Research

Intrahippocampal glutamine administration inhibits mTORC1 signaling and impairs long-term memory

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The mechanistic Target of Rapamycin Complex 1 (mTORC1), a key regulator of protein synthesis and cellular growth, is also required for long-term memory formation. Stimulation of mTORC1 signaling is known to be dependent on the availability of energy and growth factors, as well as the presence of amino acids. In vitro studies using serum- and amino acid-starved cells have reported that glutamine addition can either stimulate or repress mTORC1 activity, depending on the particular experimental system that was used. However, these experiments do not directly address the effect of glutamine on mTORC1 activity under physiological conditions in nondeprived cells in vivo. We present experimental results indicating that intrahippocampal administration of glutamine to rats reduces mTORC1 activity. Moreover, post-training administration of glutamine impairs long-term spatial memory formation, while coadministration of glutamine with leucine had no influence on memory. Intracellular recordings in hippocampal slices showed that glutamine did not alter either excitatory or inhibitory synaptic activity, suggesting that the observed memory impairments may not result from conversion of glutamine to either glutamate or GABA. Taken together, these findings indicate that glutamine can decrease mTORC1 activity in the brain and may have implications for treatments of neurological diseases associated with high mTORC1 signaling.

The mechanistic Target of Rapamycin (mTOR) is a highly conserved serine/threonine protein kinase critical for the regulation of a multitude of cellular processes, including cell growth, metabolism, protein synthesis, transcription, and autophagy (Kim et al. 2002; Laplante and Sabatini 2012; Magri and Galli 2013). mTOR exists as two distinct multiprotein complexes termed mTORC1 and mTORC2, each composed of a distinct complement of associated proteins, and differing in their cellular functions and how they are regulated. mTORC1 is rapamycin-sensitive and promotes cellular growth by increasing protein synthesis via phosphorylation of S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein (4E-BP) (Dibble and Manning 2013). In contrast, mTORC2 regulates actin polymerization and morphological changes and is insensitive to rapamycin (Cybulski and Hall 2009). mTORC1, the focus of this paper, is widely recognized to respond to a wide variety of input signals, including growth factor signaling, cellular energy state, and amino acids. In response to energy and growth factor signaling inputs, the GTPase-activating activity of the tuberous sclerosis complex (TSC) is inhibited, which releases its repression on the G-protein Rheb (Ras homolog enriched in brain), leading to mTORC1 activation (Manning and Cantley 2003). Amino acids have also been shown to modulate mTORC1 activity, but unlike energy and growth factor signaling, amino acids appear to regulate mTORC1 signaling through a TSC-independent pathway involving translocation of mTORC1 to the lysosome surface mediated by interactions with Rag-family proteins (Sancak et al. 2010; Jewell et al. 2013). This TSC-independent activation of mTORC1 may have implications in treating diseases associated with elevated mTORC1 activity by using amino acid supplementation.

Recent in vitro studies have suggested that glutamine, the most abundant amino acid found in the circulation, can modulate mTORC1 activity in cells cultured under deprived conditions by increasing the cellular uptake of leucine through the glutamine–leucine amino acid exchanger Slc7a5/Slc3a2 (Nicklin et al. 2009). However, other studies have demonstrated that glutamine can either inhibit (Nakajo et al. 2005; Deldicque et al. 2008) or stimulate (Nicklin et al. 2009; Chiu et al. 2012; Willems et al. 2013) mTORC1 activity, although the mechanism(s) through which this is accomplished are not yet clear. Although these and other studies that have examined the influence of glutamine on mTORC1 activity using cells cultured in vitro, they were typically carried out using serum- and/or amino acid-free conditions to induce a state of starvation. Furthermore, the ability of glutamine to alter mTORC1 activity in the brain under physiological conditions has not yet been determined.

In the present study, we examined if glutamine administered directly into the rat hippocampus can alter mTORC1 activity. We observed that intrahippocampal glutamine injection inhibited mTORC1 activity as indicated by decreased phosphorylation of the downstream target ribosomal protein S6. Furthermore, post-training, intrahippocampal infusion of glutamine impaired long-term spatial memory tested using the Morris water maze task. Memory impairment was not observed when glutamine and leucine were coadministered. These results suggest that changes in glutamine levels may influence memory via modulation of mTORC1 signaling.

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Results

Glutamine inhibits mTORC1 activity in vivo

Previous studies have reported that glutamine can inhibit mTORC1 activity in vitro using cultured cells deprived of serum and amino acids, a nonphysiological condition (Nakajo et al. 2005; Deldicque et al. 2008). We questioned if glutamine infused into the rat hippocampus can have similar effect in vivo, as assessed by phosphorylation of the known downstream targets ribosomal S6 kinase (S6K) and ribosomal protein S6 (S6). Guided by doses determined in our in vitro experiments using nondeprived cells (Rozas et al. 2015), rats were infused with 1.3 μL of a 194 mM solution of glutamine (37 μg glutamine) into one dorsal hippocampus while an equal volume of saline was simultaneously administered to the contralateral side of the same animal. Assuming uniform distribution and a rat dorsal hippocampus volume of 100 μL, this would result in a concentration of ∼2.5 mM, although glutamine levels are likely to be higher proximal to the site of infusion. Thirty minutes after completion of the glutamine infusion, rats were killed and their hippocampi isolated and homogenized for Western blot analysis. The representative Western blots and summary data in Figure 1A show that glutamine infusion significantly decreased the phosphorylation, but not the total level, of S6 ($P = 0.049$). We also observed decreased phosphorylation of S6K at this time point, although it did not reach statistical significance (Fig. 1B; $P = 0.16$). The phosphorylation of known upstream regulators of mTORC1 signaling: Tsc2, AMP activated protein kinase (AMPK), extracellular signal-regulated kinase (ERK), and Akt, were not significantly altered 30 min after glutamine administration, although pAMPK and pAkt were slightly elevated (Fig. 1C).

