and the door was closed to prevent reentry into the lit chamber. After training or post-training injections, rats were returned to the holding cage. Retention latencies (max of 600 s) were tested 7 days later using a similar procedure, but without the foot shock. MLA (0.5, 2, or 5 mg/kg) or DH β E (0.5, 2, or 5 mg/kg) were given i.p. 10 min prior to training. Glucose (250 mg/kg) was given i.p. immediately after training.

2.5. Foot shock perceptual thresholds

10 min after i.p. injections of saline or 2 mg/kg MLA or DH β E, rats received a series of foot shocks (each 0.5-s duration) beginning at 0.1 mA and increasing at 0.05 mA increments with a 30 s inter-shock interval until a jump response was induced. The lowest shock intensities that caused the rats to flinch and jump were recorded by two independent observers.

2.6. Spontaneous alternation testing

All training took place between 12:00 and 16:00 h. Spontaneous alternation performance was assessed in a 4-arm plus-shaped black Plexiglas maze with an open ceiling. The dimensions of each arm were 45 cm L \times 13 cm W \times 7 cm H. The dimensions of the central square-shaped area were 13 cm L \times 13 cm W \times 7 cm H. The training room contained numerous extramaze visual cues in the form of objects and wall decorations. Rats were placed in a random start arm and were allowed to freely traverse the maze for 20 min. The number and sequence of entries were recorded and alternation performance was calculated. An alternation consisted of visiting all four arms within overlapping sets of five arm visits. Using this procedure, the total number of possible alternations was equal to the number of arm entries minus 4. The percent alternation score is equal to (actual alternations/possible alternations) \times 100. Chance performance on this task is 44%. Only data from rats that made at least 10 arm choices (6 possible alternations) were included in the analyses. For experiments with i.p. injections, MLA (0.5, 2, or 5 mg/kg) or DH β E (0.5, 2, or 5 mg/kg) were administered alone or in combination with glucose (250 mg/kg) 30 min prior to testing. For experiments with intrahippocampal injections, MLA (6.75, 13.5, or 27 μ g/side) or DH β E (4, 8, or 20 μ g/side) were infused alone or in combination with glucose (16.7 nmol/side) beginning 10 min prior to training. Some of the experiments with intrahippocampal injections (i.e. those illustrated in Fig. 5) utilized a Latin square design in which each rat was tested on four separate occasions with a 3-day rest in between each test. Prior to each test, each rat received a single infusion of a drug, with doses administered in a counterbalanced order.

2.7. Perfusion and brain slicing

For analysis of infusion probe placement, brains were collected within two days of the end of behavioral testing. For pCREB immunohistochemistry, brains were collected 30 min after spontaneous alternation testing. Rats were deeply anesthetized with an overdose injection of sodium pentobarbital (i.p.; Sigma–Aldrich) and then perfused intracardially with 80 ml of 0.1 M phosphate-buffered saline followed by 80 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. Rats were decapitated and the brains were removed and placed into 4% paraformaldehyde in 0.1 M PB for \sim 72 h. The brains were transferred to 20% glycerol in 0.1 M PBS for \sim 48 h. For probe placement analysis, frozen sections (40 μ M) containing the probe infusion tracts were collected at -30 $^{\circ}$ C with a Leica 1800 cryostat (Leica Microsystems, Wetzlar, Germany). Sections were mounted onto gelatin-coated slides and stained with cresyl violet to visualize probe placements, which were determined using a dissection microscope. For immunohistochemistry, frozen sections (40 μ M) just posterior to the injection site were collected at -30 $^{\circ}$ C with a Leica 1800 cryostat. Slices were stored in a cryoprotective solution (250 mM 40 KD polyvinylpyrrolidone, 880 mM sucrose, 30% v/v ethylene glycol, 50 mM sodium phosphate) at -20 $^{\circ}$ C.

2.8. Immunohistochemistry

All steps took place at room temperature and all reactions were performed in duplicate. Slices were washed three times for 10 min each time in 0.05 M PBS initially and between all subsequent steps. Slices were first incubated in blocking solution (1% H $_2$ O $_2$, 1% normal goat serum, 0.02% triton x-100, 0.05 M PBS) for 10 min. They were transferred to a pre-incubation solution (2% NGS, 0.4% triton x-100, 0.05 M PBS) for 20 min and then incubated overnight in a solution (1% NGS, 0.4% triton x-100, 0.05 M PBS) containing a rabbit primary antibody for Ser-133 phosphorylated CREB (Millipore, Billerica, MA) diluted 1:4000. The next day, the slices

were placed for 1 h in a solution (1% NGS, 0.2% triton x-100, 0.05 M PBS) containing a goat anti-rabbit biotinylated secondary antibody (Santa Cruz, Santa Cruz, CA). They were next incubated for 30 min with ABC reagent (Vector, Burlingame, CA) in 0.05 M PBS, followed by incubation with DAB substrate (Vector) for 4 min. Slices were mounted onto slides and allowed to dry overnight. The next morning, slices were dehydrated with a graded ethanol series of washes, then coverslipped using DPX mountant (Sigma–Aldrich).

2.9. Image acquisition and analysis

Sections were imaged using a Leica DM 6000B/CTR6000 light microscope and a Leica DFC350 FX video camera, which was interfaced to a PC computer. This system was used in conjunction with Image-Pro software (Media Cybernetics, Inc., Bethesda, MD) for image acquisition and for correction of unevenness in illumination across images. Image J software (NIH, Bethesda, MA) was used to quantify the integrated optical density of pCREB staining in area CA1 and the dentate gyrus of the hippocampus. A statistical thresholding method in Image J was used to ensure that only specifically labeled cells were being measured. For each image, the optical density of a nearby region with no or little specific staining was calculated and used for background subtraction.

