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Role of β-Adrenoceptors in Memory Consolidation: β_3-Adrenoceptors Act on Glucose Uptake and β_2-Adrenoceptors on Glycogenolysis

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Noradrenaline, acting via β_2- and β_3-adrenoceptors (AR), enhances memory formation in single trial-discriminated avoidance learning in day-old chicks by mechanisms involving changes in metabolism of glucose and/or glycogen. Earlier studies of memory consolidation in chicks implicated β_3- rather than β_2-ARs in enhancement of memory consolidation by glucose, but did not elucidate whether stimulation of glucose uptake or of glycolysis was responsible. This study examines the role of glucose transport in memory formation using central injection of the nonselective facilitative glucose transporter (GLUT) inhibitor cytochalasin B, the endothelial/astrocytic GLUT-1 inhibitor phloretin and the Na^+/energy-dependent endothelial glucose transporter (SGLT) inhibitor phlorizin. Cytochalasin B inhibited memory when injected into the mesopallium (avian cortex) either close to or between 25 and 45 min after training, whereas phloretin and phlorizin only inhibited memory at 30 min. This suggested that astrocytic/endothelial (GLUT-1) transport is critical at the time of consolidation, whereas a different transporter, probably the neuronal glucose transporter (GLUT-3), is important at the time of training. Inhibition of glucose transport by cytochalasin B, phloretin, or phlorizin also interfered with β_3-AR-mediated memory enhancement 20 min posttraining, whereas inhibition of glycogenolysis interfered with β_2-AR agonist enhancement of memory. We conclude that in astrocytes (1) activities of both GLUT-1 and SGLT are essential for memory consolidation 30 min posttraining; (2) neuronal GLUT-3 is essential at the time of training; and (3) β_2- and β_3-ARs consolidate memory by different mechanisms; β_3-ARs stimulate central glucose transport, whereas β_2-ARs stimulate central glycogenolysis.
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INTRODUCTION

The process of memory formation proceeds in a series of physiological and biochemical steps from the point of input and acquisition of learning to the stage when reinforcement processes determine whether the memory will be retained (McGaugh, 2000; Gibbs and Summers, 2002a). After the initial learning experience, short-term or labile memory is consolidated into permanent memory by endogenous processes, such as arousal arising from the experience (McGaugh, 2000). This can reinforce the salience of the initial learning while this memory is still labile. Reinforcement can be provided by modulatory neurotransmitters, including noradrenaline (Bouret and Sara, 2005). Both stimulation of adrenoceptors (ARs; Kety, 1970; Berridge and Waterhouse, 2003) and glucose metabolism are crucial for this process (Messier, 2004).

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When the learning trial is constrained to a single discrete event, individual reinforcement steps can be identified and the processes followed until the memory becomes relatively permanent (Watts and Mark, 1971; Gibbs and Summers, 2002a; Izquierdo *et al*, 2006). In young chicks, a single-trial bead discrimination task allows dissection of memory into stages that are revealed by testing separate chicks at different times after training or by pharmacological intervention (Gibbs and Ng, 1977; Gibbs and Summers, 2002a). Chicks are precocial animals and soon after hatch have to function and learn about their environment for survival. Their brain development is essentially complete at hatch and they demonstrate imprinting and other learning abilities at this early age. Chicks discriminate between different colored beads and learn to avoid the bead color associated with a bitter taste, yet continue to peck at a different bead color. Dilution of the aversant on the bead creates a paradigm whereby chicks remember and will not peck at the bead color associated with the aversive taste for a short while but the memory is not permanent and fades after 30 min. Behavioral events causing arousal such as maternal hen food calls (Field *et al*, 2007), as well as pharmacological intervention by administration of stress

hormones prior to the fading of the memory, lead to the reinstatement and consolidation into long-term storage. Repeated exposure of the chick to the mildly aversive tasting bead will also trigger consolidation (Crowe *et al*, 1989, 1990). In this model of weakly reinforced training, central injection of noradrenaline promotes memory consolidation by acting on cortical and hippocampal ARs. Noradrenaline acts on a variety of different receptor subtypes: α_1-, α_2-, and β-ARs. There is differential involvement of these receptors depending on their location in the brain and the time after the initial learning trial (Gibbs and Summers, 2002a, 2005).

Training of day-old chicks on a one-trial passive avoidance task has been reported to result in a cascade of cellular events, from immediate early gene expression, through biochemical to pharmacological changes in specific brain regions, notably the intermediate medial mesopallium (IMM; Rose and Csillag, 1985; Rose and Stewart, 1999; Rose, 2000) formerly known as the IMHV (Reiner *et al*, 2004). We have also found this multimodal brain region to be important in the formation of memory in both passive avoidance and bead discrimination learning paradigms (Gibbs and Ng, 1977; Gibbs and Summers, 2002a). Memory formation is energy dependent and central injection of glucose can modulate memory (Ragozzini *et al*, 1998; Krebs and Parent, 2005; McNay *et al*, 2000, 2001). Memory formation can be inhibited by 2-deoxyglucose (DG; Gibbs and Summers, 2002b), which is not metabolized and by DAB (1,4-dideoxy-1,4-imino-D-arabinitol) an inhibitor of glycogen breakdown (Gibbs *et al* 2006a).

Glucose enters the brain across the capillary endothelial cells via facilitative glucose transporters (GLUTs) carrying glucose along a gradient, and by sodium-linked energy-dependent glucose transporters (SGLTs) that transport glucose into the endothelial cells against a concentration gradient while coupling it to Na^+ transport moving down its concentration gradient (Nishizaki *et al*, 1995; Qutub and Hunt, 2005). It has been suggested (Nishizaki *et al*, 1995) that the SGLT transporter takes up glucose from the blood into the endothelium from where it diffuses out toward the astrocytes via the facilitative GLUT1 transporter. See summary figure in discussion, which explains the relationship between the different GLUTs. The main GLUTs in the brain are GLUT-1 found on endothelial and astrocytic membranes, GLUT-3 found on neuronal membranes (Maher *et al*, 1994, Vannucci *et al*, 1997; Joost and Thorens, 2001). Here we investigated effects on memory of selective GLUT inhibitors to show that memory-dependent glucose uptake at endothelial and astrocytic sites occurs in a manner distinct from that at the neuronal site. We also show that noradrenaline, acting differentially at β_2- and β_3-ARs plays a key role in controlling glucose uptake and glycogen breakdown for memory formation in astrocytes.

MATERIALS AND METHODS

Animals and Housing

Up to 160 Rhode Island Red × New Hampshire male chicks weighing 35–40 g were obtained on the morning of each experiment from a local poultry farm (Wagner's Poultry, Coldstream, Vic.). The experimental conditions are described in detail elsewhere (Gibbs and Summers, 2002a). Briefly chicks are placed in pairs on arrival at the laboratory, each chick differentiated by their natural color variation (a dark and a light chick for each pair). The temperature in the experimental boxes is kept between 26 and 30°C by white 15 W pilot lamps above each cage and humidity is around 30%. All experimental procedures were in accordance with the guidelines approved by the Monash University Animal Ethics Committee and comply with the 1997 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All efforts were made to minimize both the suffering and the number of animals used. Chicks were killed at the completion of each experiment by CO_2 inhalation.

Learning Paradigm

The chicks are familiarized with their boxes and the presentation of beads in two trials where a small (2 mm) bead attached to a stiff wire is presented into the box. The purpose of these experiences is to reduce fear of objects being introduced into their box. Red and blue beads (5-mm diameter) are then presented to the chicks to familiarize them with the larger bead and ensure that they do not have a bias for either color. In these trials prior to the learning trial, the beads are dipped in water.

After pretraining the chicks are presented with a red bead dipped in either 100 or 20% methyl anthranilate (Sigma-Aldrich Inc., St Louis, MO, USA) for strongly or weakly reinforced training, respectively. Chicks are allowed 10 s to peck the bead, but generally do so within the first 1–2 s. Memory retention, at specified intervals after training, was measured as the discrimination ratio (DR) between pecks at clean blue and red beads—the number of pecks at the blue bead relative to the total number of pecks at the red and blue bead on the successive test trials of 10 s duration. The pecks are recorded on a hand-held recording logger decoded by computer at the completion of the experiment.

