Behavioral/Systems/Cognitive

Spatial Memory Formation and Memory-Enhancing Effect of Glucose Involves Activation of the Tuberous Sclerosis Complex–Mammalian Target of Rapamycin Pathway

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The tuberous sclerosis complex–mammalian target of rapamycin (TSC–mTOR) cascade integrates growth factor and nutritional signals to regulate the synthesis of specific proteins. Because both growth factor signaling and glucose have been implicated in memory formation, we questioned whether mTOR activity is required for long-term spatial memory formation and whether this cascade is involved in the memory-augmenting effect of centrally applied glucose. To test our hypothesis, we directly administered rapamycin (an inhibitor of mTOR), glucose, 5-aminoimidazole-4-carboxamide-1β-4-ribonucleoside (AICAR; an activator of AMP kinase), or glucose plus rapamycin into the dorsal hippocampus after we trained rats in the Morris water maze task. The results from these studies indicate that glucose enhances, whereas AICAR and rapamycin both impair, long-term spatial memory. Furthermore, the memory-impairing effect of targeted rapamycin administration could not be overcome by coadministration of glucose. Consistent with these behavioral results, biochemical analysis revealed that glucose and AICAR had opposing influences on the activation of the TSC–mTOR cascade, as indicated by the phosphorylation of ribosomal S6 kinase (S6K) and 4E binding protein 1 (4EBP1), targets of mTOR. Together, these findings suggest that memory formation requires the mTOR cascade and that the memory-enhancing effect of glucose involves its ability to activate this pathway.

Key words: **AICAR; glucose; hippocampus; rapamycin; spatial memory; tuberous sclerosis complex**

Introduction

Growth factor-induced intracellular signals play an important role in memory formation. Three major signaling pathways, extracellular signal-regulated kinase (ERK), phosphoinositide 3 kinase (PI3K), and phospholipase Cγ (PLCγ), are activated in response to the activation of growth factor receptors, and all three pathways have been implicated in memory formation (Martin et al., 1997a; Atkins et al., 1998; Blum et al., 1999; Blum and Dash, 2004; Dash et al., 2004). For example, the ERK cascade appears to be necessary for long-term, but not short-term, memory (Martin et al., 1997a; Atkins et al., 1998; Berman et al., 1998; Blum et al., 1999; Schafe et al., 2000; Thiels and Klann, 2001). Because gene expression and protein synthesis are hallmarks of long-term memory formation, identification of the intracellular pathways that enhance protein production is a subject of intense study. Recent studies focusing on elucidating the signaling pathway by which growth factors lead to enhanced protein synthesis have identified the tuberous sclerosis complex–mammalian target of rapamycin (TSC–mTOR) pathway as an integral component (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) (Inoki et al., 2005b). The TSC1–TSC2 complex acts as a negative regulator of mTOR activity, which controls the synthesis of proteins that are involved in cellular growth processes, by its activation and inactivation of ribosomal S6 kinase (S6K) and 4E binding protein 1 (4EBP1), respectively (Jefferies et al., 1997; Sonenberg and Gingras, 1998; Gao et al., 2002; Inoki et al., 2002; Jaworski et al., 2005; Kumar et al., 2005; Nobukuni et al., 2005). Because the memory-augmenting effect of hippocampal PI3K activation is associated with increased activation of S6K (Dash et al., 2004), we questioned the necessity of the TSC–mTOR pathway in long-term spatial memory formation.

In addition to arbitrating growth factor signaling, the TSC–mTOR pathway also is involved in nutrient-mediated growth processes (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) (Beck and Hall, 1999; Hardwick et al., 1999; Inoki et al., 2003). Increased glucose levels can be used as a positive influencer of memory (Gold, 1986; Azari, 1991; Kopf and Baratti, 1996; Ragozzino et al., 1996; McNay and Gold, 2001; Rashidy-Pour, 2001; Korol, 2002). In addition, it has been demonstrated that age-related memory impairments in rodents can be reversed by injection of glucose near the time of training (Messier and Destrade, 1988; McNay and Gold, 2001). Although it is thought that the memory-enhancing effect of systemic glucose administration is mediated by peripheral release of epinephrine, this mechanism is not consistent with memory enhance-

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ment that is seen after intrahippocampal or intra-amygdalal glucose infusion (Ragozzino and Gold, 1994; Li et al., 1998; Ragozzino et al., 1998; Schroeder and Packard, 2003; Canal et al., 2005; Krebs and Parent, 2005).

Because the TSC–mTOR pathway is regulated by the energy status of the cell (Inoki et al., 2003; Kimura et al., 2003), this cascade could mediate the memory-enhancing effects of glucose centrally. In the present study we determine the necessity of the TSC–mTOR cascade in long-term spatial memory formation and examine a potential mechanism for the memory-enhancing effect of glucose by combining targeted manipulation of the TSC–mTOR pathway within the hippocampus of adult rats with a post-training treatment protocol. We present data to demonstrate a role for the TSC–mTOR pathway in long-term spatial memory formation and show that the memory-enhancing effect of targeted glucose administration is associated with activation of this pathway.

Materials and Methods

Materials. Male Long–Evans rats (250–280 g) were purchased from Charles River Laboratories (Wilmington, MA). Rapamycin was purchased from Biomol (Plymouth Meeting, PA), and 5-aminoimidazole-4-carboxamide-1β-4-ribonucleoside (AICAR) was purchased from Calbiochem (La Jolla, CA). Phospho-AMP kinase-α (phospho-AMPK; Thr^{172}), AMPK-α, phospho-S6K (Thr^{421}/Ser^{424} or Thr^{389}), S6K, phospho-4EBP1 ($Thr^{37/46}$), 4EBP1, phospho-mTOR (Ser^{2448} or Ser^{2481}), and mTOR antibodies were all purchased from Cell Signaling Technology (Danvers, MA).