2.10. Statistical analyses

All analyses were performed using Statview software. Inhibitory avoidance behavioral results were analyzed using a non-parametric Kruskal–Wallis one-way analysis of variance, followed by Mann–Whitney tests for individual comparisons.

The spontaneous alternation behavioral results (except those noted below) and the optical densities of pCREB immunostaining were analyzed using one-way ANOVAs with post hoc Fisher PLSD tests where appropriate. The spontaneous alternation behavioral results that utilized a Latin square design were analyzed using repeated measures ANOVA with post hoc Fisher PLSD tests where appropriate. Correlations between behavioral results and protein expression were determined by simple linear regression.

3. Results

3.1. Inhibitory avoidance training with peripheral injections of drugs

Fig. 1 shows the effects of peripherally-administered nicotinic antagonists on retention of inhibitory avoidance training. Drugs were administered 10 min prior to training. There was a significant main effect of MLA on 7-day retention latencies ($H_{(3,28)} = 12.02$, $p < .01$). Both the 2 and 5 mg/kg doses of MLA significantly depressed 7-day retention latencies compared to those of saline-injected controls (p 's $< .05$). The 2 mg/kg dose was more effective, significantly depressing 7-day retention latencies compared to the 5 mg/kg dose ($p < .05$). There was also a significant main effect of DH β E on 7-day retention latencies ($H_{(3,28)} = 9.60$, $p < .05$). The 2 and 5 mg/kg doses of DH β E were similarly effective at depressing 7-day retention latencies compared to saline controls (p 's $< .05$). Neither MLA nor DH β E significantly altered training latencies.

To examine if glucose could attenuate drug-induced impairments in 7-day retention latencies, saline or 2 mg/kg MLA or DH β E were administered 10 min before inhibitory avoidance training in combination with immediate post-training injections of saline or glucose (250 mg/kg). The results are shown in Fig. 2. Both MLA-saline and DH β E-saline significantly reduced 7-day retention latencies compared to those of saline–saline controls (p 's $< .01$). Peripheral glucose administration attenuated these drug-induced impairments, but was more effective in reversing DH β E-induced deficits. In the MLA-glucose group, retention latencies were significantly higher than those in the MLA-saline group, but still significantly reduced compared to those in saline–saline controls (p 's $< .05$). By comparison, retention latencies in the DH β E-glucose group were similar to those in the saline–saline group ($p > .5$), with the group median reaching the 600 s maximum. Training latencies were not significantly different across groups.

MLA or DH β E-induced impairments in retention latencies may reflect an inability of rats to acquire the learned response. To examine this possibility, two experiments were performed. The first

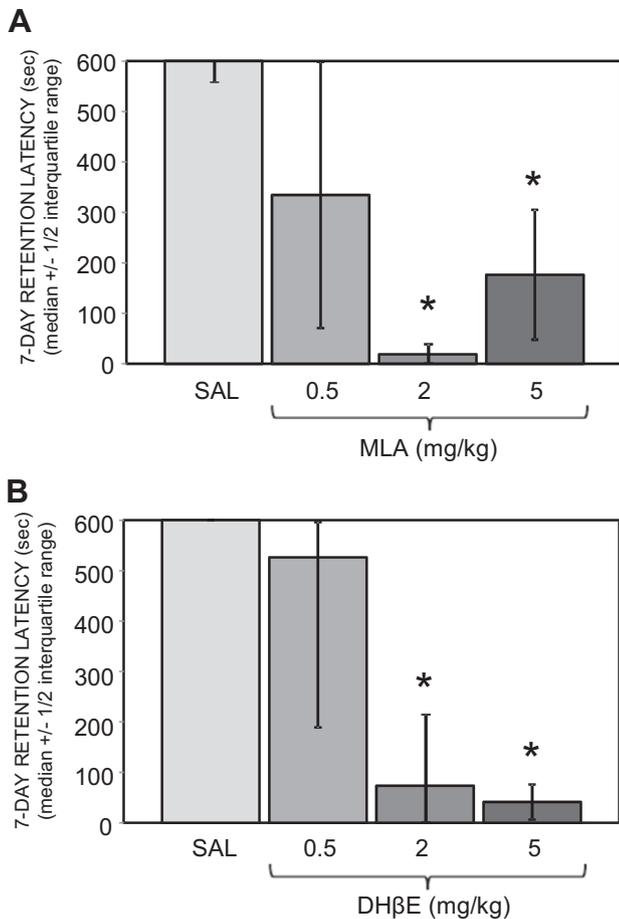


Fig. 1. Effects of peripherally-administered nicotinic antagonists on inhibitory avoidance memory tested 7 days after training. (A) MLA doses of 2 and 5, but not 0.5 mg/kg, significantly impaired memory. (*) p 's < .05 vs. saline. N 's = 8. (B) DHβE doses of 2 and 5, but not 0.5 mg/kg, significantly impaired memory. (*) p 's < .05 vs. saline. N 's = 8.

experiment assessed retention latencies using a shorter training–testing interval. Saline or 2 mg/kg MLA or DHβE was injected 10 min prior to training, and retention latencies were tested 1 h later. All three groups had median 1-h retention latencies at the maximum of 600 s, with no significant differences among groups (data not shown). The second experiment examined the effects of the drugs on foot shock perceptual thresholds. Injections of 2 mg/kg MLA or DHβE 10 min prior to foot shock did not significantly alter flinch or jump thresholds compared to those of saline-injected rats.