When a chick remembers the unpleasant taste, it avoids or gives only one or two pecks at the red bead, up to 12 pecks at the blue bead and the DR approaches 1.0. When a chick does not remember, the DR approaches 0.5, ie the chick pecks equally at red and blue beads. Individual DRs were obtained for each chick and the data presented as mean ± SEM. Chicks that did not peck the bead during the training trial (ie did not train), or avoided the blue bead on test were eliminated from the data analysis at the completion of the experiment. In some of the older experiments in this report 20 chicks were allocated to each group, but we have since refined our methodology and now allocate 16 chicks per group. With the removal of those chicks that do not peck on training or avoid the blue bead on test, the number of chicks per group may be reduced by up to 10–20%. Retention levels of chicks treated with cytochalasin B, phloretin, or phlorizin are compared with those of chicks injected with 0.9% saline. The retention levels in control chicks injected with saline are not significantly different from those seen in experiments where there were no injections (Gibbs and Ng, 1979).

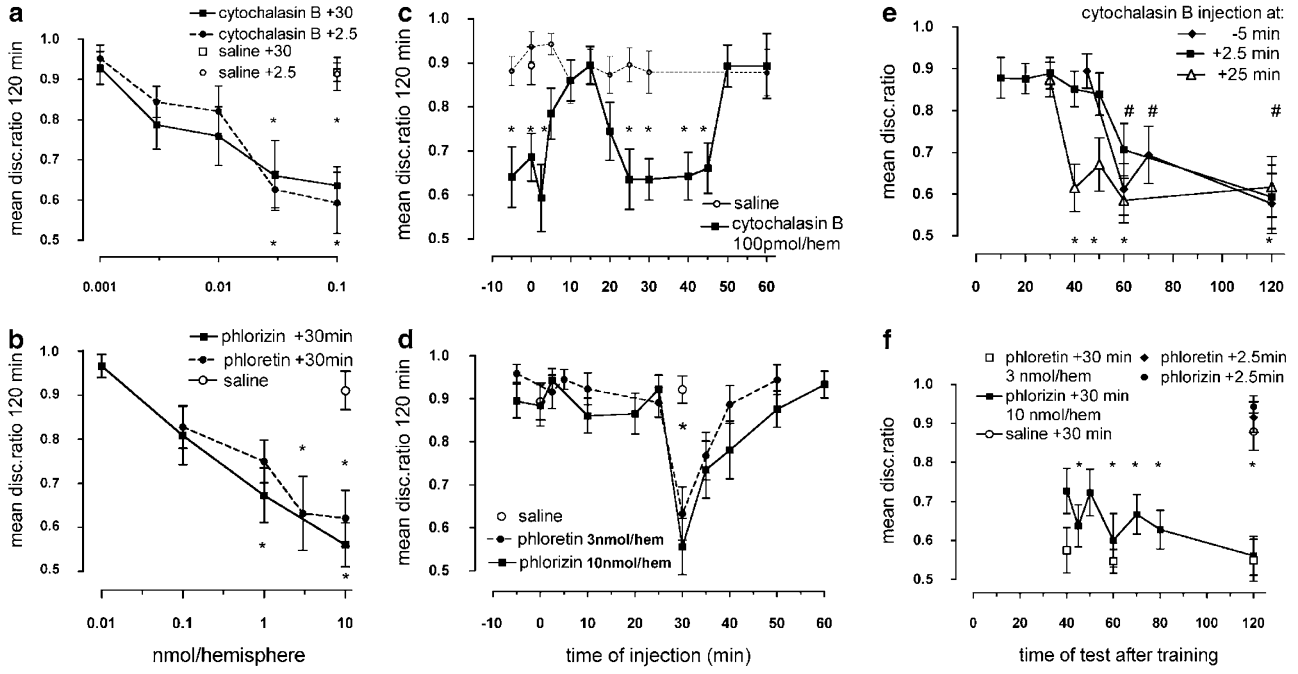

Figure 1 Inhibition of memory formation by the GLUT inhibitors. (a and b) Dose–response data for cytochalasin B injected 2.5 or 30 min (a), phlorizin and phloretin (b) injected 30 min after strongly reinforced training, with memory tested at 120 min. The dependency of the inhibition on the time of injection is seen in (c) for cytochalasin B (100 pmol per hemisphere) and (d) for phlorizin and phloretin (10 and 3 nmol per hemisphere, respectively). In Figure 1c, comparison data are included from chicks injected with saline at different times in earlier experiments. Investigation of the time at which the memory loss is first apparent is seen in (e) and (f). (e) Injection of cytochalasin B 5 min before or 2.5 min after training resulted in memory loss from 60 min, whereas injection 25 min after training resulted in memory loss, which was seen already at 40 min. (f) Following injection of phlorizin 30 min after training memory loss was apparent by 45 min. $N = 13$–20 chicks per group. $^*p < 0.05$.

Drugs and Injections

Zinterol was a gift from Bristol-Myers Squibb, Noble Park, Australia. Other chemicals were from Sigma-Aldrich Inc.: CL 316243 (disodium (R,R)-5-(2-((2-3-chlorophenyl)-2-hydroxyethyl)-amino)propyl)-1,3-benzodioxole-2,2-dicarboxylate); cytochalasin B, glucose, 2-DG, phlorizin (phloridzin dihydrate; 1-(2-(β-D-glucopyranosyloxy)-4,6-dihydroxyphenyl)-3-(4-hydroxyphenyl)-1-propanone), phloretin β-(4-hydroxyphenyl)-2,4,6-trihydroxypropiophenone), iodoacetate, and DAB. All drugs were made up and diluted in sterile 0.9% physiological saline. Doses are expressed as pmol or nmol per hemisphere for central injections or per chick for subcutaneous injections. Drugs were given centrally by direct bilateral injection into the IMM (5 μl per hemisphere) or subcutaneously (100 μl per chick) into a fold of skin ventral to the sternum.

Checks were made of the central injection sites in a proportion of animals after killing them at the completion of each experiment to confirm the accuracy of injections. The location for IMM injection is ~3 mm left or right of the midline and 4–5 mm forward of the depression between the cerebellum and the forebrain. In a typical group of chicks the mean ± SEM of the injection site was 3.30 ± 0.14 and 2.73 ± 0.14 mm to the left and right of the midline and 4.6 ± 0.18 and 5.00 ± 0.17 mm from the tegmentum ($n = 23$). The depth of the injection (3.5 mm to the tip of the needle) was controlled by a plastic sleeve on the 27-gauge needle. Injections into IMM were 10 μl except for the experiments in where iodoacetate and DAB were given in 5 μl volumes. Prior to 2005, we routinely injected drugs in 10 μl volumes but more recently we reduced these and experiments have confirmed that the same drug dose in a smaller volume produced the same effect on memory.

Experimental Design

Dose–response relationships. Dose–response curves were constructed for drugs injected either centrally into the IMM or subcutaneously, depending on the particular experiment.

The ability of agonists to enhance memory was revealed using weakly reinforced training (20% anthranilate), which in normal circumstances does not last beyond 30 min and the ability of antagonists to inhibit was revealed using strongly reinforced training (100% anthranilate). Memory, unless otherwise indicated, was tested 120 min after training. See Table 1 for details of the doses of drugs used and the timing of the injections.

Time of injection and test. Injections of the inhibitors of GLUTs were made to separate groups of chicks at times between 5 min before and 60 min after training to determine the times at which they impaired memory consolidation. With a time of injection selected on the basis of this study (Table 1), a number of groups of chicks were injected with the inhibitor and memory retention tested in separate groups of chicks at discrete time intervals after training. Other drugs and glucose were injected within previously reported windows of administration (Table 1). CL316243 and zinterol were administered subcutaneously 20 min after training.

Table 1 Selective Noradrenergic Subtype Agonists and Inhibitors of Glucose Metabolism

Drug	Site of action	Challenge dose	Window of effective administration (min)
Glucose	Metabolic substrate	10 nmol per hemisphere	−25 to +20[a]
		100 nmol per hemisphere**	
CL316243	Selective β_3-AR agonist		−5 to +25[b]
Zinterol	Selective β_2-AR agonist		0–25[b]
2-Deoxyglucose	Glucose transport and inhibitor of glycolysis	150 nmol per hemisphere	−5 and +15 to 40[a]
		250 nmol per hemisphere*	
Iodoacetate	Inhibitor of glycolysis	1 nmol per hemisphere	0 to +2.5, + 25[c] and 50[d]
		10 nmol per hemisphere*	
DAB	Glycogen phosphorylase inhibitor preventing glycogen breakdown in astrocytes	30 pmol per hemisphere	−5, +25 to 40, and +55[e]
		0.1 nmol per hemisphere*	
Cytochalasin B	Nonselective GLUT inhibitor	3 pmol per hemisphere	−5 to +2.5 and +25 to 45[f]
		100 pmol per hemisphere*	
Phloretin	GLUT-1 inhibitor of astrocytic/endothelial glucose transport	0.1 nmol per hemisphere	+30[f]
		3 nmol per hemisphere*	
Phlorizin	SGLT1-2 inhibitor sodium- and energy-dependent glucose transporter	0.1 nmol per hemisphere	+30[f]
		10 nmol per hemisphere*	

Abbreviations: AR, adrenoceptor; DAB, 1,4-dideoxy-1,4-imino-D-arabinitol; GLUT, glucose transporter.
[a]From Gibbs and Summers (2002b).
[b]From Gibbs and Summers (2000).
[c]From O'Dowd *et al* (1994).
[d]ME Gibbs, unpublished.
[e]From Gibbs *et al* (2006b).
[f]Determined in present study.
*Inhibitory dose,
**facilitatory dose.