Glutamine can be converted to glutamate and aspartate, as well as other metabolites, via the glutaminolysis pathway. Since glutamate and aspartate are neurotransmitters that can affect protein phosphorylation by activating their respective receptors, we also measured hippocampal glutamate and aspartate levels following glutamine injection by high-performance liquid chromatography (HPLC). Figure 1D shows that total glutamate and aspartate levels remained unchanged 30 min after infusion, while the glutamine content in hippocampal tissue extracts was increased 2.5-fold ($P = 0.008$). As extracellular glutamine levels in the rat brain have been reported to be ∼0.4 mM (Kanamori and Ross 2004), this 2.5-fold increase suggests that glutamine levels in the hippocampus 30 min post-infusion were ∼1.0 mM, assuming equal distribution.

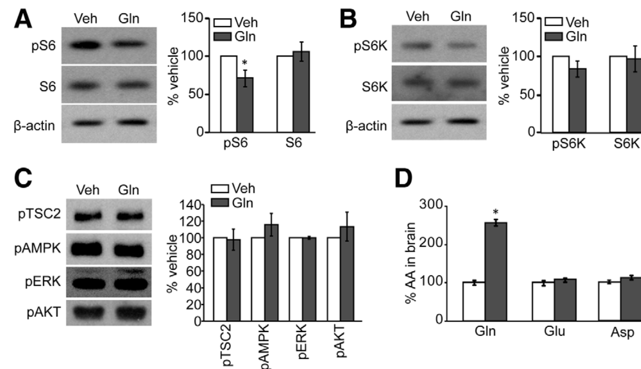

Figure 1. Intrahippocampal administration of glutamine decreases mTORC1 activity. Intrahippocampal infusions of glutamine (37 μg/hippocampus, $n = 6$) or an equal volume of vehicle (saline) were administered to animals, and samples collected 30 min later. (*A*) Glutamine administration significantly decreased the phosphorylation, but not the total levels, of S6 (two-tailed, paired Student's *t*-test; $P = 0.049$). (*B*) The phosphorylation levels of S6K were also decreased, although it did not reach significance (two-tailed, unpaired Student's *t*-test; $P = 0.16$). (*C*) Glutamine administration did not significantly affect the phosphorylation levels of Tsc2, AMPK, ERK, or Akt assessed at 30 min post-infusion. (*D*) Intrahippocampal infusions of glutamine (52 μg/hippocampus, $n = 3$) or vehicle (saline) were administered and brain extracts collected 30 min later to be analyzed by HPLC. Glutamine administration significantly increased the levels of glutamine (two-tailed, unpaired Student's *t*-test; $P = 0.008$), but not glutamate or aspartate. Sample loading was corrected by normalization to the β-actin signal. Summary data are presented as the mean ± SEM.

Glutamine does not affect hippocampal synaptic function

Although we found that total glutamate levels were not altered in the hippocampus after glutamine injection, it is possible that the additional glutamine could have influenced synaptic glutamate content, or local glutamine-to-glutamate cycling. Therefore, we recorded postsynaptic responses using horizontal brain slices to test if elevating extracellular glutamine might influence hippocampal synaptic activity. We obtained whole-cell recordings from CA1 pyramidal neurons and activated Schaffer collateral synapses by placing stimulating electrodes into the stratum radiatum at lateral distances of 200–300 μm from the recorded cell. Figure 2A shows representative excitatory postsynaptic currents (EPSCs; average of 30 trials) recorded from a single neuron during paired-pulse stimulation of Schaffer collaterals prior to (black line), and during (red line) bath application of 2.5 mM glutamine. We also measured spontaneous EPSC frequency prior to and during glutamine application (Fig. 2B). Group summary data ($n = 5$ neurons, Fig. 2C) showed that application of 2.5 mM glutamine did not lead to significant changes in evoked EPSC amplitude or paired-pulse facilitation ($EPSC_2/EPSC_1$ ratio). Furthermore, we detected no significant change in the frequency of spontaneous EPSC events following glutamine application. These data indicate that 2.5 mM glutamine does not substantially affect evoked excitatory synaptic transmission or short-term synaptic plasticity (paired-pulse facilitation).

Since glutamine can also be converted into γ-amino butyric acid (GABA) to influence inhibitory signaling (Wang et al. 2007), we also examined the effect of increased glutamine on inhibitory postsynaptic currents (IPSCs). Evoked disynaptic $GABA_A$-mediated IPSCs were recorded in the absence of glutamate receptor antagonists by holding postsynaptic CA1 neurons at 0 mV and stimulating Schaffer collaterals. Figure 3A shows that evoked IPSCs recorded prior to (black) and after (red) bath application of 2.5 mM glutamine were equivalent. Likewise, the frequency of spontaneous IPSCs did not significantly change following bath application of glutamine (Fig. 3B,C). Taken together, these results indicate that 2.5 mM glutamine does not substantially influence either excitatory (Fig. 2) or inhibitory (Fig. 3) hippocampal synaptic transmission.