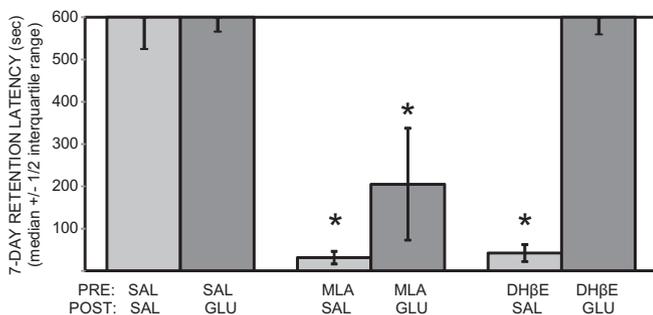


Fig. 2. Glucose attenuation of drug-induced memory impairments after inhibitory avoidance training. Pre-training administration of MLA or DHβE (2 mg/kg), together with post-training saline injections, produced deficits in memory tested 7 days after training. Post-training glucose attenuated the drug-induced memory impairments, but was more effective at reversing the impairments induced by DHβE. (*) p 's < .05 vs. saline–saline. N 's = 8.

The finch thresholds were 0.26 ± 0.01 mA (mean \pm s.e.m.) for all groups. Jump thresholds ranged from 0.63 ± 0.03 mA in the saline and DHβE groups to 0.65 ± 0.03 in the MLA group.

3.2. Spontaneous alternation testing

3.2.1. Peripheral injections of drugs

Fig. 3 shows the effects of peripherally-administered nicotinic antagonists on spontaneous alternation scores. MLA or DHβE given 30 min before testing impaired alternation scores, with effective doses similar to those observed in the inhibitory avoidance task ($F_{(3,28)} = 3.09$, $p < .05$ for MLA; $F_{(3,28)} = 3.34$, $p < .05$ for DHβE). For the MLA experiment, the intermediate dose of 2 mg/kg, but not lower or higher doses, significantly reduced alternation scores compared to saline-injected controls ($p < .05$). In contrast, MLA had no significant effect on the number of arm entries during testing, which ranged from 24.6 ± 2.8 (mean \pm s.e.m.) in the saline group to 26.1 ± 2.6 in the 2 mg/kg MLA group. For the DHβE experiment, doses of 2 and 5 mg/kg were about equally effective at impairing alternation scores compared to saline controls (p 's < .05). The number of arm entries decreased with increasing doses of DHβE, ranging from mean values of 23.3 ± 1.3 in the saline group to 18.5 ± 1.9 in the 5 mg/kg DHβE group. However, this was not a statistically significant effect.

To determine if glucose could attenuate drug-induced impairments in spontaneous alternation scores, MLA or DHβE (2 mg/kg)

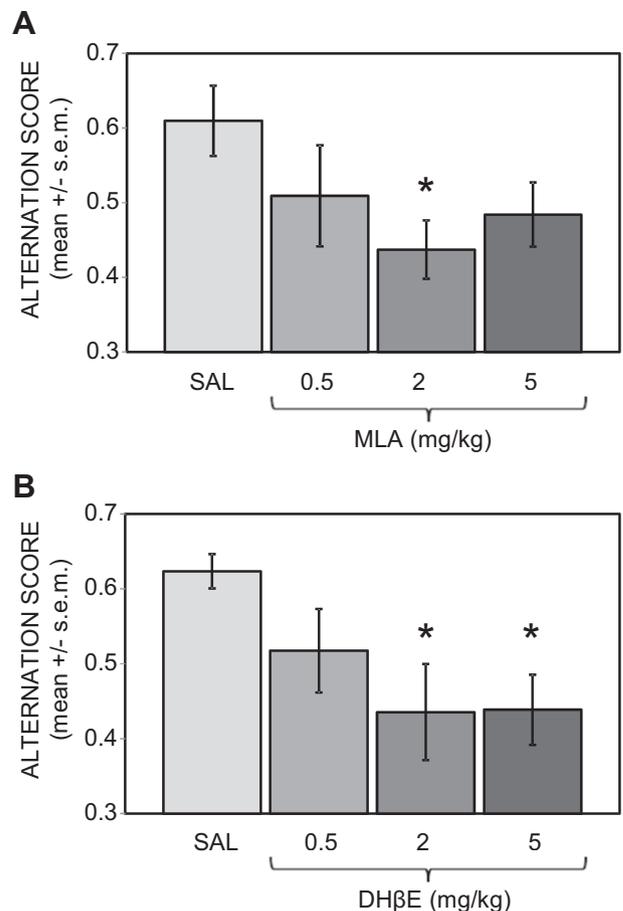


Fig. 3. Effects of peripherally-administered nicotinic antagonists on spontaneous alternation spatial working memory scores. (A) An MLA dose of 2, but not 0.5 or 5 mg/kg, significantly impaired alternation scores. (*) $p < .05$ vs. saline. N 's = 8. (B) DHβE doses of 2 and 5, but not 0.5 mg/kg, significantly impaired alternation scores. (*) p 's < .05 vs. saline. N 's = 8.

