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## mTORC2 controls actin polymerization required for consolidation of long-term memory

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### Abstract

A major goal of biomedical research has been the identification of molecular mechanisms that can enhance memory. Here we report a novel signaling pathway that regulates the conversion from short- to long-term memory. The mTOR complex 2 (mTORC2), which contains the key regulatory protein Rictor (Rapamycin-Insensitive Companion of mTOR), was discovered only recently, and little is known about its physiological role. We show that conditional deletion of *rictor* in the postnatal murine forebrain greatly reduces mTORC2 activity and selectively impairs both long-term memory (LTM) and the late (but not the early) phase of hippocampal long-term potentiation (LTP). Actin polymerization is reduced in the hippocampus of mTORC2-deficient mice and its restoration rescues both L-LTP and LTM. More importantly, a compound that selectively promotes mTORC2 activity converts early-LTP into late-LTP and enhances LTM. These findings indicate that mTORC2 could be a novel therapeutic target for the treatment of cognitive dysfunction.

### Keywords

mTOR complexes; actin assembly; spatial memory; fear memory; cognitive enhancer

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## Introduction

How memories are stored in the brain is a question that has intrigued mankind over many generations. While neuroscientists have already made great strides in identifying key brain regions and relevant neuronal circuits, many questions regarding the specialized molecular and neuronal mechanisms underlying memory formation remain unanswered. Post-translational modifications of synaptic proteins can explain transient changes in synaptic efficacy, such as short-term memory (STM) and the early phase of LTP (E-LTP, lasting 1-3 hours); but new protein synthesis is required for long-lasting ones, such as LTM and the late phase of LTP (L-LTP, lasting several hours)<sup>1-7</sup>. Changes in actin dynamics that mediate structural changes at synapses are also necessary for L-LTP and for LTM storage<sup>8-10</sup>. However, relatively little is known about the molecular mechanisms that underlie these processes.

The evolutionarily conserved mammalian target of rapamycin (mTOR) forms two complexes<sup>11-13</sup>. The first, mTORC1, consists of mTOR, Raptor and mLST8 (GβL), is sensitive to rapamycin, and is thought to regulate mRNA translation<sup>3</sup>. Although significant progress has been made in the identification of the mTORC1 pathway and understanding its function in cells and *in vivo*, much less is known about the second complex, mTORC2. mTORC2 is largely insensitive to rapamycin and contains the core components mTOR, mSIN1, mLST8 and Rictor (Rapamycin-Insensitive Companion of mTOR). Rictor is a defining component of mTORC2 and its interaction with mSIN1 appears to be required for mTORC2 stability and function<sup>14</sup>. Rictor is associated to membranes and is thought to regulate the actin cytoskeleton, but the precise molecular mechanism behind this effect remains unclear<sup>11,12,16</sup>. In addition, while little is known about mTORC2's up-stream regulation, we are beginning to understand its downstream regulation and effectors: mTORC2 phosphorylates AGC kinases at conserved motifs, including Akt at the hydrophobic motif (HM) site (Ser-473), the best characterized read out of mTORC2 activity<sup>11,12</sup>.

The key component of mTORC2, Rictor, is important for embryonic development as mice lacking *rictor* die in early embryogenesis<sup>15,16</sup>. Rictor is highly expressed in the brain, notably in neurons<sup>16</sup>, and it seems to play a crucial role in various aspects of brain development and function. For example, genetic deletion of *rictor* in developing neurons disrupts normal brain development, resulting in smaller brains and neurons, as well as increased levels of monoamine transmitters and manifestations of cerebral malfunction suggestive of schizophrenia and anxiety-like behaviors<sup>17,18</sup>.

Given that i) actin polymerization is critically required for memory consolidation<sup>8-10</sup>, ii) mTORC2 appears to regulate the actin cytoskeleton<sup>11,12,19</sup>, and iii) mTORC2's activity is altered in conditions associated with memory loss, such as aging and several cognitive disorders, including Huntington's disease, Parkinsonism, Alzheimer-type dementia and Autism Spectrum Disorders<sup>20-25</sup>, we decided to investigate its potential role in memory formation, specifically in sustained changes in synaptic efficacy (LTP) in hippocampal slices, and in behavioral tests of memory. Our results show that through regulation of actin polymerization, mTORC2 is an essential component of memory consolidation. Briefly, we

report here a selective impairment in L-LTP and LTM in mice and flies deficient in TORC2 signaling. Moreover, we have identified the up-stream synaptic events which activate mTORC2 in the brain and unraveled the detail downstream molecular mechanism by which mTORC2 regulates L-LTP and LTM, namely regulation of actin polymerization. Finally, a small molecule activator of mTORC2 and actin polymerization facilitates both L-LTP and LTM, further demonstrating that mTORC2 is a new type of molecular switch that controls the consolidation of a short-term memory process into a long-term one.

## Results

### Characterization of *ric* forebrain-specific knockout (*ric* fb-KO) mice

Pharmacological inhibitors of mTORC2 are not available, and mice lacking *ric* in the developing brain show abnormal brain development. To circumvent this problem, we conditionally deleted *ric* in the postnatal forebrain by crossing “floxed” *ric* mice<sup>16</sup> with the  $\alpha$  subunit of calcium/calmodulin-dependent protein kinase II ( $\alpha$ CaMKII)-Cre mice<sup>26</sup>, generating *ric* forebrain-specific knockout mice (*ric* fb-KO mice; see Methods and Supplementary Fig. 10). Because the  $\alpha$ CaMKII promoter is inactive before birth<sup>27</sup>, this manipulation diminishes possible developmental defects caused by the loss of *ric*.