Post-training intrahippocampal glutamine infusion impairs long-term spatial memory

Previous genetic and pharmacological studies have shown that mTORC1 signaling is critical for hippocampus-dependent long-term memory formation (Kelleher et al. 2004; Dash et al. 2006; Gong et al. 2006; Swiech et al. 2008; Costa-Mattioli et al. 2009). Since acute glutamine infusion resulted in decreased mTORC1 activity (Fig. 1), we tested if intrahippocampal infusion of glutamine might also impair long-term memory for spatial navigation. We utilized a post-training infusion paradigm to evaluate the consequences of glutamine on long-term memory in order to minimize

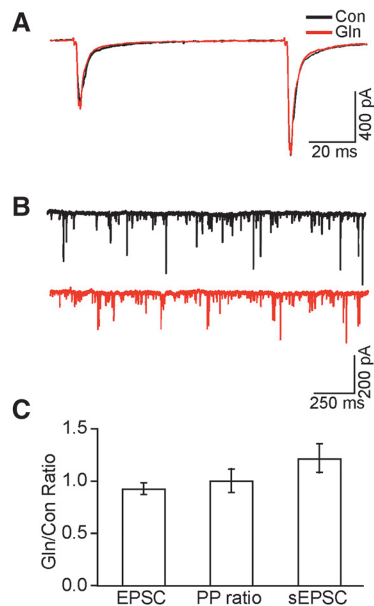

Figure 2. Glutamine bath application does not lead to significant changes in glutamatergic synaptic signaling. CA1 pyramidal neurons in horizontal hippocampal slice preparations were recorded at −65 mV in voltage-clamp mode using a cesium-based internal solution. (*A*) Representative experiment showing excitatory postsynaptic currents (EPSCs) evoked by paired pulses (10 Hz) applied to Schaffer collateral axons, prior to (black trace) and during bath application of L-glutamine (2.5 mM; red trace). Traces shown are the averages of 30 trials. (*B*) Representative trials showing spontaneous EPSCs recorded in the same CA1 pyramidal cell as in *A*, prior to (black trace) and during (red trace) application of L-glutamine (2.5 mM). (*C*) Summary data (mean ± SEM; $n = 5$ neurons) quantifing the evoked EPSC amplitude, paired-pulse ratio (EPSC2/EPSC1), and frequency of spontaneous EPSCs (sEPSCs) recorded in the presence of 2.5 mM glutamine, normalized to their respective values recorded in the control solution.

learning and state-dependent effects. Animals were trained in a one-session hidden platform version of the Morris water maze as described previously (Guzowski and McGaugh 1997; Blum and Dash 2004). Immediately after reaching criterion (average of three consecutive trials <10 sec), animals were infused bilaterally with 37 µg/hippocampus of glutamine ($n = 7$) or an equal volume of vehicle ($n = 8$). When tested for long-term memory in a retention test 48 h after training, both groups found the platform with similar latencies (Fig. 4A). However, analysis of the swimming paths revealed that the glutamine-treated group crossed the original platform location significantly fewer times (glutamine 0.74 ± 0.18 crossings; vehicle 1.87 ± 0.44 crossings, $P = 0.038$), suggesting impaired spatial localization (Fig. 4B). Consistent with this, a significant interaction of group by location was detected in the time spent in concentric circles of increasing diameter centered on the platform location ($F_{(3,36)} = 5.015$, $P = 0.005$). Post hoc analysis revealed that this interaction was due to the vehicle-infused animals spending more time in the immediate vicinity of the platform, whereas glutamine-infused animals searched in a wider area farther from the platform (indicated by *, Fig. 4D). This is visually shown by traces of the swimming paths taken by representative glutamine-treated and vehicle-treated animals during the probe test (Fig. 4C). These differences were not attributable to changes in swimming speed (Fig. 4E) or visual/motivational impairments (Fig. 4F). The memory impairments arising from glutamine infusion were dose-related. When rats were infused with 52 µg/hippocampus of glutamine, the latency to find the hidden platform during a probe trial was significantly longer than that recorded for vehicle-infused controls (Fig. 5A; glutamine 56.1 ± 2.9 sec; vehicle 34.6 ± 8.7 sec, $P = 0.04$), an effect that was not observed at the lower dose of glutamine. Similar to what was observed with the lower dose, the number of platform crossings was significantly reduced in the 52 µg/hippocampus glutamine-treated group (Fig. 5B; vehicle 0.89 ± 0.20 crossings; glutamine 0.25 ± 0.16 crossings, $P = 0.03$), and was reflected by differences in search patterns (Fig. 5C). Furthermore, the dwell time in the immediate vicinity of the hidden platform was significantly reduced in glutamine-infused animals (Fig. 5D $F_{(1,15)} = 10.052$, $P = 0.006$). No difference between the treatment groups was observed in swimming speed or latency to a visible platform (Fig. 5E,F).

Memory impairing effect of glutamine can be overcome by coadministration of leucine

It has been previously reported that leucine can enhance mTORC1 (Deldicque et al. 2008; Dodd and Tee 2012; Suryawan et al. 2012; Jewell et al. 2013; Xu et al. 2013). To examine if the mTORC1 inhibitory effect of glutamine can be neutralized by coadministration of leucine in vivo, independent groups of rats were infused with leucine (27 µg) targeted to one hippocampus while