Intrahippocampal infusion and drug preparation. All protocols involving the use of animals were in compliance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee. Rats were anesthetized with 4% isoflurane in a 2:1 N_2O/O_2 mixture and then maintained with a 2% isoflurane/2:1 N_2O/O_2 mixture via a face mask. Bilateral guide cannulas, aimed at the dorsal hippocampus (anteroposterior, −3.3 mm; lateral, ± 2.0 mm from bregma; ventral, −2.0 mm from the dura), were implanted. Then the rats were allowed to recover in their home cages for 10–12 d. During infusion the injection cannulas extended 1.75 mm beyond the tips of the guides, yielding a total depth of 3.75 mm below the dura. Stock solutions of rapamycin were prepared by initially dissolving in DMSO and then diluting in saline before infusion (final DMSO of 0.1%). AICAR and glucose were prepared in saline. All injections (1 μl/hippocampus of either drug or appropriate vehicle) were performed in freely moving animals at a rate of 0.25 μl/min via a dual syringe infusion pump (Stoelting, Wood Dale, IL).

Sample preparation and Western blotting. To examine the effect of glucose and AICAR infusion on the phosphorylation of downstream signaling molecules, we infused the animals with drug into one hippocampus and an equal amount of vehicle into the contralateral hippocampus of the same animal. At the indicated time points after infusion hippocampal punches (2 mm in diameter) surrounding the infusion site were prepared while brains were submerged in ice-cold artificial CSF [containing the following (in mM): 10 HEPES, pH 7.2, 1.3 NaH_2PO_4, 3 KCl, 124 NaCl, 10 dextrose, 26 $NaHCO_3$, and 2 $MgCl_2$] containing phosphatase inhibitors [(in mM) 2 NaF, 2 Na_2MoO_4, and 1 Na_3VO_4]. Tissues were homogenized (20 strokes) in 10 vol of a buffer containing the following (in mM): 10 Tris, pH 7.4, 1 EGTA, 1 EDTA, 0.5 DTT plus phosphatase inhibitors (0.1 μM okadaic acid and 1 mM Na_3VO_4) and protease inhibitors (1 mM PMSF and 10 μg/ml leupeptin), using a motorized Teflon glass homogenizer. The amount of protein in each sample was determined by a NanoOrange Protein Quantification Assay (Invitrogen, Carlsbad, CA), using bovine serum albumin (BSA) as the standard. Samples were boiled in Laemmli sample buffer and resolved on Tris-tricine SDS-PAGE gels. Proteins then were transferred to Immobilon-P (Millipore, Bedford, MA) membranes by using a semi-dry transfer apparatus (Millipore) and were blocked overnight in 5% BSA, followed by a 3 h incubation in primary antibody (0.5 μg/ml) at room temperature. Membranes next were washed and incubated at room temperature with alkaline phosphatase-conjugated secondary antibodies for 1 h as recommended by the vendor (Vector Laboratories, Burlingame, CA). Immunoreactivity was detected via a CDP-Star chemiluminescence system (New England Biolabs, Beverly, MA) and visualized on Kodak XAR5 film (Rochester, NY). Multiple exposures of each membrane were taken to ensure the linearity of the immunoreactive bands, which were quantified by using Image J (freely available from National Institutes of Health, Bethesda, MD). The level of phosphorylation for AMPK or S6K was normalized by reprobing stripped gels with antibodies raised against these proteins independent of their phosphorylation states. Western blot data were compiled from at least four independent animals at each time point.

Immunohistochemistry. Tissue preparation and immunohistochemistry to detect total and phosphorylated levels of proteins were performed as described by us previously (Blum et al., 1999; Dash et al., 2004). Briefly, at the designated time points after 0.9 ng of rapamycin infusion into the dorsal hippocampus or an equal volume of vehicle into the contralateral dorsal hippocampus, rats were decapitated and the brains quickly removed. Dissected hippocampi were fixed in ice-cold 4% paraformaldehyde/15% picric acid in PBS for at least 16 h. After cryoprotection in 30% sucrose in PBS the 40-μm-thick tissue sections were prepared on a cryostat. Free-floating slices were incubated overnight in an antibody that detects phosphorylated S6K (1.0 μg/ml) in PBS containing 2% BSA and 2.5% normal goat serum. Immunoreactivity was visualized via ABC and DAB kits (Vector Laboratories) by following the manufacturer's instructions. Controls for immunostaining were performed by eliminating the primary antibody from the incubation mixture. No specific immunoreactivity was observed in these control sections.

Behavioral training and testing. All behavioral experiments were performed by an experimenter who was kept blind to the treatment schedule. Cannulated animals were trained in the hidden platform version of the Morris water maze task (Schenk and Morris, 1985; Guzowski and McGaugh, 1997). For the glucose-infused animals in which augmentation of memory was anticipated, the criterion was set at three consecutive times with latency to platform averaging <15 s. For experiments involving AICAR or rapamycin administration in which impaired memory was anticipated, the criterion was set at three consecutive trials with platform latencies of <10 s. Animals that failed to reach these criteria by trial 13 were eliminated from the study. Each trial was initiated by placing the animal in one of four randomly chosen locations, facing the wall of the tank. Animals were allowed to search for the hidden platform for a period of 60 s. If an animal failed to find the platform, the animal was placed there by the experimenter. Animals were allowed to remain on the platform for a period of 30 s before being returned to a warming cage between trials. Once criterion was reached, animals were infused bilaterally with either drug or vehicle as described above. After the completion of training the animals were returned to their home cages until retention testing.

At 48 h after training the animals were tested for retention by a probe trial in which the hidden platform was removed from the maze and the animals were allowed to search for a period of 60 s. Movement within the maze was monitored by a video camera linked to tracking software (Chromotrack, San Diego Instruments, San Diego, CA). The time to platform was calculated as the latency for each animal to cross the site at which the hidden platform was located during training. Using the tracking software, we calculated swimming speed by dividing the cumulative total distance (in centimeters) traversed in each zone by the cumulative dwell time.

Statistical analysis. Statistical significance was determined by a two-tailed Student's t test for unpaired (behavioral data) or paired variables (Western blot data). Data were considered significant at $p < 0.05$.