Specificity of drug/receptor/GLUT interaction. To determine whether the memory enhancing properties of either of the β-AR agonists involved the GLUT, the action of β-AR agonists were challenged by prior administration of a fixed, low dose of selective inhibitors. These inhibitors were injected into IMM 2.5 or 5 min before the construction of dose–response curve to the agonist or glucose administered subcutaneously 20 min after the training trial. If memory enhancement is related to the activity of the GLUT then the inhibitor would be expected to shift the dose–response curve to the right, ie a higher dose of the agonist or glucose is required to enhance the memory. The timing of the administration of the low dose of inhibitor was determined in a pilot study, and it was found to be necessary to make the injection 2.5–5 min before that of the agonist. To determine whether β-AR agonists influenced memory consolidation by metabolic mechanisms known to be involved in memory, the glycolysis inhibitor iodoacetate, the glycogenolysis inhibitor DAB, or the inhibitor of glucose metabolism 2-DG were administered into IMM at predetermined times prior to the different doses of agonists given subcutaneously. The expression patterns of GLUT-1 and GLUT-3 mRNA in chicken tissues are generally similar to those in mammals (Kono *et al*, 2005), but chickens have an insulin-sensitive GLUT-8 gene in place of the GLUT-4 gene found in mammals (Seki *et al*, 2003).

Astrocyte Cultures

Measurement of incorporation of [^{3}H]-2-DG uptake. The preparation of chick astrocyte cultures and glycogen assay are described in Gibbs *et al* (2006a); Hutchinson *et al* (2007). Glucose uptake was carried out as previously described by Hamai *et al* (1999). Following 7 days of culture, cells were serum starved overnight and in the morning media was changed to 5.5 mM glucose DMEM, and 1 μM drugs (insulin, isoprenaline, CL316243, or zinterol) added for the indicated times. [^{3}H]-2-DG (50 nM) was added for the final 10 min. Reactions were terminated by aspiration and washing twice with ice-cold PBS. Cells were lysed (0.2 M NaOH, 1 h, 50 °C) and radioactivity counted. Results were calculated as a percentage of the basal glucose uptake levels, which were corrected for facilitated glucose uptake determined with cytochalasin B (10 μM), with the assumption that GLUTs in the plasma membrane do not distinguish between glucose and 2-DG (Hamai *et al*, 1999).

Data Analysis

The results for each experiment were analyzed using SPSS (Information Analysis systems SPSS Inc., Chicago, IL, USA) with one-way or two-way analysis of variance (ANOVA) for unweighted means. Dunnett's test was used for *post hoc*

analysis to identify individual group differences for the one-way ANOVAs. A significance level of $p<0.05$ was employed. Simple Main Effects analysis was used for two-way ANOVA's, the F and p values for each analysis in the results are reported in order of interaction effect and simple main effects. In the one-way ANOVA tests, the factors were DRs compared with dose, time of injection, or time of test compared to a saline control. In the challenge studies, two-way ANOVA tests compared the DRs for each dose of agonist with preadministration of saline or inhibitor.

RESULTS

Cytochalasin B, Phloretin, and Phlorizin Inhibit Strongly Reinforced Learning

Of the 13 or so identified GLUTs there are 3 of importance in the brain: GLUT-1, GLUT-3, and GLUT-4 (or the avian equivalent GLUT-8). All of these GLUTs as well as the SGLT transporter on endothelial cells are inhibited by the nonselective drug cytochalasin B. The effects of this nonspecific GLUT inhibition is contrasted by the effect of two more selective inhibitors, phloretin (Takakura *et al*, 1991; Duelli and Kuschinsky, 2001) and phlorizin (Ehrenkranz *et al*, 2005; Tyagi *et al*, 2005), that inhibit GLUT-1 and SGLT, respectively.

The effect of different doses of the GLUT inhibitors was examined by injection 30 min after strongly reinforced training, a time at which 2-DG inhibits memory (Table 1). Cytochalasin B injected at 2.5 or 30 min (Figure 1a), phloretin or phlorizin (Figure 1b) injected into IMM 30 min after strongly reinforced training, inhibited memory consolidation in a dose-dependent manner ($F_{5,92}=4.39$, $p<0.001$; $F_{5,87}=8.18$, $p<0.001$; $F_{3,66}=4.86$, $p=0.004$; and $F_{3,69}=12.67$, $p<0.001$, respectively).

Examination of the times after training when memory processing was vulnerable to interference by the transport inhibitors, revealed two periods where injection of cytochalasin B (100 pmol per hemisphere) resulted in memory loss (Figure 1c; one-way ANOVA comparing retention at different injection times with saline injected at +0 min, $F_{13,212}=4.24$, $p<0.01$): the first close to training with injections at −5 to +2.5 min ($p<0.05$) and the second 25 to 45 min after training, during Intermediate Term Memory B (ITMB) the second phase of intermediate memory from 30 to 50 min after training and following ITMA ($p<0.05$). In contrast to cytochalasin B, that inhibits all facilitative GLUT transporters, the more selective phloretin (astrocytic/endothelial GLUT-1 inhibitor (3 nmol per hemisphere)) and the Na^+- and energy-dependent SGLT inhibitor phlorizin (10 nmol per hemisphere) inhibited memory only when injected 30 min after training (Figure 1d; $F_{9,176}=5.92$, $p<0.001$; $F_{11,200}=5.77$, $p<0.001$, respectively). This suggests that the effect of cytochalasin B injected 25 to 30 min after training can be attributed, at least in part, to endothelial/astrocytic glucose uptake, whereas at the time of training the effect of cytochalasin B is likely to be due to the inhibition of a different GLUT, probably the neuronal GLUT-3.

The memory loss caused by cytochalasin B 5 min before training was still observable on test 24 h later (DR 0.59 ± 0.05, $n=15$). In addition, cytochalasin B (100 pmol per hemisphere) had no effect on memory 120 min after weakly reinforced training (DR 0.58 ± 0.05, $n=14$).

Memory loss following phlorizin or phloretin injected 30 min after training occurred by 40 min after training ($F_{7,141}=3.54$, $p=0.002$; $F_{3,63}=12.41$, $p<0.001$, respectively; Figure 1f). A similar result was seen with cytochalasin B injected at +25 min and memory loss occurring by 40 min ($F_{4,89}=3.68$, $p=0.008$; Figure 1e). However, after the earlier injections of cytochalasin B (−5 and +2.5 min), memory loss occurred at a later time, between 50 and 60 min (Figure 3; $F_{3,67}=5.47$, $p=0.002$; $F_{6,116}=4.57$, $p<0.001$) supporting the suggestion of a difference in the identity of the GLUT transporter targeted at the different times.

Any effects of drugs unrelated to effects on memory (proactive effects) and influencing pecking behavior are unlikely, since memory was still impaired 24 h after cytochalasin B, and the time between injection and memory loss is not always the same. Memory loss caused by injection of cytochalasin B, 5 min before training occurs after 60 min, whereas when cytochalasin B is injected at 25 min memory after training, loss occurs within 10 min.

Prior Administration Of Glucose Facilitates, And 2-DG Antagonizes, Responses To The β_3-AR Agonist CL316243 But They Do Not Modify The Response To The β_2-AR Agonist Zinterol

Memory after strongly reinforced training is abolished by intracerebral or systemic administration of 2-DG (Gibbs and Summers, 2002b), which competes with glucose for uptake and phosphorylation to glucose-6-phosphate, but is not further metabolized and inhibits by interrupting the glycolytic metabolic pathway or by inhibiting glucose uptake without any short-term effect on glycogenolysis.