*Ric* fb-KO mice are viable and develop normally. They show neither gross brain abnormalities nor changes in the expression of several synaptic markers (Supplementary Fig. 1). mTORC2-mediated phosphorylation of Akt at Ser473 (an established readout of mTORC2 activity<sup>11,12</sup>) was greatly reduced in CA1 and amygdala (Fig. 1a-b), but was normal in the midbrain (Fig. 1c) of *ric* fb-KO mice. By contrast, in mTORC2-deficient mice, mTORC1-mediated phosphorylation of S6K1 at Thr389 (a well-established readout of mTORC1 activity<sup>28</sup>) remained unchanged in CA1, amygdala or midbrain (Fig. 1a-c). Thus, conditional deletion of *ric* selectively reduces mTORC2 activity in forebrain neurons.

### Deficient mTORC2 activity prevents L-LTP but not E-LTP in *ric* fb-KO mice

To investigate the role of mTORC2 in synaptic function, we first showed that mTORC2 is activated in CA1 by either glutamate [*via* NMDA receptor (NMDAR)] or neurotrophins (Supplementary Fig. 2). Next, to determine whether short-term or long-term changes in synaptic potency alter mTORC2 activity, we compared the effects of one train of tetanic stimulation (100 Hz for 1s), which usually induces only short-lasting E-LTP, with that of four such trains (which typically induce a long-lasting L-LTP)<sup>1</sup>. Only an L-LTP-inducing stimulation consistently activated mTORC2 in CA1 neurons of control (Fig. 1d-f) but not *ric* fb-KO mice (Fig. 1g-h). Hence, mTORC2 is engaged selectively in long-lasting synaptic changes in synaptic strength.

We then examined whether mTORC2 deficiency affects either E-LTP or L-LTP. Whereas a single train of tetanic stimulation generated a similar E-LTP in slices from *ric* fb-KO and control littermates (Fig. 1i), four trains elicited a normal L-LTP in control littermates slices, but not in *ric* fb-KO slices (Fig. 1j). Several tests showed that the impaired L-LTP in mTORC2-deficient slices cannot be attributed to defective basal synaptic transmission

(Supplementary Fig. 3). Thus, reducing mTORC2 activity prevents the conversion of E-LTP into L-LTP.

### Deficient TORC2 activity selectively impairs long-term memory both in mice and flies

Since L-LTP-inducing stimulation increases mTORC2 activity, we then investigated whether mTORC2 is activated as a result of behavioral learning. Contextual fear conditioning, induced by pairing a context (conditioned stimulus; CS) with a foot shock (unconditioned stimulus; US), resulted in sharp temporarily increase in mTORC2 activity and phosphorylation of the p21-activated kinase PAK (a key regulator of actin cytoskeleton dynamics<sup>29,30</sup>) 15 min after training (Fig. 2a-b). In contrast, the shock-alone (US) and the context-alone (CS) failed to increase mTORC2 activity (Fig. 2c). Thus, hippocampal mTORC2 is selectively activated by behavioral learning (CS+US).

We next studied memory storage in two forms of Pavlovian conditioning, contextual and auditory fear conditioning. Contextual fear conditioning involves both the hippocampus and amygdala whereas auditory fear conditioning, in which the foot shock (US) is paired with a tone (CS), requires only the amygdala<sup>31</sup>. When mice were subsequently exposed to the same CS, fear responses (“freezing”) were taken as an index of the strength of the CS-US association. *Rictor* fb-KO mice and control littermates showed similar “freezing” behavior before training (naïve) and 2 hr after training, when their STM was measured (Fig. 2d-e). However, when examined 24 hr after training, both contextual and auditory LTM were significantly impaired in mTORC2-deficient mice (Fig. 2d-e). The less pronounced change in auditory fear LTM vs. contextual fear LTM in *rictor* fb-KO mice may be explained by the smaller reduction in mTORC2 activity in the amygdala (compare Fig. 1a vs. Fig. 1b). Spatial LTM was also deficient in *rictor* fb-KO mice when tested in the Morris water maze, where animals use visual cues to find a hidden platform in a circular pool<sup>32</sup>. Compared to controls, *rictor* fb-KO mice took significantly longer to find the hidden platform (Fig. 2f), and in the probe test, performed on day 7 in the absence of the platform, they failed to remember the platform location (target quadrant; Fig. 2g). The impaired spatial LTM was probably not caused by deficient visual or motor function since control and *rictor* fb-KO mice performed similarly when the platform was visible (Supplementary Fig. 4) and showed no significant difference in swimming speed ( $19.8 \pm 0.6$  vs.  $19.6 \pm 0.5$  cm/s for control and *rictor* fb-KO mice). Hence, mTORC2 selectively fosters long-term memory processes.

Because TORC2 is evolutionarily conserved<sup>11,12</sup>, we also wondered whether its function in memory formation is maintained across the animal phyla. To this end, we studied olfactory memory in wild-type controls (Canton-S) and *Drosophila* TORC2 (dTORC2)-deficient fruit flies. As expected, in the brain of *rictor* mutant flies (*rictor*<sup>1</sup>)<sup>33</sup> dTORC2-mediated phosphorylation of the hydrophobic motif of *Drosophila* Akt (dAkt) at Ser505 (an established readout of dTORC2 activity<sup>34</sup>) was greatly reduced (Fig. 3a). As in mammals, LTM in *Drosophila* is protein-synthesis dependent<sup>35</sup> and it is generated after “spaced” training (5 training sessions with a 15-min rest interval between each; Fig. 3b). By contrast, “massed training” (5 training sessions with no rest intervals) fails to elicit LTM but rather induces the anesthesia-resistant, protein-synthesis independent memory (ARM; Fig. 3b)<sup>35</sup>. Although responses to olfactory stimuli or electric shocks did not differ significantly

between *rictor*<sup>1</sup> and control flies (Fig. 3c-e), in *rictor*<sup>1</sup> flies LTM was blocked, whereas the short-lasting ARM was unaffected (Fig. 3f). Thus, both in fruit flies and mice TORC2 promotes LTM storage.