Results

Rapamycin impairs long-term spatial memory

Previous studies performed in our laboratory have demonstrated that activation of PI3K in the hippocampus of behaviorally trained rats improves spatial and contextual long-term memory (Dash et al., 2004). This activation was associated with an in-

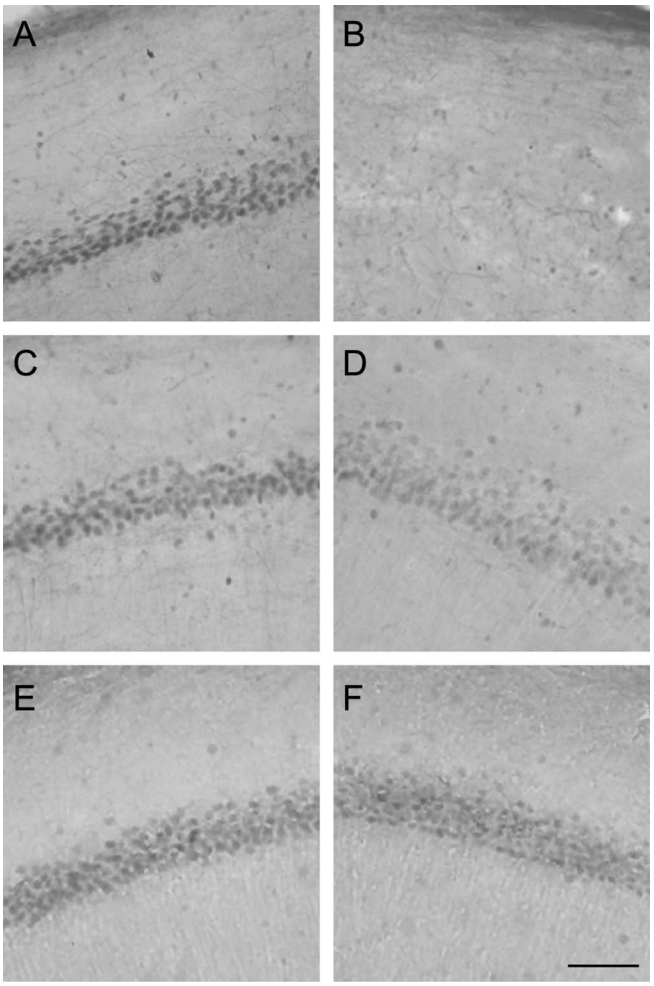


Figure 1. Intrahippocampal infusion of rapamycin decreases S6K phosphorylation. Shown are representative images of immunoreactivity for phosphorylated S6K within the CA1/CA2 subfield of the hippocampus after intrahippocampal vehicle (***A***, ***C***, ***E***) or rapamycin (***B***, ***D***, ***F***) infusion. ***A***, ***B***, Core of the infusion. ***C***, ***D***, At 1.5 mm caudal to the infusion site. ***E***, ***F***, Ventral hippocampus. Scale bar, 100 μm.

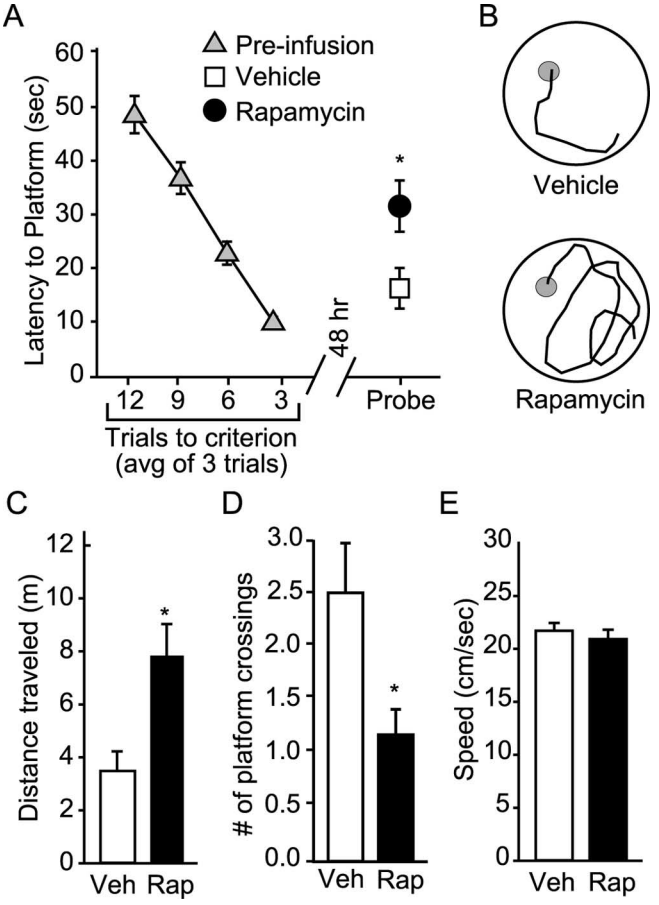


Figure 2. Post-training infusion of rapamycin impairs long-term spatial memory. ***A***, Animals were trained in the hidden platform version of the Morris water maze and then infused with either vehicle ($n = 13$) or 0.9 ng/hippocampus rapamycin ($n = 16$). Latency to the hidden platform during training and in a probe trial 48 h after training is shown. ***B***, Representative probe trial traces of a vehicle-infused and a rapamycin-infused animal showing the path taken before the first platform crossing. Shown are the mean distance traveled (***C***), the number of platform crossings (***D***), and the swimming speed (***E***) recorded during the probe trial for the two groups. Veh, Vehicle; Rap, rapamycin. Data are presented as the mean ± SEM; *$p < 0.05$.