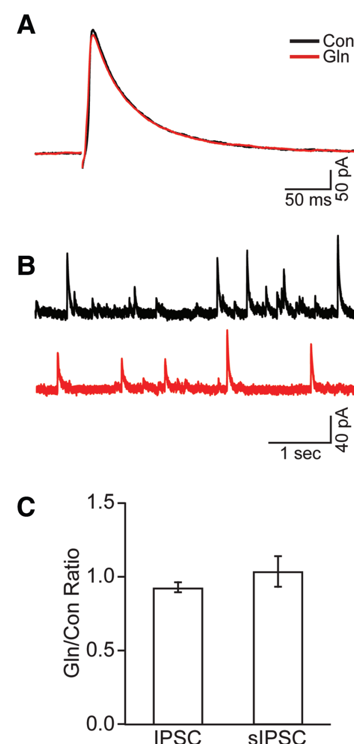

Figure 3. Glutamine application does not lead to significant changes in GABAergic synaptic signaling. CA1 pyramidal neurons were held at 0 mV using a cesium-based internal recording solution. (*A*) A representative experiment showing disynaptic inhibitory postsynaptic currents (IPSCs) evoked by Schaffer collateral stimulation, prior to (black trace) and during application of L-glutamine (2.5 mM; red trace). Traces shown are the averages of 30 trials. (*B*) Representative trials showing spontaneous IPSCs recorded in the same CA1 pyramidal cell as *A*, prior to (black trace) and during (red trace) application of L-glutamine (2.5 mM). (*C*) Summary data (mean ± SEM; $n = 5$ neurons) quantifing the evoked IPSC amplitude and frequency of spontaneous IPSCs (sIPSCs) in the presence of 2.5 mM glutamine, normalized to their respective values recorded in the control solution.

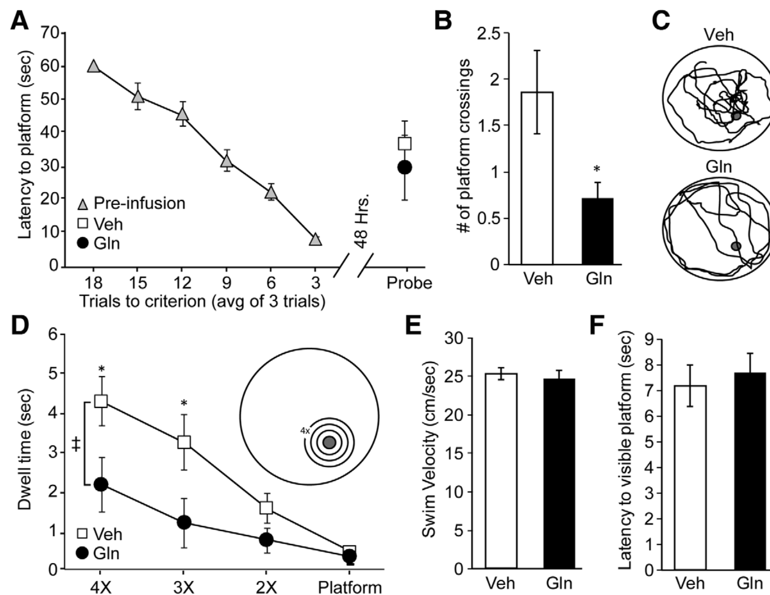

Figure 4. Post-training intrahippocampal administration of glutamine (37 μg/hippocampus) impairs long-term spatial memory. (*A*) Rats were trained in a 1-d abbreviated Morris water maze protocol and infused with either vehicle (saline; $n = 8$) or 37 μg/hippocampus of glutamine ($n = 7$) immediately after the last training trial. A probe trial was given 48 h later. (*B*) Glutamine (Gln)-infused mice had significantly fewer platform crossings during the probe trial (two-tailed, unpaired Student's *t*-test; $P < 0.038$). (*C*) Representative probe trial traces of a vehicle and a glutamine-infused animal showing their swim paths. (*D*) Dwell time in counter areas of decreasing diameters (4×, 3×, and 2× platform radius) during the probe trial was also significantly decreased in Gln-infused mice (two-way repeated-measures of ANOVA with treatment and ring number as between-subject factors: $F_{(3,36)} = 5.015$, $P = 0.005$). (*E*) There was no difference in swimming speed during the probe trial. (*F*) There was no difference in the latency to a visual platform performed after the probe trial. Data are presented as the mean ± SEM. (*) $P < 0.05$, (‡) $P < 0.01$.

an equal volume of vehicle was simultaneously infused into the contralateral hippocampus. Figure 6A,B show that acute leucine administration significantly increased the phosphorylation of the downstream targets of mTORC1, S6K ($P = 0.03$) and S6 ($P = 0.01$). However, when leucine (27 μg) and glutamine (52 μg) were coadministered, no significant differences in the phosphorylation of S6K or S6 were observed compared with vehicle-infused controls (Fig. 6C,D).

As coadministration of leucine and glutamine appeared to have offsetting effects on mTORC1 activity in the brain in vivo, we next tested the consequences of infusing the combination on long-term memory. Rats were infused bilaterally with 27 μg leucine + 52 μg glutamine per hippocampus ($n = 9$), or an equal volume of vehicle ($n = 9$), post-training. When tested for long-term memory in a retention test 48 h later, the treatment groups showed no significant difference in latency to the original platform location (Fig. 7A; Leu + Gln 29.3 ± 10.4 sec; vehicle 34.6 ± 8.7 sec, $P = 0.67$) or the number of platform crossings (Fig. 7B; Leu + Gln 1.44 ± 0.56 crossings; vehicle 0.89 ± 0.20 crossings, $P = 0.36$). Further analysis of the probe trial revealed that both groups of animals used similar search patterns that resulted in comparable times spent in the area surrounding the platform location ($F_{(3,48)} = 0.663$, $P = 0.579$; Fig. 7C).

After the completion of the behavioral studies, cannula placement was examined in animals used for the behavioral experiments. A representative image of a cresyl violet-stained section (Fig. 7D) shows the termination of an injection needle track within the hippocampus. A summary of the approximate infusion cannula termination locations for representative animals used in the behavioral studies are depicted in Figure 7E.