Using doses of CL316243 and zinterol below those necessary to enhance memory by themselves we have shown that administration of the β_3-AR agonist CL316243 5 min after glucose injection facilitates memory enhancement caused by glucose, whereas the β_2-AR agonist zinterol does not (Gibbs and Summers, 2002b). In the present experiment we challenged the response to CL316243 and zinterol with low doses of either glucose or 2-DG.

Central administration of a low dose of glucose (10 nmol per hemisphere), 2.5 min before subcutaneous administration of CL316243 at 20 min, facilitated memory enhancement produced by the β_3-AR agonist. As seen when there are no drug injections, this dose of glucose (10 nmol per hemisphere) did not enhance weakly reinforced memory at low doses of either CL316243 or zinterol (Figure 2), however it shifted the dose–response curve to subcutaneously administered CL316243 to the left in parallel (Figure 2a; drug effect $F_{1,88}=12.61$, $p=0.001$; dose effect $F_{2,88}=6.59$, $p=0.002$). In contrast, preadministration of glucose failed to shift the dose–response curve to zinterol (Figure 2b; drug effect $F_{1,124}=0.4$, $p=0.53$; dose effect $F_{1,124}=18.26$, $p<0.001$). Acute intracerebral administration of 2-DG has two effects: (1) it competes with endogenous glucose for transport and phosphorylation and (2) it accumulates within the cells, and since it cannot be metabolized beyond 2 DG glucose-6-phosphate, it reduces the metabolic flux and hence the energy supply available for memory. A dose of 250 nmol per hemisphere of 2-DG is required to inhibit

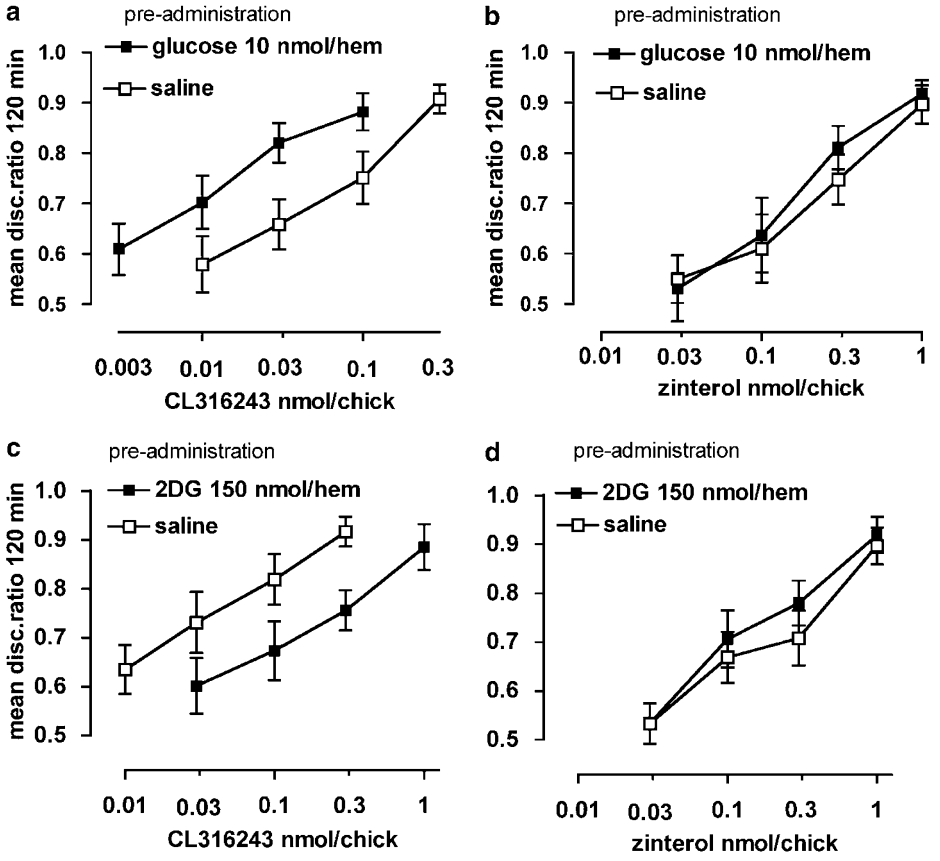

Figure 2 Effect of glucose or 2-deoxyglucose (2-DG) on the enhancement of memory produced by selective β-AR agonists. Significant shifts in the dose–response curves for memory enhancement by the β_3-AR agonist CL316243 occurred (a) to the left in the presence of a low dose of glucose and (c) to the right in the presence of a low dose of 2-DG (150 nmol per hemisphere). The response curve for the β_2-AR agonist zinterol was not altered in the presence of the low dose of either glucose (b) or 2-DG (d). Glucose and 2-DG were injected bilaterally into the IMM 17.5 and 15 min, respectively after weakly reinforced training and the selective agonists were administered subcutaneously 20 min after training. Each group contained 14–20 chicks per group. $^*p<0.05$.

memory (Gibbs and Summers, 2002b). At the lower dose of 150 nmol per hemisphere that does not inhibit memory by itself, 2-DG injected into the IMM 15 min after weakly reinforced training reduced the ability of subcutaneous CL316243 (administered 20 min after training) to enhance memory retention (Figure 2c), as indicated by the rightward shift of the dose–response curve. Both the drug and the dose effect were significant ($F_{1,105}=11.77$, $p=0.001$; $F_{2,105}=5.28$; $p=0.007$, respectively). Using a similar protocol, 2-DG failed to shift the dose–response curve to zinterol (Figure 2d). Only the dose effect was significant ($F_{3,132}=21.31$, $p<0.001$).

The memory-enhancing effect of β_3-AR activation is likely caused by increased glucose uptake (Gibbs and Summers, 2002b). β_2-AR stimulation had no effect on the ability of 2-DG to inhibit memory and therefore does not appear to facilitate memory consolidation by facilitating glucose uptake.

Incorporation of [^{3}H] 2-DG into Astrocytes

AR activation is known to increase glucose uptake in skeletal muscle through both α_{1a}- and β_2-ARs (Abe *et al*, 1993; Liu and Stock 1995; Liu *et al*, 1996; Tanishita *et al*, 1997; Nevzorova *et al*, 2002; Hutchinson and Bengtsson, 2005, 2006) by β_3-ARs in brown adipocytes (Nikami *et al*, 1996; Chernogubova *et al*, 2004; Hutchinson *et al*, 2005), and in the same tissue by β_1- and α_1-ARs in the absence of β_3-ARs (Chernogubova *et al*, 2005). The effect of the nonselective β-AR agonist isoprenaline (Figure 3a) that is known to enhance memory consolidation in weakly reinforced training (Gibbs and Summers, 2001) was compared to that of the β_2-AR agonist zinterol (Figure 3b) and the β_3-AR agonist CL316243 (Figure 3c) on 2-DG uptake into chicken astrocytes over a 2 h time period. Isoprenaline increased glucose uptake 1.5-fold after 5 min of stimulation, and this effect was maintained throughout the whole time period ($F_{4,45}=6.92$; $p=0.0002$). The early effect of isoprenaline was not mediated by β_2-ARs, as the increase in glucose uptake produced by the selective agonist zinterol only became significant at 120 min ($F_{4,20}=5.36$; $p=0.004$). On the other hand the β_3-AR agonist CL316243 increased glucose uptake 1.5- to 2-fold over the whole time period ($F_{4,45}=6.77$; $p=0.0002$).