crease in the phosphorylation of S6K, suggesting that mTOR may play a role in memory formation. To determine whether the inhibition of mTOR has any effect on long-term memory storage, we performed infusions of rapamycin, targeted to the dorsal hippocampus. Because S6K is phosphorylated and activated in response to mTOR activity, phospho-S6K immunoreactivity (Thr^{421}/Ser^{424}) was examined first to determine the effectiveness and the extent of diffusion of rapamycin infusion. Rats were infused with 0.9 ng of rapamycin into one dorsal hippocampus while an equal volume (1.0 μl) of vehicle was administered simultaneously to the contralateral dorsal hippocampus of the same animal. Assuming a volume of 100 μl for the dorsal hippocampus, it is estimated that 0.9 ng of rapamycin would give an equilibrium concentration of 100 nM. This concentration is consistent with a previous study, which demonstrated that long-term potentiation in isolated CA1 dendrites could be blocked by 200 nM rapamycin (Cracco et al., 2005). At 45 min after the completion of the infusion, the rats were decapitated and the hippocampi isolated and postfixed in ice-cold 4% paraformaldehyde containing 15% picric acid. Figure 1 shows representative photomicrographs of phospho-S6K immunoreactivity from vehicle- and rapamycin-infused animals (Fig. 1*A*,*C*,*E*, vehicle, *B*,*D*,*F*, rapamycin). The figure shows that the hippocampus infused with rapamycin has a marked decrease in phospho-S6K immunoreactivity at the infusion site (Fig. 1*A*, vehicle, vs *B*, rapamycin) with a modest decrease still visible 1.5 mm caudal of the infusion site (Fig. 1*C*, vehicle, vs *D*, rapamycin). No detectable change in S6K phosphorylation was observed in the ventral hippocampus (Fig. 1*E*, vehicle, vs *F*, rapamycin), indicating that the diffusion of the drug was limited to the dorsal hippocampus.

To test the influence of rapamycin on long-term memory storage, we performed a post-training infusion paradigm after the rats were trained in the Morris water maze task. Immediately after reaching criterion, the rats were infused bilaterally with 0.9 ng/hippocampus rapamycin ($n = 16$) or an equal volume of vehicle ($n = 13$). When they were tested for long-term memory in a retention test 48 h after training, there was a significant difference in latency to the original platform location between drug- and vehicle-infused animals, suggesting that rapamycin-infused animals had an impaired memory of the platform location (rapamycin, 31.93 ± 4.79 s; vehicle, 16.57 ± 3.80 s; $p < 0.05$) (Fig. 2*A*). Representative traces of the swimming paths taken by a drug- and a vehicle-infused animal during the transfer test are shown in Figure 2*B*. In addition to a latency difference to the first platform crossing, the rapamycin-infused animals had a significantly

longer distance traveled to the first platform crossing (rapamycin, 784.2 ± 128.4 cm; vehicle, 346.3 ± 80.7 cm; $p < 0.05$) (Fig. 2*C*) and significantly crossed the original platform location less frequently than did the vehicle-infused animals (rapamycin, 1.25 ± 0.23 crossings; vehicle, 2.62 ± 0.45 crossings; $p < 0.05$) (Fig. 2*D*). These differences seen in the transfer test are not attributable to a difference in swimming speed (rapamycin, 21.20 ± 0.87 cm/s; vehicle, 21.91 ± 0.63 cm/s; not significant) (Fig. 2*E*).

Intrahippocampal administration of glucose activates the mTOR cascade

The TSC–mTOR pathway has been demonstrated to regulate protein synthesis via the integration of growth factor and cellular energy signals (Inoki et al., 2005b). Because long-term memory is critically dependent on the *de novo* synthesis of proteins, we investigated the ability of glucose to activate the TSC–mTOR pathway and enhance long-term spatial memory. AMPK is phosphorylated and activated in response to increases in the AMP/ATP ratio. Activation of AMPK increases the inhibitory action of the TSC complex on mTOR, thereby reducing S6K phosphorylation (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Using antibodies that specifically recognize phosphorylated AMPK (Thr^{172}), mTOR (Ser^{2448} or Ser^{2481}), S6K (Thr^{389}), and 4EBP1 ($Thr^{37/46}$), we performed Western blots to determine the linearity of detection. All antibodies gave rise to linear immunoreactivity between 5 and 20 μg of total protein loaded on the gel, with the exception of the phospho-mTOR Ser^{2448} antibody that had detectable immunoreactivity only between 10 and 20 μg (Fig. 3*A*). Although linear, immunoreactivity for phospho-4EBP1 was found to triple in response to a doubling in protein amount. Thus the immunoreactivity obtained for phospho-4EBP1 was normalized against the standard curve before statistical evaluation. To determine the effect of intrahippocampal administration of glucose, we loaded equal amounts (10 μg) of total protein from hippocampi infused with either vehicle or 20 μg of glucose, as described in Materials and Methods. This dose was chosen on the basis of previous studies by Schroeder and Packard (2003), who used 10 μg of glucose for intra-amygdala (a relatively smaller structure) injection. Preliminary time course experiments revealed that biochemical changes could be detected as early as 10 min after glucose injection. Figure 3*B* shows that glucose (20 μg) significantly decreased the phosphorylation of AMPK by 10 min after infusion as compared with the levels detected in the contralateral saline-infused hippocampus of the same animal ($n = 5$). No change in the total level of AMPK was observed.

Using two commercially available antibodies to phosphorylated mTOR (Ser^{2448} and Ser^{2481}), we next examined whether glucose infusion altered the phosphorylation of this kinase. Figure 3*C* shows representative Western blots and summary data ($n = 4$/condition) indicating that mTOR phosphorylation is not altered significantly in response to glucose infusion (30 min post-infusion data shown). However, consistent with an increase in mTOR activity as a result of reduced AMPK activity, a significant increase in the phosphorylation, but not the total levels, of the translational regulators S6K and 4EBP1 was detected by 30 min after infusion (Fig. 3*D*). A modest, but significant, increase in phospho-S6K immunoreactivity also was observed in the 10 min samples used to evaluate phospho-AMPK (data not shown).