Discussion

Studies in many different model systems have shown that long-term memory formation requires protein synthesis (Barondes and Cohen 1968; Davis and Squire 1984; Montarolo et al. 1986; Cracco et al. 2005; Sutton and Schuman 2006; Costa-Mattioli et al. 2009; Buffington et al. 2014). mTORC1 is a critical cellular energy and nutrient status sensor that integrates signals from multiple pathways to regulate protein, lipid, and nucleotide synthesis, and it has also been identified as an important modulator of learning and memory (Dash et al. 2006; Gkogkas et al. 2010; Hoeffer and Klann 2010; Graber et al. 2013; Santini et al. 2014). Previous studies using cultured cells deprived of serum/amino acids have shown that glutamine can either increase or decrease mTORC1 activity (Deldicque et al. 2008; Nicklin et al. 2009; Jewell and Guan 2013; Kim et al. 2013). However, as these in vitro studies were carried out under serum- and/or amino acid-deprived culture conditions, the physiological relevance of these findings remains uncertain. We reasoned that changes in glutamine levels may affect mTORC1 signaling in the brain to modulate learning and memory in vivo. By performing direct glutamine infusions into the rat hippocampus, the present study made three key findings: (1) high extracellular glutamine inhibits mTORC1 activity in the hippocampus, (2) post-training, intrahippocampal infusion of glutamine impaired long-term spatial memory in a dose-dependent fashion, and (3) memory impairment after glutamine administration was overcome by simultaneous infusion of leucine, a known mTORC1 activator. These results suggest that glutamine can modulate memory formation via inhibition of mTORC1 activity, a finding that may have implications for the treatment of disorders associated with elevated brain mTORC1 activity.

The experimental results presented in this manuscript show that when glutamine was infused into the hippocampus of freely moving, awake animals, it reduced mTORC1 activity, as indicated by its effect on the phosphorylation of downstream targets. This reduction in mTORC1 activity was associated with the impairment of long-term spatial memory. There are several possible mechanisms whereby supplementing glutamine could affect long-term memory. In addition to influencing mTORC1 activity, glutamine is a precursor used in the synthesis of the neurotransmitters glutamate, aspartate, and GABA (Wang et al. 2007; Albrecht et al. 2010). It has been previously shown that glutamine can play an important role in regulating synaptic signaling, having impacts on both excitatory (Albrecht et al. 2010; Uwechue et al. 2012; Tani et al. 2014) and inhibitory (Liang et al. 2006; Wang et al. 2007) synaptic signaling through its effects on glutamate and GABA synthesis and release. As shown in Figure 1, we did not observe any significant change in the overall hippocampal levels of glutamate or aspartate 30 min post-glutamine infusion, although changes in the synaptic levels of these neurotransmitters could not be excluded. To examine possible effects of glutamine on excitatory synaptic transmission, we measured CA1

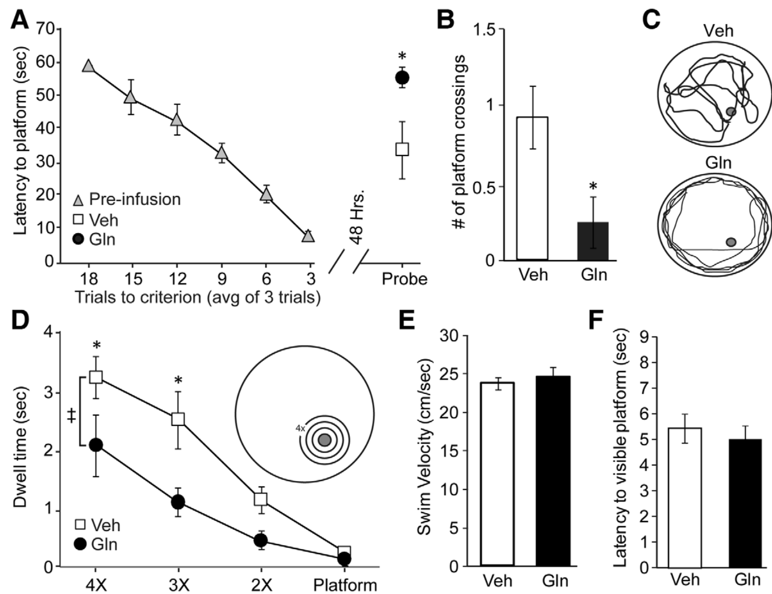

Figure 5. Post-training intrahippocampal administration of glutamine (52 μg/hippocampus) impairs long-term spatial memory. Rats were infused with either vehicle solution ($n = 9$) or 52 μg/hippocampus of glutamine ($n = 8$) immediately after the last training trial. A probe trial was given 48 h later. Glutamine infused animals (*A*) required significantly longer search-time to find the hidden platform (two-tailed, unpaired Student's *t*-test; $P = 0.04$) and (*B*) crossed the platform fewer times than did vehicle-infused controls (two-tailed, unpaired Student's *t*-test; $P = 0.03$). (*C*) Representative probe trial traces of a vehicle and a glutamine-infused animal during the probe trial showing the paths taken. (*D*) Dwell time in counter areas of decreasing diameters (4×, 3×, and 2× platform radius) during the probe trial was also significantly decreased in Gln-infused mice (two-way repeated-measures of ANOVA with treatment and ring number as between-subject factors: group main effect $F_{(1,15)} = 10.052$, $P = 0.006$). (*E*) There was no difference in swimming speed during the probe trial. (*F*) There was no difference in latency to a visual platform performed after the probe trial. Data are presented as the mean ± SEM, (*) $P < 0.05$, (‡) $P < 0.01$.