Insulin has been shown to increase glucose uptake in astrocyte and glial cultures in some studies (Kum *et al*, 1992; Clarke *et al*, 1984; Werner *et al*, 1989) but not others (Hara *et al*, 1989; Hamai *et al*, 1999). Here we examined the ability of insulin to increase 2-DG uptake in chick astrocytes over a 2 h time period as a control (Figure 3d). Insulin significantly increased glucose uptake ~2-fold over basal

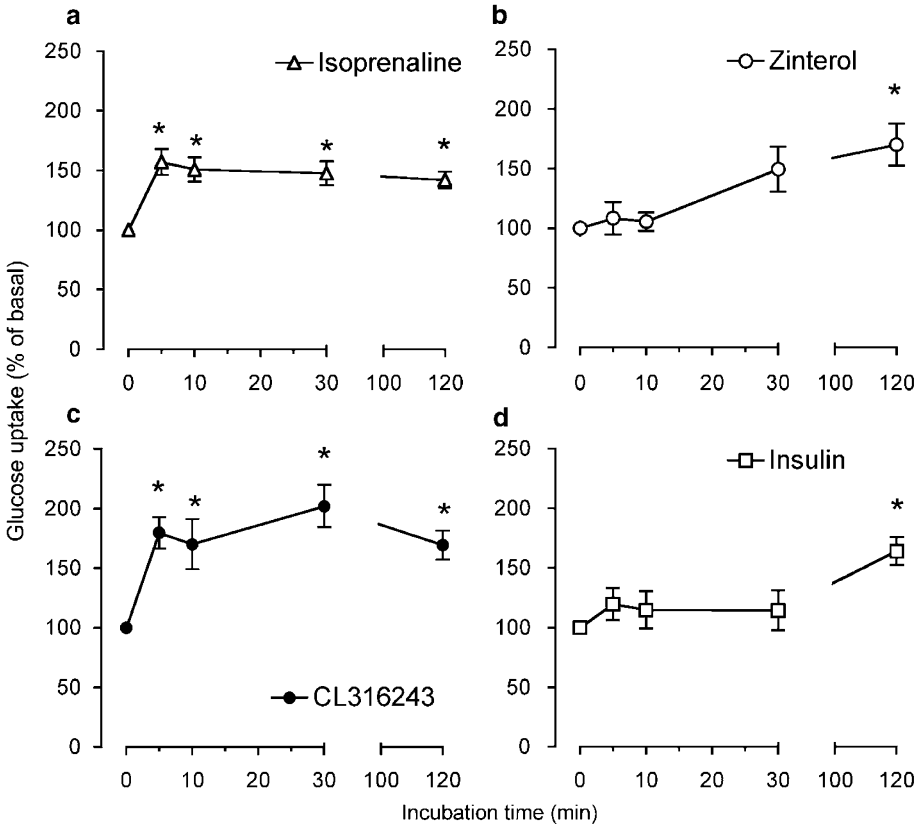

Figure 3 Comparison of the effect of CL316243 and zinterol on incorporation of [^{3}H] 2-deoxyglucose into astrocytes at different times of exposure to the agonists from 5 to 120 min. Mean [^{3}H]2 DG uptake ± SEM expressed as percentage of basal. Controls incubated with 1 μM insulin (a), 1 μM isoprenaline (b), 1 μM CL316243 (c), or 1 μM zinterol (d). A total of 4–6 astrocyte cultures per group. *$p<0.01$ 5–10 cultures per incubation time.

levels following 2 h of stimulation ($F_{4,34}=3.67$; $p=0.014$) but not at other time points examined.

Selectivity of Action of Cytochalasin B on Memory Enhancement Caused by CL316243 and Glucose

Injection of cytochalasin B 5 min before glucose administration challenged memory enhancement caused by glucose (Figure 4a; $F_{3,73}=4.627$, $p=0.005$). When the inhibitor was injected 10 or 15 min earlier, it did not affect glucose facilitation of memory, suggesting that the inhibitor only remained at a high enough concentration at the site of action for less than 10 min. To challenge the enhancement of memory after weakly reinforced training in response to glucose, CL316243 or zinterol injected at 20 min, a low dose of cytochalasin B (3 pmol per hemisphere) was injected intracerebrally 5 min before subcutaneous administration of glucose or AR agonists. Cytochalasin B shifted the dose–response curve to subcutaneously injected CL316243 (Figure 4c) and glucose (Figure 4b) to the right. To enhance memory, a higher dose of the agonist was required in the presence of the GLUT inhibitor (CL316243, $F_{1,112}=7.98$, $p=0.006$; glucose, $F_{1,107}=9.70$, $p=0.002$), further supporting the concept that the β_3-AR agonist enhances glucose transport. However, there was no shift in the dose–response curve to zinterol in the presence of cytochalasin B (Figure 4d; $F_{1,146}=0.23$, $p=0.635$) suggesting that the action of the β_2-AR agonist was not associated with glucose uptake.

Phloretin and Phlorizin Influence Memory Enhancement Produced by CL316243 and Glucose, but not Zinterol

Phloretin and phlorizin inhibited strongly reinforced training when injected at high doses 30 min after training. These compounds are GLUT inhibitors that are more selective than cytochalasin B, and block GLUT-1 and SGLT glucose transport respectively. Preadministration (+15 min) of a low dose of phlorizin (0.1 nmol per hemisphere; Figure 5a) or phloretin (0.1 nmol per hemisphere; Figure 5c) into IMM shifted the dose–response curve to CL316243 (phlorizin $F_{1,100}=14.94$, $p<0.001$; phloretin $F_{1,100}=20.66$, $p<0.001$) or glucose (Figure 5b; phlorizin $F_{1,108}=9.00$, $p=0.003$; phloretin $F_{1,97}=8.18$, $p=0.005$), when they were administered subcutaneously at +20 min. However, neither phlorizin nor phloretin shifted the dose–response curve to zinterol (Figure 5d, $F_{2,128}=1.16$, $p=0.32$). Although these two inhibitors had less effect on memory in terms of time of susceptibility, at least some of the action of β_3-AR agonist on memory appears to involve GLUT-1 and the SGLT at the time of memory consolidation. As with cytochalasin B, there was no effect of these inhibitors on the dose–response relationship to zinterol.

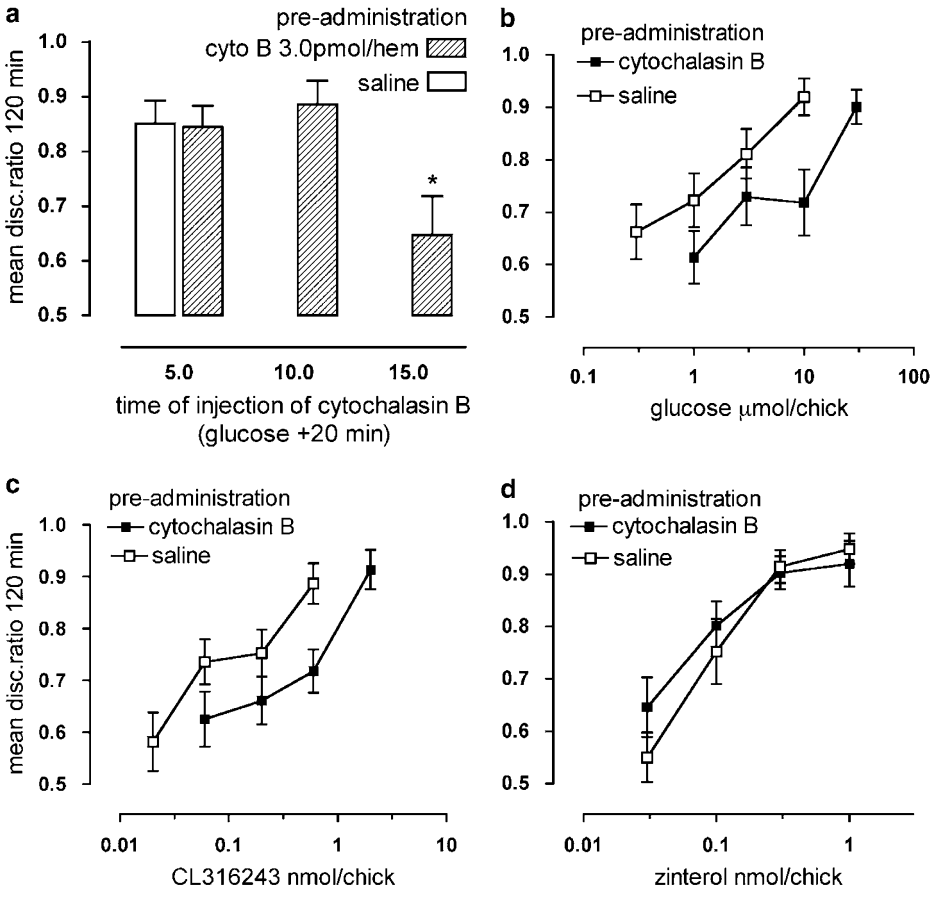

Figure 4 Selectivity of memory enhancement by CL316243 and glucose involves glucose transport. (a) Cytochalasin B (3 pmol per hemisphere) has to be injected within 5 min of the glucose injection to challenge enhancement of memory consolidation. Injection of inhibitor 10 or 15 min before glucose injection did not prevent glucose facilitation of memory. The dose-dependent enhancement of memory consolidation by (b) subcutaneous glucose or (c) CL316243 at 20 min is reduced by intracerebral administration of a suboptimal dose of cytochalasin B 5 min earlier, whereas cytochalasin B had no effect on the dose–response to zinterol (d). Each group contained 17–20 chicks per group. $^*p<0.05$.