### Deficient actin dynamics and Rac1-GTPase-mediated signaling in CA1 neurons of *rictor* fb-KO mice

We also probed the molecular mechanism by which mTORC2 regulates L-LTP and LTM by first testing whether mTORC2 deficiency impairs actin dynamics in CA1 neurons *in vivo*. Actin exists in two forms: monomeric globular actin (G-actin) and polymerized filamentous actin (F-actin) composed of aggregated G-actin. The transition between these two forms is controlled by synaptic activity<sup>8,9</sup>. The ratio of F-actin to G-actin, which reflects the balance between actin polymerization and depolymerization, was significantly reduced in CA1 of *rictor* fb-KO mice (Fig. 4a-b). Since Rho-GTPases have been identified as key intracellular signaling molecules that regulate actin dynamics at synapses<sup>36</sup>, we measured the activity of Rho-GTPases in CA1 of mTORC2-deficient mice. Rac1 (Ras-related C3 botulinum toxin substrate 1) and Cdc42 (cell division cycle 42), two major Rho GTPases, induce actin polymerization by promoting PAK and Cofilin phosphorylation<sup>8</sup>. Rac1-GTPase activity (but not Cdc42 activity) and the phosphorylation of PAK and Cofilin were greatly diminished in CA1 neurons of *rictor* fb-KO mice (Fig. 4c-f). Moreover, we found that the Rac1-specific guanine nucleotide-exchange factor (GEF) Tiam1 links Rictor (mTORC2) to Rac1 signaling (Fig. 4g-k). Finally, dendritic spine density in CA1 pyramidal neurons was significantly reduced in *rictor* fb-KO mice (Fig. 4l). Thus, in the adult hippocampus, mTORC2 regulates actin dynamics-mediated changes in synaptic potency and architecture.

### Restoring actin polymerization rescues the impaired L-LTP and LTM caused by mTORC2 deficiency

If L-LTP is impaired in mTORC2-deficient slices because actin polymerization is abnormally low, increasing the F-actin/G-actin ratio should convert the impaired, short-lasting LTP elicited by four tetanic trains into a normal L-LTP. We therefore predicted that jasplakinolide (JPK), a compound which directly promotes actin polymerization<sup>37</sup>, should restore the normal function. Indeed, a low concentration of JPK (50 nM), raised the low F-actin/G-actin ratio and restored L-LTP in *rictor* fb-KO slices (Fig. 5a-b), but had no effect on wild-type (WT) slices (Fig. 5a, c) or on baseline synaptic transmission in *rictor* fb-KO slices (Supplementary Fig. 5a). In addition, cytochalasin D, an inhibitor of actin polymerization, blocked L-LTP in WT slices (Fig. 5c), but had no effect on the short-lasting LTP evoked either by a single tetanic train in control slices (Supplementary Fig. 5b) or by repeated tetanic stimulation in mTORC2-deficient slices (Fig. 5b). The deficient L-LTP in mTORC2-deficient slices is therefore primarily caused by impaired actin polymerization.

To determine whether deficient actin dynamics underlie the impaired LTM in *rictor* fb-KO mice, we bilaterally infused JPK into the CA1 region (Supplementary Fig. 6), at a low dose (50 ng) that promoted F-actin polymerization only in *rictor* fb-KO mice (Supplementary Fig. 7a). JPK infused immediately after training boosted contextual LTM (Fig. 5d), but had no comparable effect on hippocampus-independent auditory LTM (Fig. 5e) in *rictor* fb-KO mice or on contextual LTM in WT mice (Fig. 5f). These pharmacogenetic rescue

experiments provide strong evidence that deficient actin dynamics account, at least in part, for the impaired LTM in *rictor* fb-KO mice.

### Direct stimulation of actin polymerization promotes L-LTP and enhances LTM

Given that i) a short-lasting LTP is evoked by either repeated tetanic stimulation in mTORC2-deficient slices (Fig. 1i) or by a single tetanic train in control slices (Fig. 1h) and ii) JPK restored the deficient L-LTP in mTORC2-deficient slices (Fig. 5b), we predicted that JPK would facilitate the induction of L-LTP in control slices. Combining JPK with a *weak* stimulation, that normally elicits only a short-lasting E-LTP, we found that indeed JPK lowers the threshold for the induction of L-LTP in WT slices (Fig. 5g).

Having shown that boosting actin polymerization converts short-lasting LTP into long-lasting LTP, we next wondered whether this JPK-facilitated L-LTP depended on new protein synthesis. We found that the sustained LTP induced by a single train at 100 Hz in combination with JPK was blocked by the protein synthesis inhibitor anisomycin (Supplementary Fig. 8a). Furthermore, JPK could not rescue the impaired L-LTP induced by four trains at 100 Hz in the presence of anisomycin (Supplementary Fig. 8b). These data indicate that the actin cytoskeleton-mediated facilitation of L-LTP depends on protein synthesis.

We then bilaterally-infused JPK or vehicle into CA1 of WT mice immediately after a *weak* Pavlovian fear conditioning training (a single pairing of a tone with a 1s, 0.7 mA foot-shock). This protocol generated only a relatively weak memory in vehicle-infused mice, as measured 24 hr after training (Fig. 5h). In contrast, in JPK-infused mice the same protocol induced a greatly enhanced contextual fear LTM (Fig. 5h). As expected, JPK had no effect on contextual fear STM (Fig. 5i) or hippocampus-independent auditory fear LTM (Supplementary Fig. 7b). Because JPK acts directly on actin itself by increasing its polymerization<sup>37</sup>, our data support the idea that actin polymerization is an essential mechanism for the consolidation of L-LTP and LTM.