pyramidal neuron EPSCs in hippocampal slices in response of Schaffer collateral stimulation (Fig. 2). We observed that sustained exposure of hippocampal slices to 2.5 mM glutamine did not significantly alter evoked EPSC amplitude, paired-pulse ratio, or spontaneous EPSC frequency. These results show that a relatively high level of glutamine (estimated to achieve >6 times the extracellular glutamine levels reported by Kanamori and colleagues) did not significantly change either spontaneous or evoked glutamate release onto CA1 dendrites, or short-term plasticity (Kanamori and Ross 2004). Furthermore, it has previously been demonstrated that NMDA and metabotropic agonists increase mTOR activity (Gong et al. 2006), an effect opposite to that reported here. Given these findings, local conversion of glutamine to glutamate in presynaptic terminals and its subsequent release does not appear to be a likely mechanism underlying the behavioral effects we observed.

Since glutamine can also serve as an upstream precursor for GABA production, it is possible that the increase in extracellular glutamine may have led to increased GABA synthesis and release, resulting in reduced neuronal activity and decreased mTORC1 activity. Results from our intracellular recordings of CA1 neurons (Fig. 3) showed that sustained exposure of hippocampal slices to 2.5 mM glutamine did not significantly alter either evoked or spontaneous IPSCs. Furthermore, a recent study showed that in vivo activation of $GABA_B$ receptors in the presence of NMDA antagonist increased mTORC1 activity (Workman et al. 2013), which is opposite to the decrease in mTORC1 activity we observed after intrahippocampal glutamine infusion (Fig. 1). Taken together, these findings suggest that altered synaptic transmission may not be the mechanism by which the infused glutamine impaired long-term memory.

Furthermore, the behavioral consequences of administering glutamine, versus coadministration of glutamine + leucine, in rats are not consistent with altered GABA or glutamate synthesis and release. Leucine plays an important metabolic role in the brain, helping to maintain overall nitrogen, amino acid, and neurotransmitter homeostasis in the brain. Leucine is an important source of the nitrogen used to generate glutamate in the brain, directly contributing between 25% and 50% of glutamate's amine groups (Yudkoff 1997; Kanamori et al. 1998), which can then indirectly enter the GABA synthesis pathway (Sakai et al. 2004). Thus, increasing leucine would be expected to facilitate the production of glutamate and/or GABA. If increased glutamate and/ or GABA signaling was a major mechanism mediating the

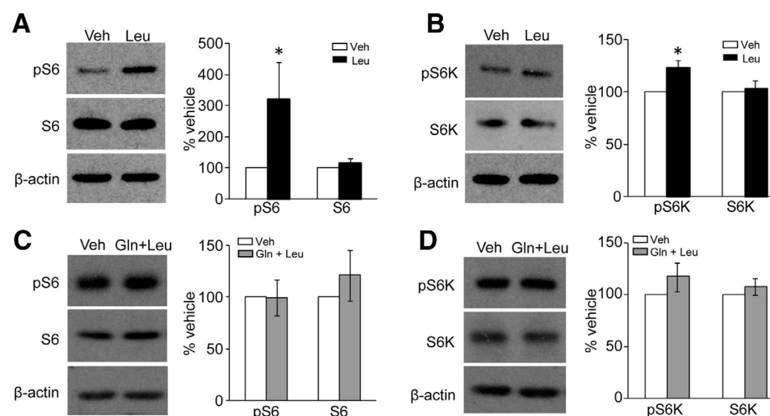

Figure 6. Intrahippocampal coadministration of leucine and glutamine resulted in no net change in mTORC1 activity. (*A,B*) Intrahippocampal infusions of leucine (27 μg/hippocampus, $n = 5$) or vehicle (saline) were administered and samples collected 30 min later. Leucine administration significantly increased the phosphorylation of mTORC1 targets (*A*) S6 (two-tailed, paired Student's *t*-test; $P = 0.01$) and (*B*) S6K (two-tailed, paired Student's *t*-test; $P = 0.03$). (*C,D*) Intrahippocampal coinfusion of leucine and glutamine (27 μg/hippocampus, 52 μg/hippocampus, respectively, $n = 5$) or vehicle (saline) were administered and samples collected 30 min later. There were no significant effects on the levels of *C* phosphorylated S6K or total S6K. (*D*) There were no significant effects of glutamine and leucine coadministration on the phosphorylation or total levels of S6. Sample loading was normalized to the β-actin signal. Summary data are presented as the mean ± SEM, (*) $P < 0.05$.