was administered alone or in combination with glucose (250 mg/kg) 30 min before testing (Fig. 4). MLA and DH β E significantly impaired alternation scores compared to saline-injected controls (p 's < .01). Glucose had no significant effect on attenuating MLA-induced deficits; rats treated with MLA-glucose had alternation scores similar to those in the MLA group (p > .5) and significantly lower than those of saline controls (p < .05). However, glucose reversed DH β E-induced impairments in alternation performance, raising scores well above those of rats administered DH β E alone (p < .05). The numbers of arm entries were significantly reduced in the DH β E (17.5 \pm 1.5) and DH β E-glucose (17.1 \pm 0.8) groups compared to saline controls (23.4 \pm 1.4; p 's < .01). Therefore, the effect of glucose was restricted to DH β E effects on alternation scores and did not extend to drug effects on locomotor activity. The numbers of arm entries in the other groups were not significantly different from those in the saline group.

3.2.2. Intrahippocampal injections of drugs

Fig. 5 shows the effects of intrahippocampally-administered nicotinic antagonists on spontaneous alternation scores. To reduce the number of rats subjected to surgical procedures, a Latin square design was used here but not in subsequent experiments with glucose. Drugs were infused beginning 10 min before testing. Both MLA and DH β E infusions significantly impaired alternation scores ($F_{(3,24)} = 3.02$, p < .05 for MLA; $F_{(3,24)} = 3.17$, p < .05 for DH β E). MLA doses of 6.75 and 13.5 μ g/side were equally effective at impairing alternation scores compared to those of aCSF-injected controls (p 's < .05). However, MLA lost its effectiveness in impairing alternation scores at the highest dose of 27 μ g/side. DH β E was ineffective at the lowest dose of 4 μ g/side, but significantly impaired alternation performance at higher doses of 8 and 20 μ g/side (p 's < .05 vs. aCSF). Neither MLA nor DH β E infusions significantly altered the number of arm entries. The mean numbers of arm entries were slightly lower than those seen with peripheral injections, ranging from 19.4 \pm 2.8 to 23.1 \pm 2.7 in the MLA experiment and 19.1 \pm 2.4 to 21.2 \pm 1.4 in the DH β E experiment.

Using the Latin square design, each rat received an intrahippocampal injection on four separate occasions, which could have progressively increased damage at the infusion site. To examine this possibility, repeated measures ANOVAs were conducted to determine if the number of injections affected alternation scores, revealing no main effects in either the MLA ($F_{(3,24)} = 1.02$, p > .2) or DH β E ($F_{(3,24)} = 2.23$, p > .1) experiments.

All infusion cannulae were placed in the dorsal hippocampus, as shown schematically in Fig. 5. After the four infusions, the area near the tip did not display unusual damage and appeared similar to that seen in other groups in this and in past experiments.

The effects of intrahippocampal glucose on attenuating drug-induced deficits largely paralleled the results seen with

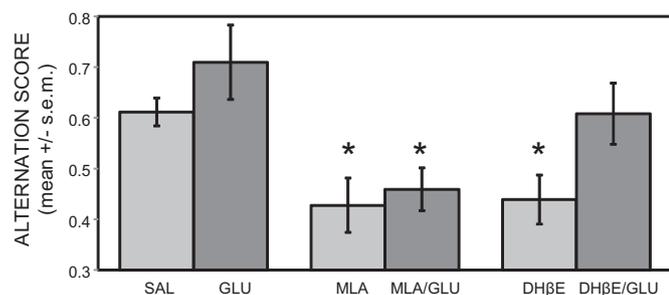


Fig. 4. Glucose attenuation of drug-induced working memory impairments in the spontaneous alternation task. MLA or DH β E (2 mg/kg) produced deficits in alternation scores. Co-administration of glucose with the antagonists attenuated DH β E- but not MLA-induced deficits. (*) p 's < .05 vs. saline. N 's = 8.

peripheral injections. Glucose (16.7 nmol/side) was combined with the lowest effective doses of the nicotinic antagonists and infused 10 min before alternation testing. As shown in Fig. 6, glucose had no effect on attenuating MLA-induced impairments in alternation performance. Both the MLA and MLA-glucose groups had significantly depressed alternation scores (p 's < .05 vs. aCSF), which were not significantly different from each other (p > .5). In contrast, glucose reversed DH β E-induced impairments in alternation scores. Although DH β E alone produced significant deficits in alternation scores (p < .05 vs. aCSF), the DH β E-glucose group had similar scores compared to aCSF (p > .5). Unlike the pattern of results seen with peripheral injections of drugs, there was a trend for enhancement in alternation scores following infusion of intrahippocampal glucose alone (p < .06 vs. aCSF). The number of arm entries were similar across groups, ranging from 19.1 \pm 3.0 in the DH β E-glucose group to 24.6 \pm 3.8 in the MLA-glucose group.

3.3. pCREB staining

Immunostaining levels of pCREB were examined 30 min after spontaneous alternation testing in rats receiving intrahippocampal infusions of drugs (corresponding to Fig. 6). As shown in Fig. 7, pCREB staining levels in area CA1 paralleled the behavioral results. Both MLA and DH β E impaired pCREB staining compared to aCSF-injected controls (p 's < .05). Glucose reversed the DH β E- but not MLA-induced pCREB deficits (p > .5 DH β E vs. aCSF; p < .05 MLA vs. aCSF). Unlike the behavioral results, there was no trend for enhancement of pCREB staining by intrahippocampal glucose alone (p > .5). In contrast to the results for area CA1, there were no significant effects of drug treatments on pCREB staining levels in the dentate gyrus (data not shown).