Both β_2- and β_3-AR Agonists Affect Memory Formation by Mechanisms Involving Glycolysis that are Inhibited by Iodoacetate

Inhibition of glyceraldehyde-3-phosphate dehydrogenase by iodoacetate stops further steps in glycolysis and thereby inhibits degradation of both glucose and glycogen. Iodoacetate inhibits memory when given at two points during the first 30 min after training (Table 1), one around the time of training (+ 0 and + 2.5 min) and the other 25 min later. Iodoacetate had a 10 min period where it reduced the memory enhancement by zinterol (Figure 6b, $F_{2,47}=4.108$, $p=0.023$). A low dose of iodoacetate selected from the data in Figure 6a (1 nmol per hemisphere) injected centrally 5 min before the subcutaneously administered β-agonists zinterol (Figure 6c) or CL316243 (Figure 6d) at 20 min after training (ie 15 min after training) shifted the dose–response curves to both agonists to the right ($F_{1,102}=8.62$, $p=0.004$; $F_{1,94}=17.65$, $0<0.001$, respectively). However, iodoacetate injected 5 min before training did not affect the dose–response to zinterol injected 2.5 min after training (Figure 6e). ($F_{1,131}=0.07$, $p=0.787$), indicating that the metabolic events around the time of training are not modulated by β_2-*ARs*. The importance of timing in the mechanisms involved in memory processing is clearly illustrated by the difference between Figure 6c and Figure 6e, and relates to the time at which these processes are involved in memory processing.

β_2-AR Agonists affect Memory Formation by Activating Glycogenolysis

Inhibition of glycogenolysis by the glycogen phosphorylase inhibitor DAB abolishes memory when administered around the time of training and at the time of memory consolidation, although glucose metabolism still occurs (Gibbs *et al*, 2006a). Glycogen content in the brain of the day-old chick is high (O'Dowd *et al*, 1994; Hertz *et al*, 2003; Nahorski *et al*, 1975), and glycogenolysis in cultured chick astrocytes is stimulated by the β_2-AR agonist zinterol (Gibbs *et al*, 2006a). During glycogenolysis, each glucosyl unit is released as glucose-1-phosphate, converted to glucose-6-phosphate and further degraded to pyruvate along the same glycolytic pathway as glucose. Recent evidence indicates that glucose and glycogen metabolites are not interchangeable, but play different roles in memory formation (Gibbs *et al*, 2007).

As noradrenaline promotes glycogenolysis (Magistretti, 1988; Subbarao and Hertz, 1990; Sorg and Magistretti, 1991; Coopersmith and Leon, 1995), the action of β-AR agonists

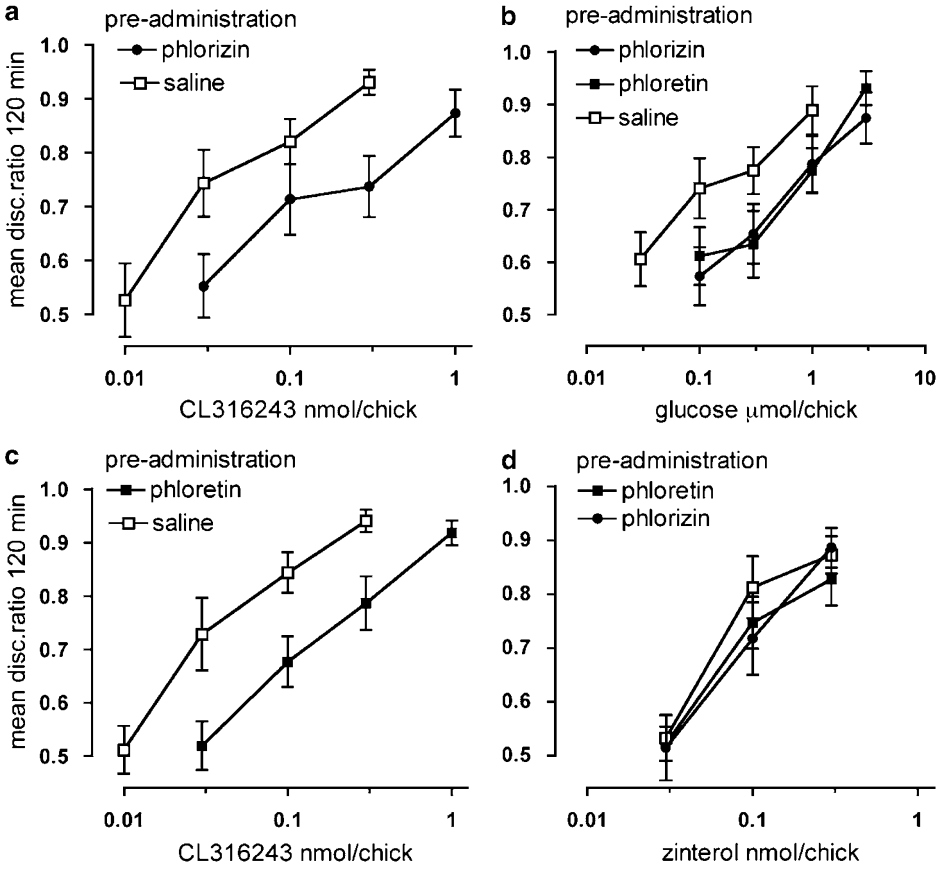

Figure 5 Selectivity of action of the GLUT inhibitors phlorizin and phloretin on the memory enhancing effects of the β-AR agonists and glucose. The dose-dependent action of CL316243 administered subcutaneously at 20 min is shifted significantly to the right in the presence of a suboptimal dose of (a) phlorizin (0.1 nmol per hemisphere) or (c) phloretin (0.1 nmol per hemisphere) injected into the IMM 5 min earlier. The response to glucose (b) is shifted to the right by phlorizin and phloretin but the response to zinterol is not shifted by either phloretin or phlorizin (d). Each group contained 14–20 chicks per group. $^{*}p<0.05$.

was challenged with the glycogen phosphorylase inhibitor DAB that prevents glycogen breakdown (Andersen *et al*, 1999; Andersen and Westergaard, 2002). We have shown that administration of DAB interferes with memory processing (Gibbs *et al*, 2006a). Preadministration of 30 pmol per hemisphere, a low, noninhibitory dose of DAB (Gibbs *et al*, 2006a) 20 min after training and 5 min before systemic injection of either CL316243 or zinterol following weakly reinforced training demonstrated an interaction between glycogenolysis and β_2- but not β_3-AR activation. DAB shifted the dose–response curve to zinterol to the right, reducing the ability of zinterol to enhance memory (Figure 7b; $F_{1,84}=15.74$, $p<0.001$) but did not shift the dose–response curve to CL316243 (Figure 7a; $F_{1,125}=0.28$, $p=0.600$). This strongly suggests that the β_2-AR agonist zinterol, enhances memory consolidation by increasing glycogen breakdown.

Glycogen Content in Astrocytes after Stimulation with Adrenergic Agonists

We have reported (Gibbs *et al*, 2006a) that following 2 h incubation, glycogen levels were reduced by zinterol, and this reduction was prevented by DAB. The present results confirm this finding and add the new information that incubation in the presence of CL316243 did not alter glycogen levels with or without DAB (Figure 8).

DISCUSSION

Noradrenaline modulates memory consolidation and its effect depends upon specific receptor subtypes, location of the receptors in different brain regions and the time after the learning experience. In this paper we show that some of the ways that noradrenaline influences memory relate to specific metabolic effects: memory enhancement by β_3-AR agonists depends on activation of GLUTs, whereas memory enhancement by β_2-AR agonists depends on stimulating glycogenolysis. We also show that blockade of glucose transport into cells in the brain inhibits memory formation at discrete times after training and the stage of memory affected, revealed by both the time of test after injection and training and the time memory is tested, is dependent on the particular GLUT transporter (Figure 9).