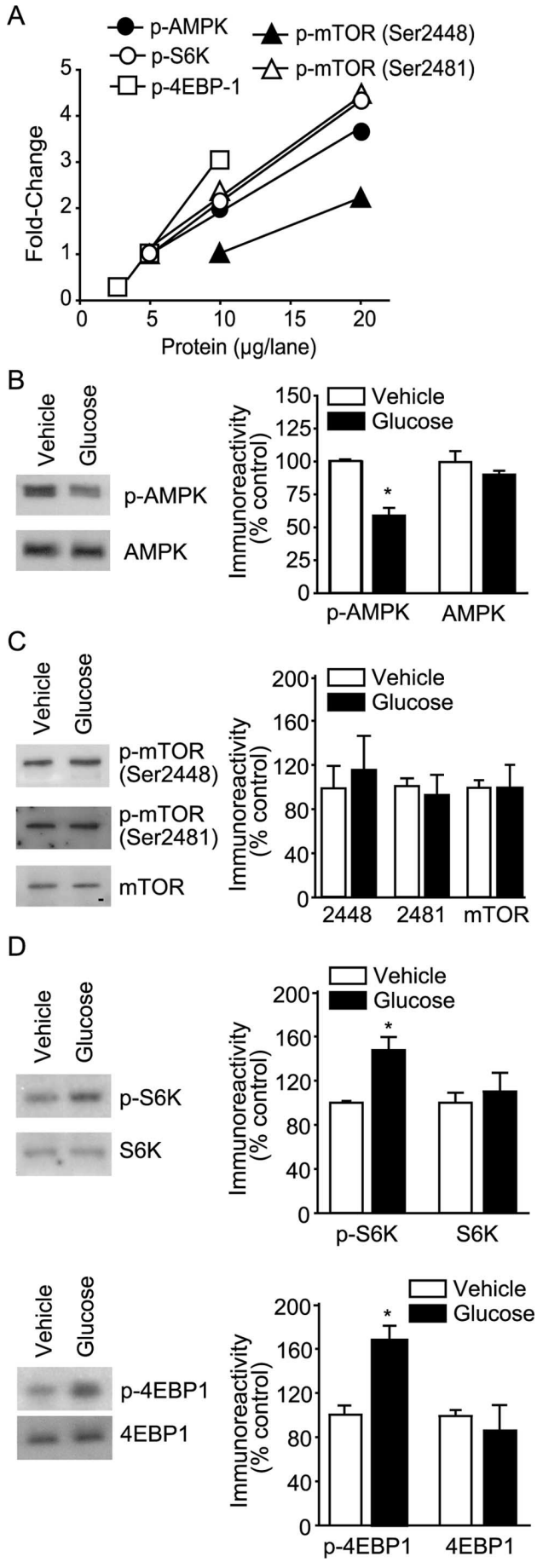
Post-training intrahippocampal administration of glucose enhances long-term spatial memory

Because glucose was anticipated to cause a memory improvement, Morris water maze training was performed by using a soft criterion (three consecutive trials with an average of <15 s platform latency) and a limited number of training trials (maximum of nine trials). According to our experience, this training paradigm elicits poor memory performance in saline-infused controls (our unpublished observations). Immediately after reaching criterion the rats were infused bilaterally with 20 μg/hippocampus glucose ($n = 10$) or an equal volume of vehicle ($n = 9$). When they were tested for long-term memory during a retention test 48 h after training, there was a significant difference in latency to the original platform location between drug- and vehicle-infused animals, suggesting that glucose-infused animals had an enhanced memory of the platform location (glucose, 22.11 ± 5.34 s; vehicle, 41.94 ± 7.75 s; $p < 0.05$) (Fig. 4*A*). Representative traces of the swimming paths to the platform taken by a glucose-infused and a vehicle-infused animal during the probe trial are shown in Figure 4*B*. In addition to a latency difference to the first platform crossing, the glucose-infused animals had a significantly shorter distance traveled to the first platform crossing (glucose, 483.7 ± 113.8 cm; vehicle, 871.5 ± 162.3 cm; $p < 0.05$) (Fig. 4*C*) and crossed the platform location significantly more times (glucose, 1.80 ± 0.39 crossings; vehicle, 0.67 ± 0.29 crossings; $p < 0.05$) than did the vehicle-infused animals (Fig. 4*D*). These differences were not attributable to changes in swimming speed (glucose, 22.89 ± 1.14 cm/s; vehicle, 20.47 ± 0.99 cm/s; not significant) (Fig. 4*E*).

AICAR decreases mTOR activity and attenuates spatial memory

The association of memory enhancement with activation of the TSC–mTOR cascade by glucose suggests that agents that activate AMPK may be detrimental to memory. To test this possibility, we examined the biochemical and behavioral consequences of intrahippocampal administration of the AMPK activator AICAR. Numerous studies have used AICAR as a specific activator of AMPK (Sullivan et al., 1994; Corton et al., 1995; Bolster et al., 2002; McCrimmon et al., 2004; Lee et al., 2005). AICAR (260 ng) was infused into one hippocampus, and an equal volume of vehicle was infused into the contralateral hippocampus of the same animal ($n = 4$). Using 30 min post-infusion extracts, we made comparisons between hippocampi infused with AICAR and the contralateral hippocampi infused with vehicle. Figure 5*A* shows representative Western blots and summary data demonstrating that AICAR significantly decreased the phosphorylation of S6K and 4EBP1. No changes in the total levels of S6K or 4EBP1 were detected.

To determine whether the inhibition of mTOR by AICAR has any effect on long-term memory storage, we again performed the Morris water maze task. Animals were trained and, immediately after reaching criterion (three consecutive trials <10 s), were infused bilaterally with 260 ng/hippocampus AICAR ($n = 9$) or an equal volume of vehicle ($n = 8$). When they were tested for long-term memory in a retention test 48 h after training, there was a significant difference in latency to the original platform location between AICAR-infused and vehicle-infused animals, suggesting that AICAR-infused animals had an impaired memory of the platform location (AICAR, 41.53 ± 7.13 s; vehicle, 17.51 ± 3.95 s; $p < 0.05$) (Fig. 5*B*). Representative traces of the swimming paths to the first platform crossing taken by a drug- and a vehicle-infused animal during the transfer test are shown in