As shown in Fig. 8, there was a significant positive correlation between area CA1 pCREB staining levels and spontaneous alternation scores in rats receiving MLA-glucose injections ($r = 0.90$, p < .01), and a trend for significance in rats receiving MLA alone ($r = 0.69$, $p = .09$). There were no correlations or trends in any of the other groups (p 's > .2).

4. Discussion

4.1. Effects of nicotinic antagonists on memory

Peripheral injections of MLA or DH β E impaired memory when tested 7 days after inhibitory avoidance training, supporting the view that inhibition of cholinergic signaling through either α 7 or α 4 β 2 nicotinic receptors inhibits the formation of long-lasting memories. Previous studies in rats and mice have shown that pre-training peripheral administration of the centrally-acting nicotinic antagonist mecamylamine impairs later retention for inhibitory avoidance training, whereas the peripherally-acting nicotinic antagonist hexamethonium has no effect (Chiappetta and Jarvik, 1969; Glick and Greenstein, 1972; Goldberg et al., 1971; Ragozzino and Gold, 1991; Rush and Streit, 1992). The present results extend these findings, suggesting that mecamylamine could exert its behavioral effects through inhibition of α 4 β 2 and/or α 7 receptors in the brain.

In the present study, the administration of the drugs prior to instead of after inhibitory avoidance training leaves open the possibility that the drugs produced apparent amnesia by altering factors other than memory, such as attention, motivation, or sensorimotor processes. However, a few pieces of evidence indicate that behavioral differences due to effects on non-mnemonic variables are unlikely. First, neither MLA nor DH β E altered training latencies or thresholds for foot shock sensitivities, suggesting that these measures of sensorimotor processes were intact during

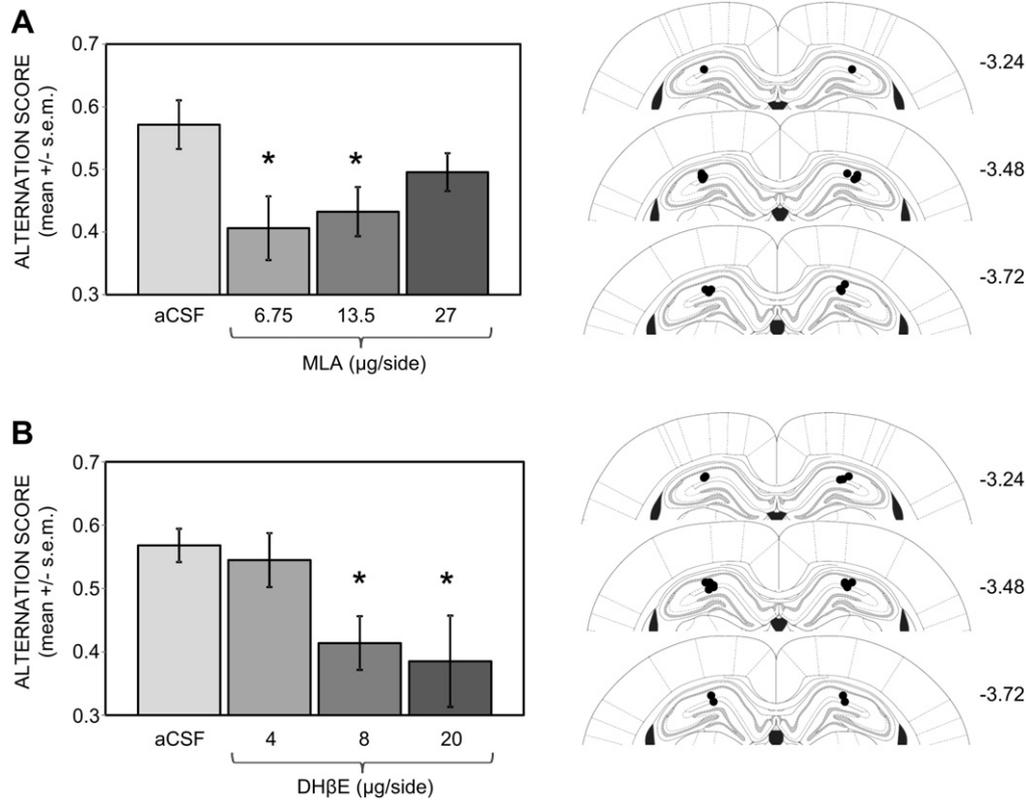


Fig. 5. Effects of intrahippocampally-administered nicotinic antagonists on working memory assessed in a spontaneous alternation task. (A) MLA doses of 6.75 and 13.5, but not 27 µg/side, significantly impaired alternation scores. (*) p 's < .05 vs. aCSF. $N = 9$. (B) DHβE doses of 8 and 20, but not 4 µg/side, significantly impaired alternation scores. (*) p 's < .05 vs. aCSF. $N = 9$. To the right of each graph is an illustration of the infusion sites targeting the dorsal hippocampus for each experiment. Filled circles represent tips of infusion tracts. Numbers refer to distance in mm posterior to bregma. Adapted with permission from Paxinos and Watson (2003).

training. Second, the high memory scores in MLA- or DHβE-injected rats tested 1 h after training indicate the drugs did not interfere with the ability of the rats to learn and initially remember the training experience. However, differences between saline- and drug-injected groups could have been masked by a ceiling effect, given that the median 1-h retention latencies in all groups reached the maximum of 600 s.