### Selective activation of mTORC2 by A-443654 facilitates L-LTP and enhances LTM

We then reasoned that direct activation of mTORC2 signaling should convert short- to long-lasting memory processes (for both LTP and LTM). To test this hypothesis, we employed a small molecule (A-443654), which increases mTORC2-mediated phosphorylation of Akt at Ser473 (independently of mTORC1<sup>38</sup>). We found that A-443654 promoted mTORC2 activity, PAK phosphorylation, as well as actin polymerization in WT slices (Fig. 6a-b), but not in mTORC2-deficient slices (Fig. 6d-e). More importantly, A-443654 converted a short-lasting E-LTP into a sustained L-LTP in WT slices (Fig. 6c), but it failed to do so in *rictor* fb-KO slices (Fig. 6f). Thus, the facilitated L-LTP induced by combining A-443654 and a single high frequency train is mediated by mTORC2.

If mTORC2 is involved in learning and memory, acute activation of mTORC2 should also promote LTM. Indeed, in WT mice, an intra-peritoneal (i.p.) injection of A-443654 increased the activity of both mTORC2 and PAK in the hippocampus (Supplementary Fig. 9). In addition, when WT mice were i.p.-injected with either vehicle or A-443654



immediately after a *weak* Pavlovian fear conditioning training (a single pairing of a tone with a 1s, 0.7 mA foot-shock; Fig. 7a), we found that 24 hr later the A-443654-injected mice froze nearly twice as often as vehicle-injected controls, indicating that their contextual LTM was enhanced (Fig. 7b). By contrast, A-443654 failed to enhance contextual LTM in mTORC2-deficient mice (Fig. 7c), confirming the selectivity of A-443654. The facts that A-443654 was injected post-training and that contextual STM is not altered by A-443654 (Fig. 7d) argue against non-specific responses to fear. Taken together these pharmacogenetic data provide compelling evidence that A-443654's enhancing effect on synaptic plasticity and behavioral learning is crucially dependent on mTORC2.

## Discussion

### mTORC2 regulates actin polymerization-dependent long-term in synaptic strength and memory

Changes in actin dynamics are required for long-lasting synaptic plasticity and memory consolidation<sup>8-10</sup>. Although changes in synaptic actin dynamics are thought to occur during learning, how the synaptic actin cytoskeleton controls memory storage remains poorly understood. Our findings reveal that the recently discovered mTOR complex 2 (mTORC2) *bidirectionally* controls the actin polymerization that is required for the conversion of a short-term synaptic process (E-LTP, STM) into a long-lasting one (L-LTP, LTM). Specifically, we found that genetic inhibition of mTORC2 activity blocks actin polymerization, actin regulatory signaling (Fig. 4) and selectively suppresses LTM and L-LTP (Fig. 1 and 2). Conversely, activation of mTORC2 by A-443654 promotes actin polymerization, actin signaling and enhanced L-LTP and LTM in WT mice (Fig. 6a-c and Fig. 7a-b) but not in mTORC2-deficient mice (Fig. 6d-f and Fig. 7c).

More importantly, restoring pharmacologically actin polymerization in mTORC2-deficient mice reverses the impaired L-LTP and LTM (Fig. 5b, 5d). We speculate that the stabilization of the actin cytoskeleton in mTORC2-deficient neurons leads to a morphological reorganization of the synapse which, in response to activity, could facilitate the trafficking and insertion of AMPA receptors clustered at the Postsynaptic Density (PSD). Consistent with this notion, structural plasticity was found to be altered in mTORC2-deficient hippocampal neurons (Fig. 4l). Alternatively, the restoration of actin dynamics in mTORC2-deficient synapses could induce a functional rather than a morphological change as AMPA receptors insertion in the hippocampus during LTP might occur independently of changes in spine shape<sup>39</sup>. Another interesting possibility is that changes in actin remodeling could regulate changes in gene expression at synapses which are required for L-LTP and LTM (see below).

Other lines of evidence further support the hypothesis that mTORC2 promotes long-term changes in synaptic strength by promoting actin polymerization. *First*, L-LTP induction is associated with an increase in the F-actin/G-actin ratio<sup>40-42</sup> as well as with changes in synaptic morphology and actin signaling<sup>43</sup>. *Second*, inhibitors of actin polymerization block the late-phase of LTP, leaving the early phase of LTP intact<sup>44-46</sup>. Consistent with these data, only stimulation that induces a stable L-LTP reliably increases F-actin at spines<sup>45</sup>. *Third*, direct activation of actin polymerization by JPK converts E-LTP into L-LTP and enhances

LTM (Fig. 5g-h). *Fourth*, the disruption of actin filaments in CA1 impairs the consolidation of contextual fear LTM<sup>47</sup>. *Fifth*, inhibition of actin polymerization and/or actin regulatory protein signaling in the lateral amygdala blocks auditory fear LTM but not STM<sup>48,49</sup>. *Finally*, mTORC2 is activated during learning (Fig. 2a-c), but only by protocols that induce late-LTP (Fig. 1d-f).

### Temporal and structural aspects of LTP and memory consolidation: protein synthesis vs. actin cytoskeleton polymerization

According to the prevailing view of memory consolidation, LTM is distinguished from STM by its dependence on protein synthesis<sup>1-7</sup>. Consequently, all the “molecular switches” identified so far are transcription or translation factors that regulate gene expression (from CREB<sup>50</sup> to eIF2 $\alpha$ <sup>51</sup> to Npas4<sup>52</sup>). However, like protein synthesis, mTORC2-mediated actin polymerization determines whether synaptic and memory processes remain transient or become consolidated in the brain. The evolutionary conservation of this new model, indicated by our comparable findings in *Drosophila* (Fig. 3), also suggests that our findings may be relevant to the study of memory consolidation in higher mammals, including humans.