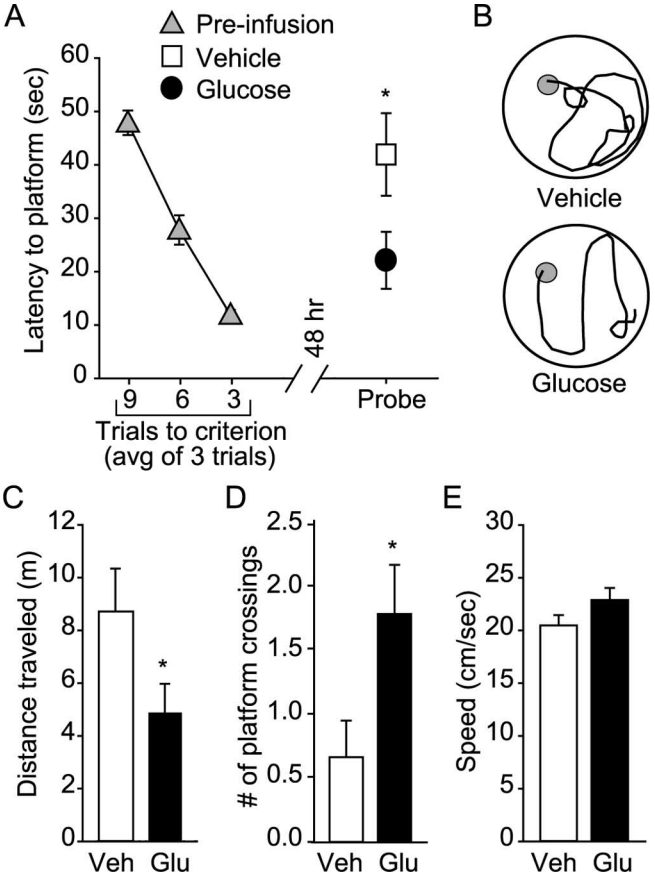


Figure 4. Post-training infusion of glucose augments long-term spatial memory. ***A***, Animals were trained in the hidden platform version of the Morris water maze by using a soft criterion and then were infused with either vehicle ($n = 9$) or 20 μg/hippocampus glucose ($n = 10$). Training and probe trial performance (latency) are shown. ***B***, Representative probe trial traces of a vehicle-infused and a glucose-infused animal showing the path taken before the first platform crossing. Glucose (Glu)-infused animals had a reduced mean distance traveled (***C***) and an increased number of platform crossings (***D***) when compared with vehicle (Veh)-infused rats. ***E***, Swimming speed recorded during the probe trial for the two groups. Data are presented as the mean ± SEM; $^*p < 0.05$.

Figure 5*C*. In addition to a latency difference to the first platform crossing, the AICAR-infused animals had a significantly longer distance traveled to the first platform crossing (AICAR, 1050.9 ± 189.4 cm; vehicle, 252.7 ± 38.70 cm; $p < 0.05$) (Fig. 5*D*) and crossed the original platform location less frequently than did the vehicle-infused animals (AICAR, 1.11 ± 0.54 crossings; vehicle, 2.13 ± 0.44 crossings; $p < 0.06$) (Fig. 5*E*). These differences seen in the transfer test were not attributable to a difference in swimming speed (AICAR, 24.96 ± 1.10 cm/s; vehicle, 22.24 ± 1.38 cm/s; not significant) (Fig. 5*F*).

Memory-impairing effect of rapamycin cannot be overcome by coadministration of glucose

To examine whether intrahippocampal administration of glucose is able to overcome the spatial memory deficit seen with

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Figure 3. Intrahippocampal administration of glucose activates the TSC–mTOR pathway. ***A***, Standard curve showing the linear relationship between immunoreactivity and increasing amounts of protein as determined by Western blot analysis for the phospho-S6K and phospho-AMPK antibodies. Shown are representative Western blots and summary data for phospho- and total AMPK (***B***), phospho- and total mTOR (***C***; 30 min after infusion), and phospho- and total S6K and 4EBP1 (***D***) after intrahippocampal infusion of either vehicle or 20 μg of glucose. Data are presented as the mean ± SEM; $^*p < 0.05$.