Peripheral administration of MLA or DHβE also impaired performance in the spontaneous alternation task. These results were remarkably similar to the effects of the antagonists on retention performance in the inhibitory avoidance task, and were consistent with past findings showing that peripheral injections of mecamylamine, but not the peripherally acting hexamethonium,

impair alternation performance (Ragozzino and Gold, 1991). In both the spontaneous alternation and inhibitory avoidance tasks, MLA-induced impairments followed a U-shaped pattern, in which the middle dose of 2 mg/kg was most effective at impairing behavioral performance. In contrast, both 2 and 5 mg/kg doses of DHβE were similarly effective at impairing performance in these behavioral tasks. The U-shaped pattern observed for MLA-induced deficits may relate to non-specific effects of the drug at higher doses.

Direct infusions of MLA or DHβE into the dorsal hippocampus also impaired performance in the spontaneous alternation task, suggesting that the drugs work directly in the brain to impair spatial working memory processes. These results are consistent with prior behavioral studies of spatial working memory in the appetitive radial arm maze task, where infusions of MLA or DHβE directly into the hippocampus, amygdala, or frontal cortex, significantly increased working memory errors (Addy et al., 2003; Bettany and Levin, 2001; Chan et al., 2007; Levin et al., 2002; Nott and Levin, 2006). Interestingly, infusions of DHβE into the mediadorsal thalamic nucleus actually reduced working memory errors during radial arm maze testing (Cannady et al., 2009). Opposing effects in different brain regions of MLA or DHβE have also been observed in trace fear conditioning (Raybuck and Gould, 2010), suggesting some regional variability in the actions of these drugs on learning and memory. In the present study, the effects of peripheral administration of MLA or DHβE on alternation scores were similar to those seen with intrahippocampal infusions, consistent with the possibility that the effects on memory of the peripherally-injected drugs may be mediated by hippocampal actions.

An interesting and unexpected result was the lack of any dissociation between the effects of MLA and DHβE on behavioral performance. Similar doses of each drug impaired performance in

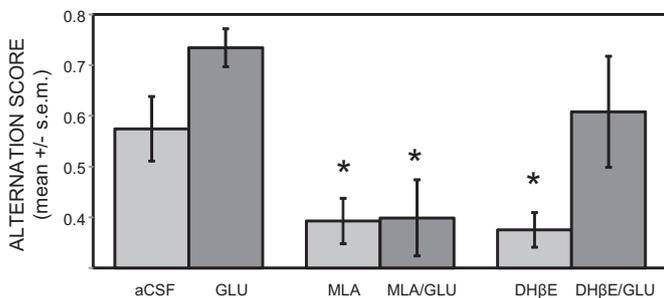


Fig. 6. Glucose attenuation of impairments in spontaneous alternation memory scores produced by intrahippocampally-administered antagonists. MLA (6.75 µg/side) or DHβE (8 µg/side) produced deficits in alternation scores. Co-administration of glucose with the antagonists attenuated DHβE- but not MLA-induced deficits. There was a trend for enhancement in alternation scores following administration of glucose alone. (*) p 's < .05 vs. aCSF. N 's = 8 for aCSF and GLU. N 's = 7 for all other groups.

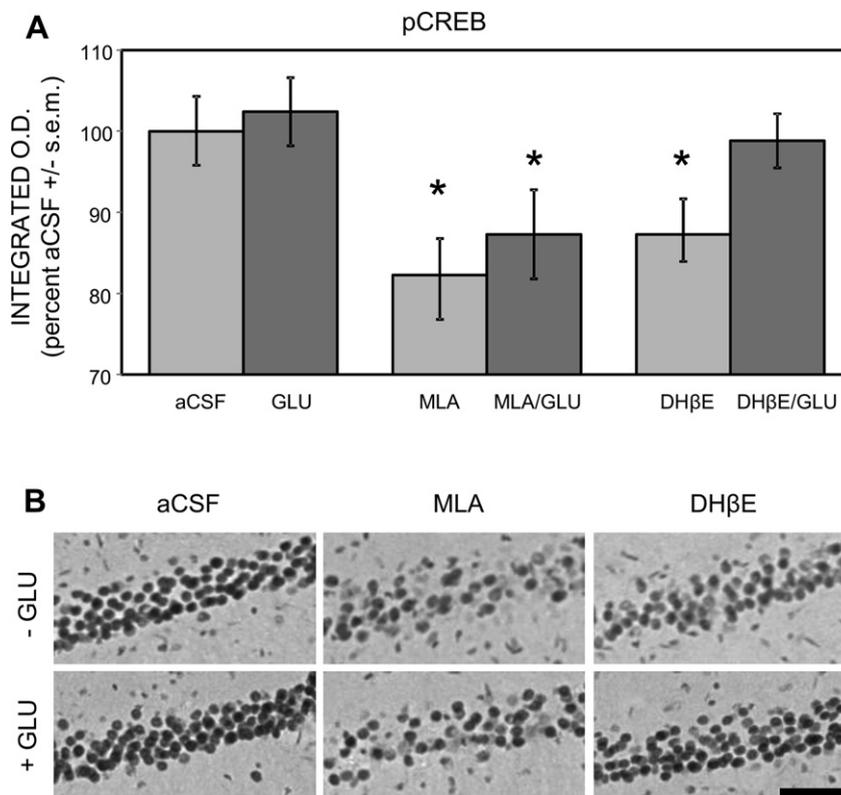


Fig. 7. Differences in pCREB immunoreactivity in area CA1 following spontaneous alternation testing with intrahippocampal administration of drugs. (A) MLA (6.75 $\mu\text{g}/\text{side}$) or DH β E (8 $\mu\text{g}/\text{side}$) reduced pCREB levels. Co-administration of glucose with the antagonists attenuated DH β E- but not MLA-induced pCREB deficits. (*) p 's < .05 vs. aCSF. N 's = 8 for aCSF and GLU. N 's = 7 for all other groups. (B) Representative photomicrographs of pCREB immunostaining in area CA1. Scale bar = 100 microns.