Whether actin-mediated changes in synaptic strength depend on, or are perhaps triggered by, changes in gene or protein expression is not immediately clear. Nevertheless, a step towards clarifying the link between actin polymerization and protein synthesis during L-LTP is the finding that actin polymerization, triggered by LTP-inducing stimulation, induces the *synthesis* of the PKMzeta<sup>53</sup>, a kinase necessary for the maintenance of L-LTP<sup>54</sup>. In agreement with these data, we found that the JPK-facilitated L-LTP induced by one tetanic train was blocked by anisomycin (Supplementary Fig. 8a). These results support the idea that actin polymerization is up-stream of protein synthesis. However, we cannot rule out the possibility that protein synthesis and actin polymerization are parallel processes during L-LTP and LTM. Another intriguing possibility is that changes in actin polymerization could directly affect changes in gene expression. For example, actin polymerization promotes the shuttling of the myocardin-related transcription factor (MRTF) protein MKL to the nucleus where it interacts with the Serum Response factor (SRF), thus inducing activity-dependent gene expression in neurons<sup>55,56</sup>. Alternatively, incorporation of G-actin into F-actin filaments could alter local translation at synapses, by modulation the trafficking of ribosomes, translation initiation factors, RNA bindings proteins or even specific mRNAs<sup>57</sup>. If so, the facilitated L-LTP induced by promoting actin polymerization should be insensitive to transcriptional inhibitors.

Our results suggest that neurons have evolved a remarkable bimodal strategy that allows them to control L-LTP and LTM storage both temporally (through regulation of protein synthesis) and structurally (through control of actin dynamics). In this respect, given that mTOR regulates two key processes of L-LTP and LTM - namely mTORC1-mediated protein synthesis<sup>3,5</sup> and mTORC2-mediated actin cytoskeleton dynamics - we propose that mTOR is a key regulator of memory consolidation, controlling “distinct” aspects, the *temporal* through mTORC1 and the *structural* through mTORC2.



Dysregulation of mTORC1 and mTORC2 signaling appears to have a crucial role in memory disorders, such as the cognitive deficit associated with Autism Spectrum Disorder (ASD). Interestingly, the activity of mTORC2 is altered in the brain of ASD-patients harboring mutations in *PTEN* and/or *TSC1/2* (two upstream negative regulators of mTORC1)<sup>58,59</sup>. In addition, in *PTEN* and *TSC2* ASD-mouse models, prolonged rapamycin treatment *in vivo*, which indeed ameliorates the ASD-like phenotypes and restores mTORC1 activity, also corrects the abnormal mTORC2 activity<sup>23,60</sup>. Our new findings that mTORC2 plays a crucial role in memory consolidation raises the intriguing possibility that the neurological dysfunction in ASD is caused by dysregulation of mTORC2 rather than mTORC1 signaling.

In conclusion, our study not only helps to define key basic cellular and molecular mechanisms of physiological learning and memory but it points towards a new therapeutic approach to the treatment of human memory dysfunction in cognitive disorders or even aging, in which mTORC2 activity is known to be abnormally low.