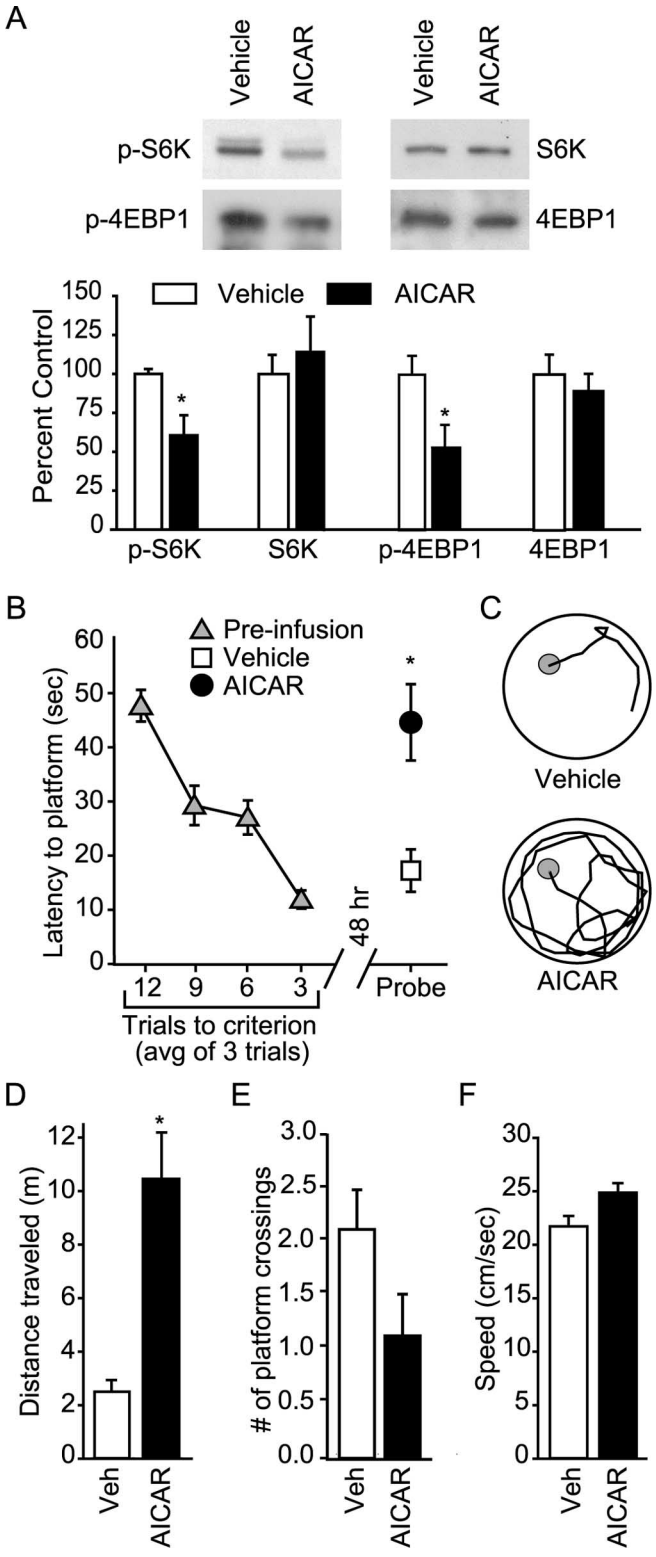

Figure 5. AICAR decreases mTOR activity and impairs long-term spatial memory. ***A***, Representative Western blots and summary data showing that intrahippocampal administration of 260 ng of AICAR decreases phospho-S6K and phospho-4EBP1, but not total S6K or 4EBP1, immunoreactivities at 30 min after infusion as compared with the contralateral vehicle-infused hippocampus. ***B***, Latencies during water maze training and probe trial for animals infused with either vehicle ($n = 8$) or 260 ng/hippocampus AICAR ($n = 9$). ***C***, Representative probe trial traces of a vehicle-infused and an AICAR-infused animal showing the path taken before the first platform crossing. AICAR-infused animals had an increased mean distance traveled (***D***) and a decreased number of platform crossings (***E***) during the probe trial. ***F***, Swimming speed was not different between the two groups. Data are presented as the mean ± SEM; $^*p < 0.05$.

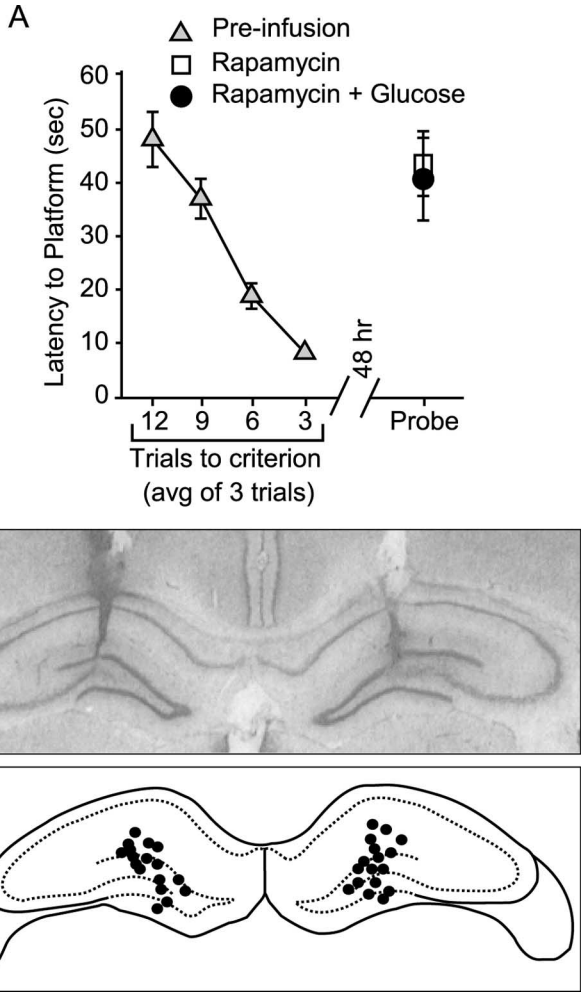

Figure 6. Post-training administration of glucose cannot overcome the memory impairment caused by the coadministration of rapamycin. ***A***, Performance during training and testing in the water maze task for animals infused with either 0.9 ng of rapamycin/hippocampus or 0.9 ng of rapamycin plus 20 μg of glucose/hippocampus. Error bars indicate SEM. ***B***, Representative photomicrograph of a cresyl violet-stained tissue section showing the infusion track in one of the cannulated animals used in the behavioral studies. ***C***, Illustration indicating the infusion sites (black circles) for the animals used in the behavioral studies. Non-redundant infusion sites are shown.

rapamycin, we performed a coadministration of rapamycin and glucose after Morris water maze training of the rats. Immediately after reaching criterion, the animals were infused bilaterally with 0.90 ng of rapamycin and 20 μg/hippocampus glucose ($n = 9$) or 0.90 ng/hippocampus rapamycin alone ($n = 9$). When they were tested for long-term memory in a retention test 48 h after training, there was no difference in latency to the original platform location between either group of animals, suggesting that glucose was unable to overcome the rapamycin-induced memory deficit previously demonstrated (rapamycin plus glucose, 40.32 ± 7.70 s; rapamycin, 43.20 ± 6.01 s; not significant) (Fig. 6*A*). There was no difference between the path lengths taken to reach the platform nor in the swimming speed between groups (data not shown).

Infusion sites

After the completion of behavioral testing the animals were killed, and the brains were analyzed for infusion site accuracy. Figure 6*B* shows a photomicrograph of a representative cresyl

violet-stained tissue demonstrating that the infusion tracks terminate in the dorsal hippocampus. Figure 6*C* shows the locations of the intrahippocampal infusion sites for representative animals used in the Morris water maze behavioral studies. All animals had infusion sites (black circles) that terminated in the dorsal hippocampus. Only novel infusion site locations are represented.

Discussion

The findings from the present study demonstrate that the TSC–mTOR pathway, a major downstream cascade recruited by growth factors and nutrient signals, is required for spatial memory formation. Post-training intrahippocampal blockade of this pathway (by rapamycin or AICAR) impairs, whereas its stimulation (by glucose) facilitates, memory formation. Four key findings support these conclusions: (1) inhibition of the TSC–mTOR pathway impairs spatial memory in normal animals, (2) activation of AMPK reduces mTOR activity and impairs normal memory, (3) administration of glucose enhances spatial memory and activates the TSC–mTOR pathway, and (4) inhibition of the TSC–mTOR cascade precludes the memory-enhancing effect of glucose.