both the inhibitory avoidance and spontaneous alternation tasks, suggesting that both $\alpha 7$ and $\alpha 4\beta 2$ receptors may be important in modulating memory in these tasks. These results underscore the diverse role of nicotinic acetylcholine receptors in learning and memory processes, supporting the view that multiple interconnected or parallel nicotinic signaling mechanisms can support memory formation.

4.2. Ability of glucose to reverse drug-induced memory impairments

In the inhibitory avoidance task, post-training injections of glucose were more effective at reversing DH β E- than at reversing

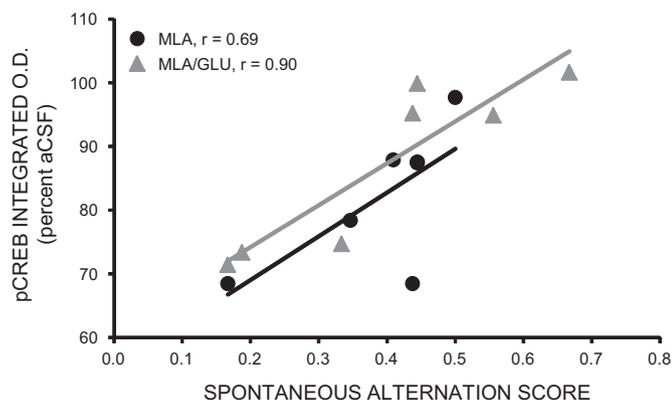


Fig. 8. Correlations between area CA1 pCREB immunostaining and spontaneous alternation scores. There was a significant correlation in rats receiving intrahippocampal injections of MLA-glucose ($p < .01$), and a trend for a correlation in rats receiving MLA alone ($p = .09$).

MLA-induced memory deficits. Likewise, in the spontaneous alternation task, co-administration of glucose with the antagonists reversed DH β E- but not MLA-induced memory impairments, regardless of whether the drug/glucose combinations were administered peripherally or directly into the hippocampus. Previous work has shown that glucose reverses the memory-impairing effects of mecamylamine in both the inhibitory avoidance and spontaneous alternation tasks. However, glucose does not attenuate mecamylamine-induced decreases in locomotor activity, such as reductions in the number of arm entries during spontaneous alternation testing (Ragozzino and Gold, 1991; Ragozzino et al., 1994). In the present study, the results obtained with peripheral DH β E administration parallel those seen previously with mecamylamine. DH β E produced memory impairments and decreased the number of arm visits during spontaneous alternation testing; glucose attenuated the memory but not motor deficits. In contrast, MLA-induced memory impairments were not attenuated by glucose and followed a U-shaped dose–response function not seen previously with mecamylamine. MLA also had no significant effect on spontaneous alternation arm entries. The similarities in the behavioral effects of mecamylamine and DH β E but not MLA may be a consequence of the functional properties of mecamylamine. Although mecamylamine is a nonselective nicotinic antagonist, it is a more potent and long-lasting inhibitor of β subunit-containing receptors (Papke et al., 2001). Therefore, the behavioral effects of mecamylamine are more likely mediated by inhibition of $\alpha 4\beta 2$ and/or other β subunit-containing receptors, as opposed to $\alpha 7$ receptors.

The present findings indicate that glucose may enhance memory by differentially modulating nicotinic receptor signaling through distinct receptor subtypes. Previous studies indicate that glucose is remarkably effective at attenuating or reversing memory

impairments produced by a variety of centrally-acting pharmacological agents, including mecamylamine, scopolamine, morphine, and muscimol (Blanchard and Duncan, 1997; Jafari et al., 2004; Kopf and Baratti, 1994; Krebs and Parent, 2005; Krebs-Kraft and Parent, 2008; Ragozzino and Gold, 1991; Ragozzino et al., 1994, 1992; Stone et al., 1995, 1991, 1988). Glucose also reverses age-related memory impairments in inhibitory avoidance, spontaneous alternation, and other tasks (McNay and Gold, 2001; Morris et al., 2010; Salinas and Gold, 2005). The present study illustrates a rare case in which glucose was ineffective at reversing a pharmacologically-induced memory deficit. The ineffectiveness of glucose at reversing MLA-induced behavioral deficits suggests that the memory-improving actions of glucose may rely more on activation of $\alpha 7$ - as opposed to $\alpha 4\beta 2$ -mediated signaling processes. For example, glucose may selectively up-regulate acetylcholine release in regions with high densities of $\alpha 7$ receptors. Previous studies suggest that glucose is targeted to particular neural systems in response to task demands, where it may enhance local acetylcholine release (cf. Gold, 2004). There are high concentrations of $\alpha 7$ receptors in brain areas in which direct glucose injections lead to memory improvement. The hippocampus has particularly dense levels of $\alpha 7$ receptors, which are found both pre- and post-synaptically throughout the strata of the CA subfields and dentate gyrus. In the hippocampus, $\alpha 7$ receptors often form clusters at GABAergic and other synaptic sites, and have a distinct regional localization and cell type distribution compared to $\alpha 4\beta 2$ receptors, which tend to be more diffusely distributed (Fabian-Fine et al., 2001; Gotti and Clementi, 2004; Kawai et al., 2002; Tribollet et al., 2004; Zarei et al., 1999). By contrast, Pych et al. (2006) showed that injections of glucose into the dorsal striatum, which has relatively low levels of $\alpha 7$ receptors but an abundance of $\alpha 4\beta 2$ receptors (Gotti and Clementi, 2004; Tribollet et al., 2004), failed to enhance learning of a striatum-sensitive maze in which learning is based on egocentric responses.