## Methods

Methods and supplemental figure legends are available in the online supplementary material of the paper.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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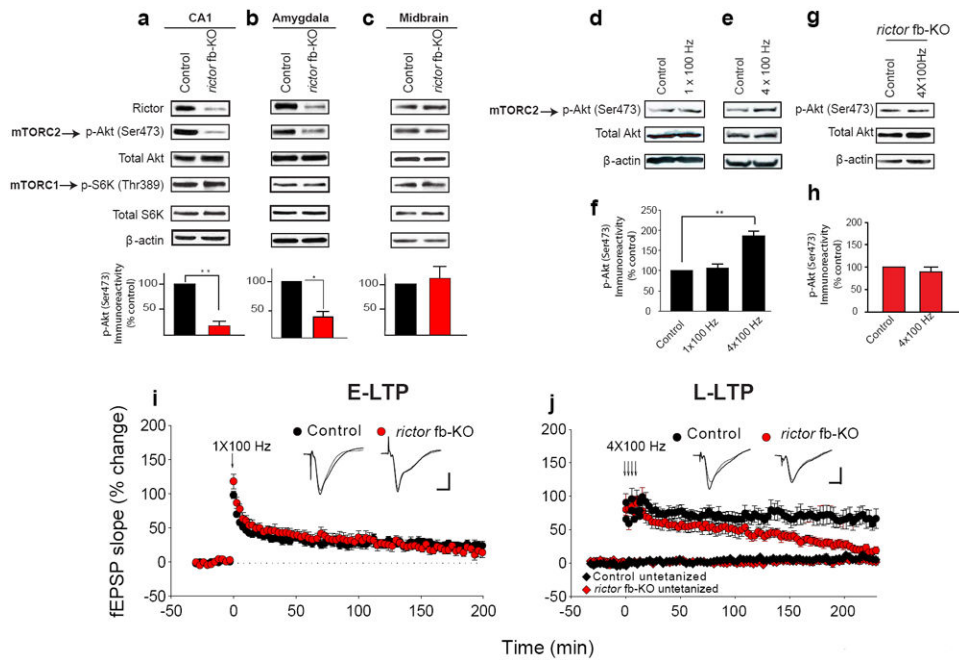
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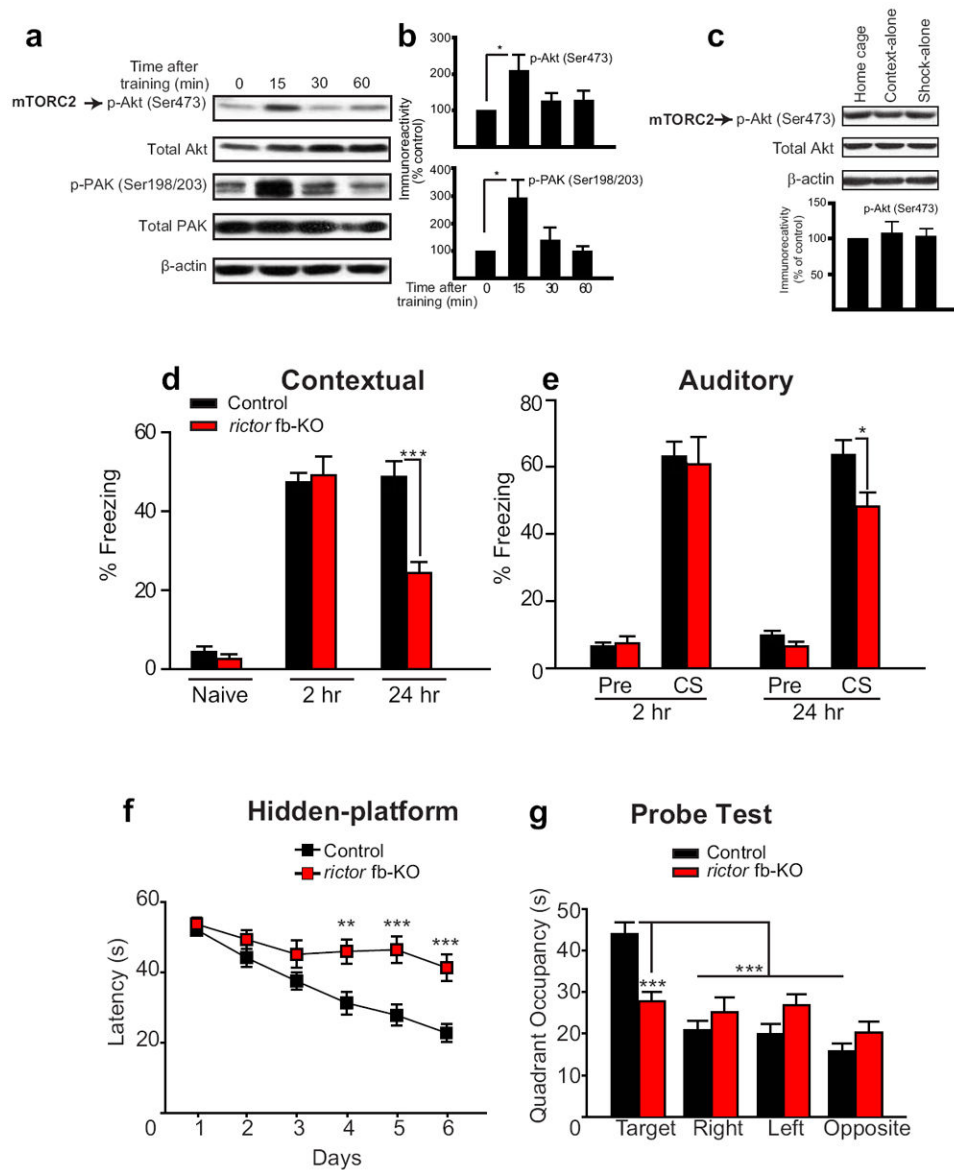
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**Fig. 1. L-LTP, but not E-LTP, is impaired in mTORC2-deficient slices**