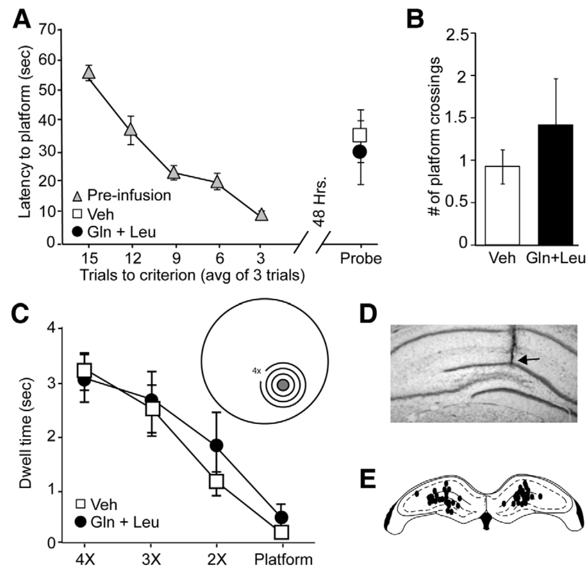

Figure 7. Post-training intrahippocampal coadministration of leucine and glutamine resulted in no net effect on long-term spatial memory formation. (*A*) Rats were trained in a 1-d Morris water maze protocol and infused with either 27 μg/hippocampus of leucine + 52 μg/hippocampus of glutamine ($n = 9$) or vehicle solution ($n = 9$) immediately after the last training trial. A probe trial was given 48 h after training detected no difference in either the (*B*) number of crossings or the (*C*) dwell time in counter areas of decreasing diameters (4×, 3×, and 2× platform radius). (*D*) Representative image of a cresyl violet-stained section showing an infusion needle track (arrow) that terminates within the hippocampus. (*E*) Summary representation of cannula placements for the animals used in this experiment. Data are presented as the mean ± SEM.

memory impairments we observed, one would have expected animals coadministered leucine + glutamine to experience memory impairments similar to animals receiving glutamine. However, the inclusion of leucine, a well-documented activator of mTORC1, in the infusion mix blocked the spatial memory impairments observed in glutamine-only infused animals. As the glutamine doses used in this report were relatively high, particularly locally at the site of infusion, another possible mechanism for impaired memory after glutamine infusion is increased osmolarity resulting in nonspecific neuronal dysfunction at or near the site of infusion. However, animals showed no memory impairments following the coadministration of glutamine + leucine (Fig. 7), suggesting that the memory impairments observed following the infusion of glutamine alone are unlikely to be due to increased osmolarity. While we cannot rule out contributions from other mechanisms, our results support the conclusion that high glutamine negatively impacts long-term memory at least in part by reducing mTORC1 activity.

Although it has been demonstrated in many different cell types that mTORC1 can be activated when serum-starved cells are exposed to high levels of amino acids, the mechanism by which mTORC1 activity is regulated by amino acids is only just beginning to be understood (Wang and Proud 2011). Lysosomal localization of mTORC1 under conditions of high amino acid availability appears to be critical for activation (Bar-Peled and Sabatini 2014). This model proposes that high amino acid levels in lysosomes change the interaction of v-ATPase (an enzyme that acidifies lysosomes, though this function does not appear to be required for amino acid level sensing) with a guanine exchange factor (GEF) referred to as Ragulator. This interaction activates Rag heterodimers, which subsequently recruit mTORC1 to the lysosomal membrane where it can then be activated by GTP-bound Rheb (Ras homolog enriched in brain). In contrast, when amino acid levels are limiting, GATOR1 (a Rag GAP) switches active Rag heterodimers to their inactive state which results in mTORC1 being released from the lysosomal surface and away from GTP-bound Rheb. A recent study indicates that Rag GTPases also recruit TSC2 to the lysosomal surface where it can inhibit Rheb, suggesting that amino acids can indirectly alter intracellular localization of the TSC regulatory complex (Demetriades et al. 2014). As the TSC complex inhibits Rheb via its GAP (GTPase-activating protein) activity, simultaneous stimulation with growth factors seems to be required for amino acid stimulation of mTORC1 activity (Menon et al. 2014). Thus, inactivation of both Rag GTPases and Rheb appears to be required for release of mTORC1 from the lysosome.

It is not known if high levels of glutamine can cause the release of mTORC1 from the surface of lysosomes, leading to the inhibition of mTORC1 activity that we observed. However, a study by Averous et al. (2014), reports that mTORC1 activity does not correlate with its lysosomal localization in serum-fed cells. Other mechanisms for glutamine regulation of mTORC1 have been proposed, including increasing the influx of leucine via the heterodimeric cotransporter SLC7A5–SLC3A2 (Nicklin et al. 2009), increasing the levels of α-ketoglutarate (Yao et al. 2012), and decreasing Akt activity (Deldicque et al. 2008). The results of our biochemical evaluation of several pathways known to regulate mTOR did not reveal a clear candidate signaling pathway that was responsible for the observed effects. Further research will be required to identify the signaling pathway(s) responsible for the decrease in brain mTORC1 activity that we observed.

Materials and Methods

Materials

Male Long-Evans rats (250–280 g) were purchased from Charles River Laboratories (Wilmington, MA). C57/BL6 mice were obtained from breeding stocks maintained at the University of Texas Health Science Center at Houston. All protocols involving the use of animals were in compliance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" and were approved by the UTHealth Institutional Animal Care and Use Committee. L-glutamine and L-leucine were purchased from Sigma-Aldrich. Antibodies for phosphorylated (Serine 240/244) and total S6, phosphorylated (Threonine 389) and total S6 Kinase, phosphorylated (Serine 473) and total Akt, phosphorylated (Threonine 1462) and total TSC2, phosphorylated (Threonine 172) and total pAMPK, phosphorylated (Threonine 202/Tyrosine 204), and total ERK1/2, were purchased from Cell Signaling Technology.

Intrahippocampal infusion and drug administration

Rats were anesthetized with 5% isoflurane in a 1:1 O_2/air mixture and then maintained with 2.5% isoflurane in 1:1 O_2/air mixture via a nose cone. Bilateral guide cannulae aimed at the dorsal hippocampus (anteroposterior, −3.6 mm, lateral, ±2.0 mm from bregma; ventral, −2.0 mm from the skull surface), were implanted. Animals were allowed to recover in their home cages for 10 d. Injection cannulas extended 1.75 mm beyond the tips of the guides, yielding a total depth of 3.75 mm below the dura. L-glutamine (194 mM) and L-leucine (137 mM) were dissolved in saline before infusion. All injections (1.3–1.5 μL/hippocampus of either drug or saline) were performed in freely moving animals at a rate of 0.2 μL/min using dual syringe infusion pumps (Stoelting). After completion of the behavioral experiments, correct cannula placement was verified histologically. All rats examined for these studies had needle tracks that terminated within the targeted region of the hippocampus.