Growth processes leading to morphological changes in neurons are a hallmark of long-term memory (Greenough et al., 1985; Bailey and Kandel, 1993). The TSC1–TSC2 complex integrates two of the most important extracellular signals involved in the regulation of growth: growth factors and glucose (Li et al., 2004). Although the involvement of growth factor receptor-initiated signals in memory formation has been well established, it is not known which of these signals mediates its influence on spatial memory via the TSC–mTOR cascade. Both the PI3K and ERK cascades have been shown to phosphorylate and inactivate TSC2 (Tee et al., 2003; Ma et al., 2005), leading to disruption of the TSC1–TSC2 complex. Disinhibition of the TSC complex results in increased mTOR activity, which then controls the synthesis of proteins that are involved in cellular growth processes. Placed into a context of neuronal plasticity, this suggests that mTOR activity may be required for long-term protein synthesis-dependent synaptic changes. Consistent with this notion, it has been demonstrated that rapamycin-sensitive protein synthesis is required for synaptic capture of long-term facilitation in isolated *Aplysia* sensory motorneuron cultures (Casadio et al., 1999). Similarly, long-term potentiation in hippocampal slices, as well as in isolated dendrites of hippocampal CA1 pyramidal neurons, requires rapamycin-sensitive protein synthesis (Raymond et al., 2002; Tang et al., 2002; Cracco et al., 2005), as does the consolidation of auditory cortex-dependent memories (Tischmeyer et al., 2003). Activation of the TSC–mTOR pathway at specific synapses during learning can give rise to local protein synthesis at these synaptic sites and synapse-specific long-term plasticity. As hypothesized previously, synapse-specific protein synthesis is thought to serve as a tag for the maintenance of long-term plasticity (Frey and Morris, 1997; Martin et al., 1997b). A recent study reports that Eker rats, heterozygous for *tsc2* ($tsc^{+/-}$, which would cause modest activation of the mTOR cascade) show enhanced episodic-like memory (Waltereit et al., 2006). The present study supports these previous findings and additionally implicates the TSC–mTOR pathway in hippocampal-dependent long-term memory storage. Limitations for this interpretation are that the involvement of mTOR is based on the use of rapamycin and that our attempts to examine the activation of mTOR within the hippocampus (by assessing mTOR and S6K phosphorylation levels by immunohistochemistry) after training were inconclusive (data not shown). This lack of a detectable change in mTOR activity was attributable to poor immunoreactivity by the phospho-mTOR antibodies and the relatively high percentage of cells already displaying phosphorylated S6K in untrained controls. Because numerous studies have shown that rapamycin is a highly selective inhibitor for mTOR, with no other target for rapamycin being identified (Heitman et al., 1991), its interference with normal memory storage suggests that mTOR activity is required for hippocampal-dependent memory.

It has been well established that glucose can modulate memory processes. The memory-enhancing effect of increases in blood glucose is thought to be mediated by peripheral release of epinephrine that, in turn, increases acetylcholine levels in the hippocampus and amygdala, two structures involved in memory (Cracco et al., 2005). Although systemic administration of glucose has been suggested to modulate memory via this peripheral mechanism, it has been shown that extracellular hippocampal glucose concentration decreases in rats when they perform a spatial navigation task, suggesting a central mechanism of action for glucose (McNay et al., 2000). Consistent with this suggestion, a few studies have indicated that direct injection of glucose into specific brain structures (e.g., amygdala or the hippocampus) can facilitate memory (Ragozzino and Gold, 1994; Ragozzino et al., 1998; Schroeder and Packard, 2003; Canal et al., 2005; Krebs and Parent, 2005). It is thought that centrally applied glucose may facilitate memory formation by restoring cellular energy levels that were reduced as a result of learning and/or by increasing the release of acetylcholine. The present findings show that, in addition to (or as a result of) restoration of cellular energy, glucose activates the TSC–mTOR pathway as indicated by increased S6K and 4EBP1 phosphorylation. Although we observed significant changes in the phosphorylation of these two downstream markers of mTOR activity, we did not observe corresponding changes in the phosphorylation of mTOR itself. Although the reason for this lack of change is unknown, recent studies have brought into question the role of mTOR phosphorylation in modulating mTOR activity (Peterson et al., 2000; Sekulic et al., 2000; Bolster et al., 2003; Mothe-Satney et al., 2004; Yonezawa et al., 2004).

Involvement of the TSC–mTOR pathway for glucose use and memory formation may have clinical implications. TSC is an autosomal dominant disorder that is characterized by the development of tumor-like growths, named hamartomas, in the kidneys, heart, skin, and brain. Disease-causing mutations (or genetic deletion) of TSC1 or TSC2 are associated with increased phosphorylation of S6K and 4EBP1. In a recent population-based study it was revealed that ~31% of TSC patients had an estimated IQ < 21, with only 55% having an IQ > 70 (Prather and de Vries, 2004). Deficiencies are most frequent in the domains of executive control and memory. In particular, autism and attention deficit hyperactivity disorder have been reported in association with TSC mutations at much higher rates than in the normal population. The present results implicating the TSC–mTOR pathway in memory formation may account for some of the behavioral and memory problems seen in patients carrying mutations in either *tsc1* or *tsc2* genes. In addition to TSC, gene mutations causing human syndromes (e.g., Peutz–Jeghers syndrome and Lhermitte–Duclos disease) have been mapped to the TSC–mTOR pathway (Inoki et al., 2005a). These syndromes may have associated memory and other cognitive dysfunctions (or enhancement), depending on the degree of mTOR activation caused by the disease-causing mutations. Because glucose stimulates the TSC–mTOR pathway, a reduced activation of this pathway in disease conditions may impair memory formation. For example, calculation of cerebral glucose use with [^{18}F]-2-fluoro-deoxy-D-glucose

positron emission tomography (FDG-PET) has shown hypometabolism in the hippocampus and other limbic structures, even in the early stages of AD when cognitive impairments are mild (Nestor et al., 2003). Furthermore, blood flow measurements with functional MRI show reduced flow and (by extrapolation) decreased glucose availability. Similarly, a number of vascular and cardiovascular conditions can cause cerebral hypoperfusion, development of vascular cognitive impairments, and vascular dementia (Roman et al., 2002). It is tempting to speculate that the reduced blood flow and decreased glucose availability in these conditions may, in part, cause memory impairments as a result of diminished TSC–mTOR activation.

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