It is well documented that following learning, increases in glucose uptake occur in brain regions associated with memory (Bontempi *et al*, 1996, 1999; Rose and Csillag, 1985; McNay *et al*, 2000, Krebs and Parent, 2005), and it is clear from our results that this effect is dependent on GLUTs

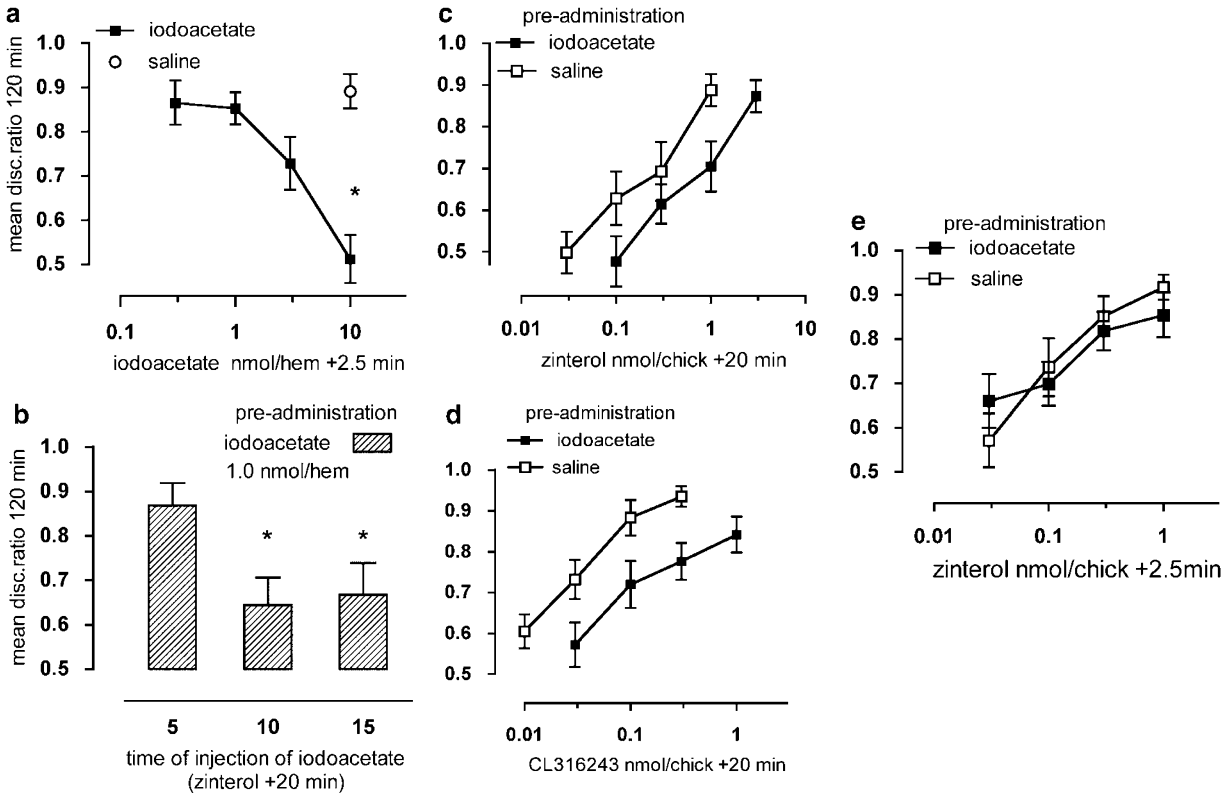

Figure 6 Inhibitory action of the glycolytic inhibitor iodoacetate on the effects of the selective β_2- and β_3-AR agonists. The memory enhancing action of both zinterol (c) and CL316243 (d) administered subcutaneously 20 min after training are significantly impaired by injection of 1 nmol per hemisphere of iodoacetate into IMM 10 min after training. However, the enhancing effect of zinterol at 2.5 min (e) is not influenced by iodoacetate given 5 min before training. (a) this dose of iodoacetate by itself does not impair memory. The administration time of iodoacetate prior to the agonists was determined by injection at 5, 10, or 15 min before zinterol (b). Injection 5 or 10 min before zinterol prevented the memory enhancement, whereas injection 15 min before did not. Each group contained 14–20 chicks per group. $^*p < 0.05$.

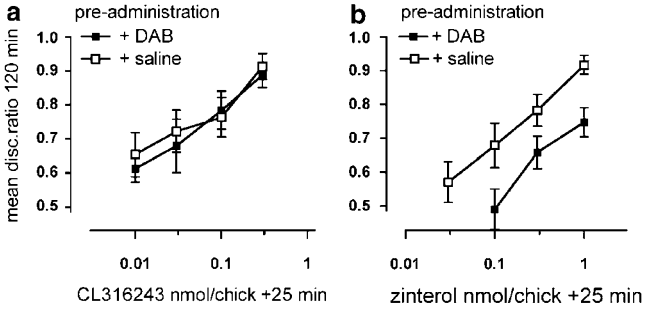

Figure 7 Selectivity of action of zinterol on glycogenolysis. (a) The dose–response curve to CL316243, injected subcutaneously at 25 min after training was not affected by administration of 30 pmol of DAB (1,4-dideoxy-1,4-imino-D-arabinitol) injected into the IMM 5 min earlier, whereas (b) the dose–response curve to zinterol was shifted to the right. Each group contained 14–19 chicks per group.

localized on different cell types. By comparing the temporal profile of different selective *vs* nonselective drugs, we provide evidence for a specific role for astrocytic/endothelial glucose transport 30 min after learning. This role is not apparent at the time of training and we speculate that glucose uptake at this time is neuronal.

The endothelial/astrocytic GLUT-1 is abundant in frontal and motor cortices in areas where there is high energy demand. GLUT-3 is found in neuropil and neuronal perikarya suggesting a role in synaptic energy provision and neurotransmitter synthesis (Choeiri *et al*, 2002). Increases in hippocampal and sensorimotor cortex GLUT-1 immunoreactivity and mRNA after operant conditioning in mice suggest that GLUT-1 mRNA responds rapidly to changes in cellular metabolic demands (Choeiri *et al*, 2005). We show that inhibition of GLUT-1 or SGLT produces identical effects on memory. Both transporters are expressed in cultured endothelial cells from cerebral cortical arteries and the difference between them is that GLUT-1 transports glucose rapidly along its concentration gradient, whereas SGLTs can transport glucose into cells against a concentration gradient. A SGLT-like GLUT may thus contribute to glucose uptake into the endothelium at the blood–brain barrier whereas GLUT-1 transports glucose from endothelial cells to astrocytes (Nishizaki and Matsuoka, 1998; Qutub and Hunt, 2005). The extent to which phloretin-mediated inhibition of memory is associated with astrocytic energy demand is unknown, but an increase in energy flux is essential prior to the formation of long-term memory (Gibbs *et al*, 2006b).

Interference with Glucose Transport Inhibits Strongly Reinforced Memory

This study provides further evidence that glucose improves learning. To our knowledge it is the first demonstration that

long-term memory formation is abolished by inhibition of GLUT or SGLT transporters, and is one of few studies that clearly indicates a physiological role for SGLT in brain. Inhibition of glucose uptake into astrocytes or neurons by the nonselective GLUT inhibitor cytochalasin B interfered with memory in a similar manner to the nonmetabolized glucose analogue 2-DG (Gibbs and Summers, 2002b) or the glycogenolysis inhibitor DAB (Gibbs *et al*, 2006a). Interference with the glycolytic pathway generating pyruvate occurs after administration of any of these three inhibitors and it appears that both glucose uptake and glycogen breakdown are needed as energy and/or substrate sources for memory processing.

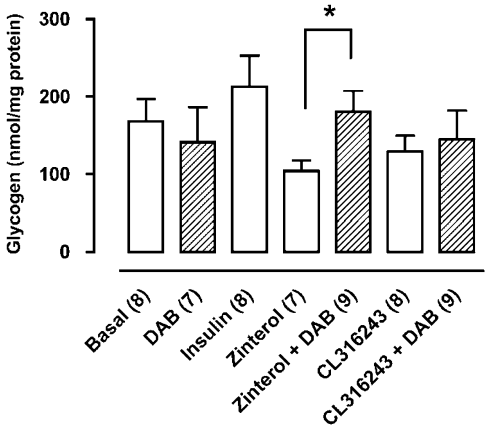

Figure 8 The effect of DAB (1,4-dideoxy-1,4-imino-D-arabinitol) on basal and zinterol and CL316243 stimulated glycogen levels in cultured astrocytes. Astrocytes were incubated for 2 h under basal conditions with no drug added, or for 2 h in the presence of the β_2-AR agonist zinterol (1 μM) or the β_3-AR agonist CL316243 (1 μM). The DAB (10 μM) was added 20 min prior to the 2 h incubation. Results are means ± SEM values; number of cultures per group indicated. *$p < 0.05$.