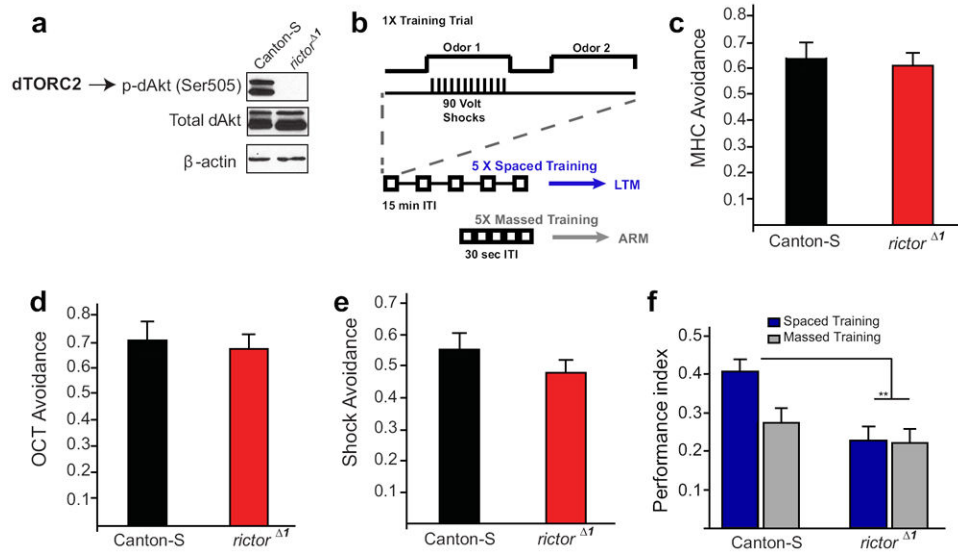
**a-c**) Western blots show selective decrease in Rictor and mTORC2 activity (p-Akt Ser473) in CA1 (**a**) and amygdala (**b**) but not in midbrain (**c**) of *riCTOR* fb-KO mice. Below: normalized data (**a**; n=4 per group,  $t=9.794$ ,  $**p<0.01$ ; **b**; n=5 per group,  $t=2.976$ ,  $*p<0.05$ ; **c**; n=4 per group,  $t=0.470$ ,  $p=0.663$ ). **d-e**) In CA1 extracts from control mice 30 min post-stimulation mTORC2 activity was consistently increased with four tetanic trains, but not a single train. Hippocampal slices were stimulated at 0.033 Hz (control), tetanized by one train (100 Hz for 1 s; **d**), or four such trains at 5 min intervals (**e**). **f**) Normalized mTORC2 activity (n=5 per group, 1 X 100 Hz:  $t=0.31$ ,  $p=0.23$ ; 4 X 100 Hz:  $t=6.01$ ,  $**p<0.01$ ). **g**) In CA1 from *riCTOR* fb-KO mice repeated trains failed to increase mTORC2 activity 30 min after stimulation. **h**) Normalized data (n=5 per group,  $U=5.00$ ,  $p=0.151$ ). **i**) Similar E-LTP was elicited in control (n=9) and *riCTOR* fb-KO slices (n=8) (LTP at 30 min:  $41 \pm 5.6\%$  for controls and  $44 \pm 5.7\%$  for *riCTOR* fb-KO,  $F_{(1, 14)}=0.130$ ,  $p=0.724$ ; LTP at 180 min:  $23.7 \pm 5.3\%$  for controls and  $24.7 \pm 8.5\%$  for *riCTOR* fb-KO,  $F_{(1, 15)}=0.011$ ,  $p=0.917$ ). **j**) L-LTP elicited by four trains in *riCTOR* fb-KO slices (n=11) was impaired vs. control slices (n=14; LTP was similar at 30 min, control  $72 \pm 11.3\%$  and *riCTOR* fb-KO  $67 \pm 13.2\%$ ,  $F_{(1, 23)}=0.811$ ,  $p=0.368$ ; but at 220 min L-LTP was only  $21 \pm 10.8\%$  for *riCTOR* fb-KO slices vs.  $70 \pm 14.8\%$  for controls;  $F_{(1, 23)}=23.4$ ,  $p<0.01$ ). Superimposed single traces were recorded before and 180 min after tetani (**i**) or before and 220 min after tetani (**j**). Calibration: 5 ms, 2 mV.



**Fig. 2. Long-term, but not short-term, fear memory is impaired in mTORC2-deficient mice**  
**a** In Western blots of control dorsal hippocampus, phosphorylation of both Akt at Ser473 and PAK is transiently enhanced 15 min after fear conditioning. **b** Normalized data (top,  $n=6$  per condition,  $t=2.599$ ,  $*p<0.05$ ; bottom,  $n=5$  per condition,  $t=2.930$ ,  $*p<0.05$ ). **c** Compared to home-cage mice, either context-alone (CS) or shock-alone (US) failed to increase mTORC2 activity ( $n=4$  per group,  $F_{(2,9)}=0.127$ ,  $p=0.882$ ). In the context-alone group (CS) mice were treated identically but were not given foot shocks whereas in the shock alone group (US) mice were given two foot-shocks and were immediately removed from the chamber. **d** For contextual fear conditioning, freezing was assessed in control ( $n=22$ ) and *riCTOR* fb-KO mice ( $n=14$ ) during a 2 min period before conditioning (naïve) and then during a 5 min period at 2hr (STM) and 24 hr (LTM) after a strong training protocol (two pairings of a tone with a 0.7 mA foot-shock, 2s). **e** For auditory fear conditioning, freezing was assessed 2 hr and 24 hr after training, for 2 min before the tone presentation