Glucose may also selectively modulate $\alpha 7$ -mediated signaling processes due to the unique functional properties of these receptors. $\alpha 7$ receptors have the highest calcium permeability of any known nicotinic receptor subtype (Castro and Albuquerque, 1995; Séguéla et al., 1993; Shen and Yakel, 2009). Calcium influx through these receptors plays an important role in the presynaptic release of GABA, glutamate, and other neurotransmitters in the brain (Shen and Yakel, 2009; Sher et al., 2004; Wonnacott et al., 2006). Glucose may potentiate these responses through actions mediated by presynaptic ATP-dependent potassium (K-ATP) channels. A number of studies indicate that regulation of K-ATP channel activity is important to the memory-enhancing effects of glucose (Stefani and Gold, 2001, 1998; Stefani et al., 1999; Rashidi-Pour, 2001). Another unique property of $\alpha 7$ receptors is that they have a high affinity for choline, which functions as a full and selective agonist of these receptors (Alkondon et al., 1997; Mike et al., 2000; Uteshev et al., 2003). Glucose may modulate the activity of $\alpha 7$ receptors by regulating local extracellular choline levels. Previous studies indicate that glucose regulates high-affinity choline uptake and can act synergistically to improve memory when administered together with choline (Kopf et al., 2001; Micheau et al., 1995; Messier et al., 1990). However, the effects of glucose on extracellular choline levels, in the context of memory enhancement, have not been directly investigated.

There are a number of other possible explanations for why glucose was ineffective at reversing MLA-induced behavioral impairments. The present study only tested one peripheral and one intrahippocampal dose of glucose. These doses were similar to or the same as those used in previous studies, in which glucose reversed pharmacologically-induced or age-dependent memory impairments. However, testing a range of glucose concentrations

may reveal dose–response interactions with MLA or DH β E that would alter the interpretations of results. Another possibility is that MLA but not DH β E impaired behavioral performance by affecting cognitive or other processes that are not modulated by glucose. For example, there is some evidence that systemically administered MLA plays a greater role than DH β E in altering attentional processes (Hahn et al., 2011).

4.3. Effects of nicotinic antagonists and glucose on CREB activation

Intrahippocampal injections of MLA or DH β E reduced pCREB levels in area CA1 but not the dentate gyrus, perhaps because of insufficient diffusion of the drugs to this hippocampal region. Co-administration of glucose reversed DH β E- but not MLA-induced pCREB deficits in area CA1, paralleling the behavioral results and providing converging evidence for the effects of these antagonists on memory processes. The ability of glucose to reverse DH β E-induced deficits in pCREB activation supports prior work showing that peripheral glucose or intrahippocampal lactate administration modulates hippocampal CREB phosphorylation in the context of memory enhancement (Morris and Gold, *in press*; Suzuki et al., 2011). Glucose infusions into area CA1 have also been shown to activate components of the mTOR pathway while improving memory (Dash et al., 2006).

Glucose did not attenuate MLA-induced deficits in pCREB activation in area CA1, and levels of pCREB in MLA-injected rats were correlated with individual differences in alternation performance. These results support the hypothesis that $\alpha 7$ signaling pathways involving CREB activation may be important to the memory-enhancing effects of glucose. Previous studies have shown that chronic changes in CREB levels were correlated with spatial working memory performance in the radial arm maze (Rendeiro et al., 2012; Williams et al., 2008). However, based on the time of drug administration in the present study, it is unlikely that the acute changes in CREB phosphorylation could initiate transcriptional events quickly enough to alter behavioral performance. More likely, CREB activation here serves as a good marker of upstream molecular processes that may play a more direct role in modulating spatial working memory processes.

4.4. Conclusions

The present results indicate that glucose reverses deficits in memory and CREB phosphorylation produced by the $\alpha 4\beta 2$ receptor antagonist DH β E but not the $\alpha 7$ receptor antagonist MLA. These findings support the view that memory enhancement by glucose involves interactions with certain nicotinic receptor subtypes more than others. The ineffectiveness of glucose at reversing MLA-induced deficits in both behavioral and molecular measures suggests that the memory-enhancing effects of glucose may involve its ability to activate downstream $\alpha 7$ receptor signaling. Although $\alpha 4\beta 2$ receptors appear to be less important in this regard, the current results do not rule out the possibility that other acetylcholine receptor subtypes (e.g. muscarinic receptors) or neurotransmitter systems may also play a role. Given the central function of glucose in brain metabolism, it seems likely that glucose modulates cognition by multiple mechanisms, perhaps dependent on the interaction of brain area, memory task, and other variables.

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