These experiments are interpreted in terms of a three-stage model of memory (Gibbs and Ng, 1977; Gibbs and Summers, 2002a) where strongly reinforced training progresses through short-term memory and ITM to long-term memory (retained memory on test 120 min later), whereas weakly reinforced training results in a labile memory (retained ~30 min). Memory processing is increasingly being recognized as a three-stage process in many animal models (Matthies, 1989; Stough *et al*, 2006), and we argue that memory consolidation occurs midway through intermediate memory at the transition from ITMA to ITMB approximately 30 min after learning. The memory stages are quite discrete and the timing is not altered by increasing the dose of the inhibitor or changing the time of drug administration (within limits; Mark and Watts, 1971; Watts and Mark, 1971; Gibbs and Ng, 1977). It is likely that different animal models have different memory stage durations; the exact timing depending on the parameters of the learning task. We have consistently found in our paradigm that the transition occurs around 30 min after training. This is evidenced by labile, weakly reinforced memory lasting only for this time and unless triggered by modulatory neurotransmitters, arousal, or behavioral triggers (Field *et al*, 2007).

There are two periods where interference with glucose transport or inhibition of glycogenolysis inhibits memory: at the time of training and around the time of long-term memory formation. These times are common to 2-DG, cytochalasin B, and DAB, although inhibition by 2-DG

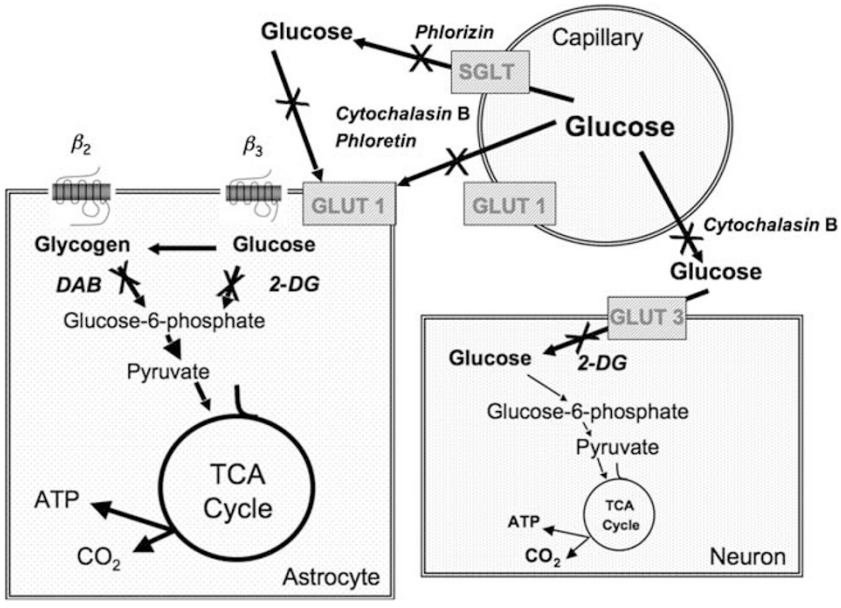

Figure 9 Cartoon of glucose metabolism and transporters in astrocytes, neurons, and capillaries. Glucose is transported across the capillary endothelial cells by the equilibrative glucose transporter (GLUT)-3 and the Na+-dependent and potentially concentrative SGLT (sodium-linked energy-dependent glucose transporter), into neurons by GLUT-3 and into astrocytes by the equilibrative GLUT-1. All transporters are inhibited by cytochalasin B, and SGLT is inhibited by phlorizin and GLUT-1 by phloretin. Glucose is metabolized with neurons and astrocytes via glucose-6-phosphate to pyruvate, which is further metabolized in the TCA cycle, generating CO_2, H_2O (not shown), and ATP. In astrocytes glucose is also used for synthesis of glycogen, which is metabolized to glucose-6-phosphate by a different route than glucose and from there to pyruvate by the same pathway as glucose. Glucose-6-phosphate formation from glucose is inhibited by 2-DG and glucose-6-phosphate formation from glycogen by DAB (1,4-dideoxy-1,4-imino-D-arabinitol).

occurred with injections 5 min earlier than the other inhibitors at both vulnerable periods, suggesting that has to be transported into the cell before it can be incorporated into the glycolytic pathway and interfere with memory. After cytochalasin B, memory loss following injection at −5 min occurred between 50 and 60 min later at the end of ITMB, whereas loss following injection at +25 min occurred 10 min later at the beginning of ITMB. This suggests that different mechanisms are involved at the two times.

We have suggested that glucose is required by neurones at the time of training and by astrocytes at the time of memory consolidation (Gibbs and Summers, 2002b; Gibbs *et al*, 2006b). The results with cytochalasin B support such a view, although they do not discriminate between neuronal, endothelial, or astrocytic GLUT-dependent glucose uptake. However, interference with GLUT-1 or SGLT transporters only inhibited memory when they were administered at the time of consolidation, ie 30 min after training, but not when injected around the time of training or during short-term memory, suggesting that memory loss with cytochalasin B was not due to GLUT-1 or SGLT inhibition. This strongly suggests that inhibition of the neuronal GLUT-3 occurs at the time of learning. The rescue of weakly reinforced learning by lactate immediately after training suggests a need for metabolically derived energy at this time (Gibbs *et al*, 2006a). Moreover, 2-DG administered subcutaneously interferes with memory when injected at the time of consolidation but not at the time of learning, although injection into the mesopallial area of the brain inhibits at both times (Gibbs and Summers, 2002b). This also points to neuronal glucose uptake occurring at the time of learning, whereas astrocytic/endothelial glucose uptake seems to be important at the time of consolidation. It may appear peculiar that neuronal energy demand is not accompanied by glucose transport into the brain, but the high affinity GLUT-3 can maintain the glucose supply to the neurons even at low interstitial glucose concentrations (Duelli and Kuschinsky, 2001). Decreases in extracellular glucose levels occur in the hippocampus during the learning of a spatial task and systemic administration of glucose blocks this decrease and enhances learning (McNay *et al*, 2000, 2001).

Interactions between β_2- and β_3-ARs and Glucose Metabolism

The current studies suggest that 2-DG exerts its effect by competing with glucose uptake, since cytochalasin B, phloretin, or phlorizin, all interfered with consolidation of weakly reinforced memory by CL316243. Conversely, glucose administration potentiated the effect of the β_3-AR agonist. In contrast, none of these metabolic inhibitors affected the ability of the β_2-AR agonist zinterol to consolidate weakly reinforced memory. This effect was counteracted by DAB, suggesting that zinterol acts by stimulating glycogenolysis (Nahorski *et al*, 1975; Magistretti, 1988; Subbarao and Hertz, 1990; Coopersmith and Leon, 1995). There was no interaction between CL316243 and DAB. Iodoacetate, which inhibits pyruvate formation from glucose (glycolysis) or from glycogen, interfered with consolidation of weakly reinforced memory by both CL316243 and zinterol.

Besides pinpointing metabolic process stimulated by β_2-AR and β_3-ARs, our studies show that enhanced glycogenolytic activity cannot compensate for reduced glycolytic activity, and vice versa, although both pathways enhance pyruvate production. This supports the idea that glycolysis and glycogenolysis are compartmentalized in astrocytes (Sickmann *et al*, 2005; Gibbs *et al*, 2007).

The interactions between β_2- and β_3-ARs and glucose metabolism were studied shortly before memory consolidation (20–25 min posttraining). Although iodoacetate (1 nmol per hemisphere) shifted the dose–response curve for zinterol to the right there was no interaction between iodoacetate and zinterol 2.5 min after training. Changes in the rate of glycogenolysis occurring at this time (Hertz *et al*, 2003) must be controlled cellular mechanisms not dependent on β_2-AR activation.

We found that insulin increases glucose uptake 1.5- to 2-fold in chicken astrocytes, a result similar to that reported in cultured mouse astrocytes (Kum *et al*, 1992). Isoprenaline, which increases glucose uptake in glial cells (Hsu and Hsu, 1990), also increased glucose uptake in chicken astrocytes. The increased breakdown of glycogen in response to zinterol gives additional support to our behavioral findings. The early component of glucose uptake is mediated by the β_3-AR agonist CL316243, whereas the β_2-AR agonist zinterol increased glucose uptake at the later times, suggesting that while both AR subtypes increase glucose availability, they do so by different mechanisms.

In conclusion, this study shows that glucose uptake at the time of learning is controlled by cellular mechanisms that differ from those operating at the time of memory consolidation. At the time of learning, glucose uptake is via neuronal GLUT-3, whereas 30 min later, glucose uptake is via endothelial/astrocytic (GLUT-1), and the SGLTs at the blood–brain barrier. Our evidence also suggests that CL316243 enhances glucose uptake via astrocytic transport, whereas zinterol enhances memory by mechanisms requiring the breakdown of stored glycogen in astrocytes.

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DISCLOSURE/CONFLICT OF INTEREST

The author(s) declare that except for income received from the primary employer no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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