Sample preparation and Western blotting

For examining the influence of glutamine on mTORC1 activity, rats were infused with glutamine into one hippocampus and an equal volume of vehicle into the contralateral hippocampus of the same animal. At the indicated time points after infusion, animals were killed by decapitation, the hippocampi quickly dissected and hippocampal punches (2 mm in diameter) surrounding the infusion site were taken under ice-cold artificial CSF containing phosphatase inhibitors (2 mM NaF, 2 mM Na_2MoO_4, and 1 mM Na_3VO_4). Tissues were disrupted by 2 sec, 20% amplitude sonication pulses (Vibra Cell, Ultrasonic processor 130 W, 20 KHz) (Sonics, Newtown, CT) in homogenization buffer (10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.5 DTT, 0.1 μM okadaic acid, 1 mM Na_3VO_4, 1 mM PMSF and 10 μg/mL leupeptin). After an equal volume of 2× Laemmli buffer was added, the protein concentration was determined by NanoOrange Protein Quantification Assay (Invitrogen), using bovine serum albumin (BSA) as the standard. Samples were resolved on Tris-glycine SDS-PAGE gels. Proteins were then transferred to Immobilon-P membranes (Millipore) and blocked for 30 min in 5% BSA in Tris-buffered saline (TBS). Primary antibodies were incubated overnight at 4°C in TBS containing 5% BSA and 0.1% Tween-20. Membranes were then washed and incubated at room temperature with alkaline phosphatase-conjugated secondary antibodies (Vector Laboratories) for 1 h. Immunoreactivity was detected using a CDP-Star chemiluminescence system (New England Biolabs) and visualized on Kodak XAR5 film. All antibody signals were normalized against β-actin immunoreactivity. Western blot data were compiled from at least five independent animals.

Hippocampal slice preparation

Hippocampal horizontal slices (400 μm) were obtained from C57BL/6 mice, aged P15-16. Animals were anesthetized with isoflurane and decapitated. Slices were cut using a vibratome (Leica VT1200S) in an ice-cold sucrose-containing solution consisting of 212 mM sucrose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 10 mM $MgSO_4$, 26 mM $NaHCO_3$, 11 mM glucose, and 0.5 mM $CaCl_2$, saturated with 95% O_2–5% CO_2. Slices were incubated at 34°C for 50 min in saline solution containing 126 mM NaCl, 26 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 10 mM glucose, 2 mM $CaCl_2$, and 2 mM $MgCl_2$, saturated with 95% 0_2-5% CO_2. Slices were then maintained at room temperature until used for experiments.

Electrophysiological recordings

Recordings were performed at 33°C–35°C using an in-line heater (Warner Instruments) while perfusing the recording chamber with saline solution. Visualized recordings were performed under infrared-differential interference contrast visualization using an Olympus BX51WI microscope (Olympus Optical) and an IR-1000 camera (Dage MTI). Whole-cell voltage-clamp recordings from CA1 pyramidal neurons were obtained using glass pipettes of 2–4 MΩ filled with an internal solution containing 120 mM $CsMeSO_3$, 10 mM CsCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 1 mM QX-314, 11 mM EGTA, 2 mM Mg-ATP, and 0.3 mM Na-GTP, adjusted to 295 mOsm and pH 7.3. To record spontaneous and evoked EPSCs, cells were held at −65 mV. For IPSC recordings, cells were voltage-clamped at 0 mV. Extracellular stimulation of Schaffer collateral axons (10–60 μA) was carried out with pipettes pulled from theta glass (10–15 μm tip diameter, WPI Inc.), filled with saline solution, and placed at 200–300 μm lateral distance from the recorded neuron.

Morris water maze

All behavioral experiments were performed by an experimenter who was kept blind to the treatment schedule. Male rats were cannulated and allowed to recover for 10 d. Animals were trained to criterion (three consecutive trials with platform latencies of <10 sec) in the hidden platform version of the Morris water maze task as described previously (Schenk and Morris 1985; Guzowski and McGaugh 1997; Blum and Dash 2004). Each trial started by placing the animal in one of four randomly chosen locations, facing the wall of the tank. Animals were allowed to search for the platform for 60 sec. If an animal failed to find the platform, it was placed there by the experimenter. Animals were allowed to remain on the platform for a period of 30 sec before being returned to a warming cage between trials. Once criterion was reached, animals were infused bilaterally with either an amino acid solution or vehicle. At 48 h after training the animals were tested for memory 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 sec. Animals were monitored by a video camera linked to tracking software (Ethovision, Noldus). A total of 41 animals were used in the behavior experiments shown in Figures 4, 5, and 7. The animals in Figures 5 and 7 were run concurrently, and therefore used the same vehicle control groups to reduce the number of required animals.

Statistical analysis

In all experiments, data collected from the same animal with one or more factors, such as dwell time, was subjected to repeated-measures (RM) ANOVA. Data comparing only one factor between groups, such as probe trial data analysis and Western blot data, were subjected to a two-tailed Student's t test for unpaired (behavioral data) or paired variables (Western blot data). Data comparing >2 groups were subjected to one-way ANOVA. Data were considered significant at $P < 0.05$.

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Competing interest statement

The authors declare no competing financial interests.

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