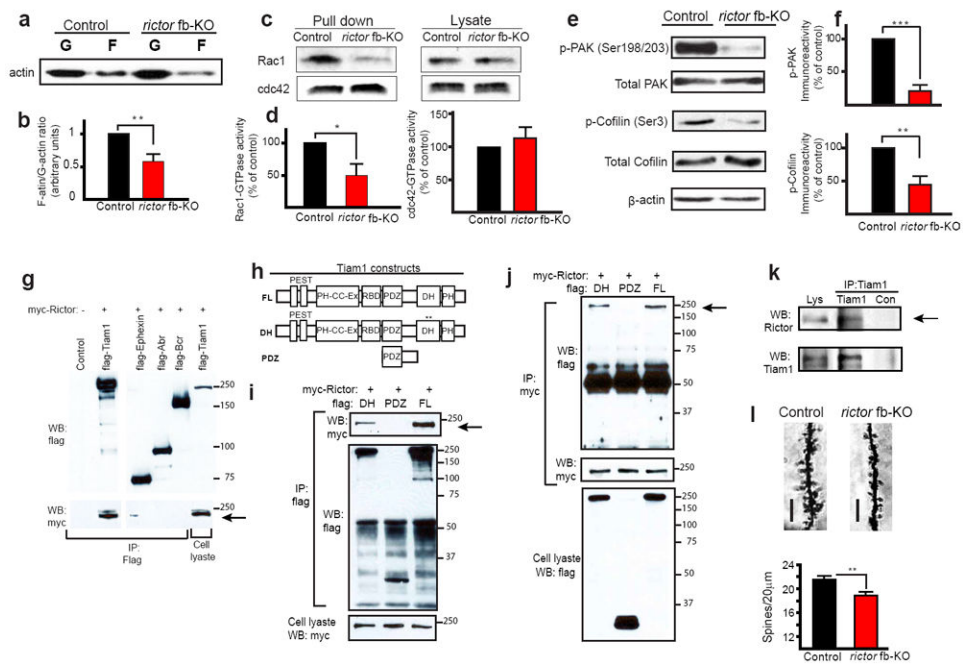


(pre-CS) and then during a 3 min period while the tone sounded (CS). Decreased freezing at 24 hr after training indicates deficient fear LTM in *ric1* fb-KO mice (**d**,  $F_{(1, 34)}=20.253$ ,  $***p<0.001$ ; **e**,  $F_{(1,34)}=4.704$ ,  $*p<0.05$ ). **f-g**) Spatial LTM is impaired in *ric1* fb-KO mice. **f**) In the hidden-platform version of the Morris water maze, on days 4, 5 and 6 escape latencies were significantly longer for *ric1* fb-KO mice ( $F_{(1,37)}=8.585$ ;  $**p<0.01$ ;  $F_{(1,37)}=14.651$ ;  $***p<0.001$ ,  $F_{(1,37)}=18.101$ ,  $***p<0.001$ ). **g**) In the probe test on day 7, only control mice showed preference for the target quadrant (control vs. *ric1* fb-KO mice;  $F_{(1,37)}=15.554$ ,  $***p<0.001$ ; within control group  $F_{(3, 96)}=28.840$ ,  $***p<0.001$ ).



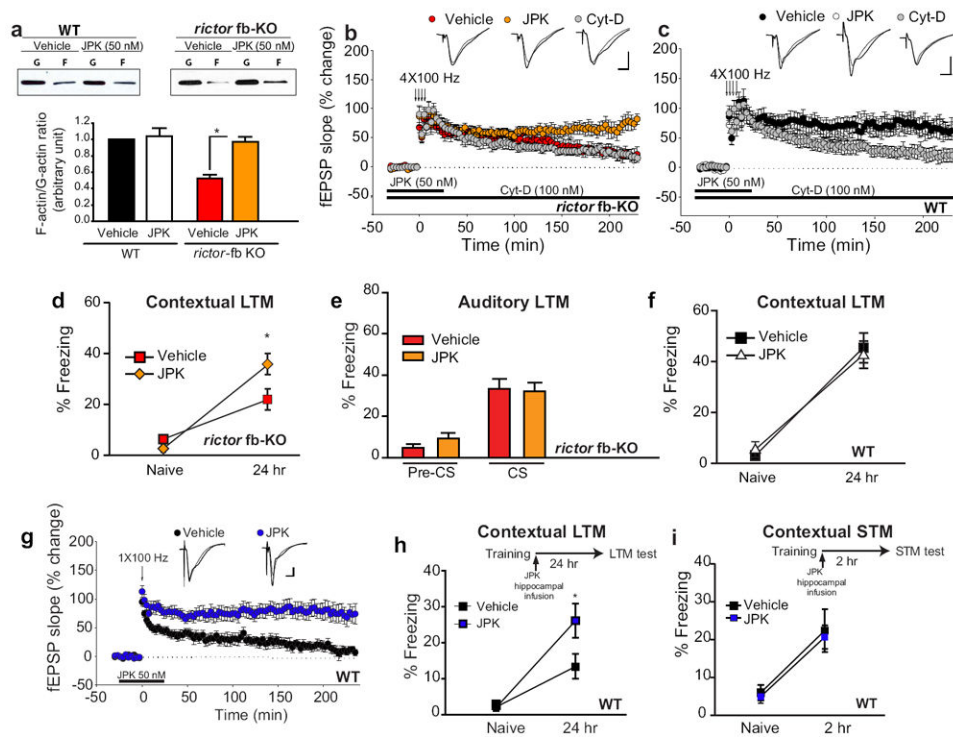
**Fig. 3. In TOR2-deficient *Drosophila*, long-term spaced memory (but not massed) is impaired**

**a)** Western blotting for p-dAkt (Ser505), total dAkt and  $\beta$ -actin in the brain of control Canton-S and *rictor*<sup>1</sup> mutant flies. **b)** In olfactory conditioning, a single training trial consists of 12 electric shocks delivered during the presentation of an odor, while a second odor is explicitly unpaired with the shocks. LTM of the conditioned odor is generated when flies are given five such training trials at 15 min intervals (“spaced training”). However, if the five training trials are given with at much shorter (30 sec) intervals (“massed training”) only a shorter-lasting Anesthesia Resistant Memory (ARM) is formed, but not LTM. **c-d)** WT Canton-S and *rictor*<sup>1</sup> flies did not significantly differ in the avoidance of 0.12% methylcyclohexanol (MCH; **c**;  $t=0.47$ ,  $p=0.647$ ) and 0.2% octanol (OCT; **d**;  $t=1.86$ ,  $p=0.99$ ). **e)** Both Canton-S and *rictor*<sup>1</sup> flies similarly avoided the T-maze arm with 90V electric shocks ( $t=1.86$ ,  $p=0.17$ ). For each sensory control experiment, an “n” was at least 9 for either genotype. Performance index was calculated as described<sup>61</sup>. **f)** In *Drosophila* olfactory memory tests, spaced training-induced LTM was selectively impaired in *rictor*<sup>1</sup> flies ( $t=4.37$ ,  $**p<0.01$ ). In contrast, massed training elicited a similar performance in of both Canton-S (controls) and *rictor*<sup>1</sup> flies ( $t=1.10$ ,  $p=0.3$ ). Spaced training did not significantly improve the performance of *rictor*<sup>1</sup> mutants over that achieved through massed training protocols ( $t=0.19$ ,  $p=0.85$ ).  $n=6$  for each group.



**Fig. 4. Actin dynamics, Rac1-GTPase activity and signaling are impaired in CA1 of *rictor fb-KO* mice**

**a-f)** Western blotting shows that the ratio of F-actin/G-actin (**a**), Rac1-GTPase activities (**c**), p-PAK and p-Cofilin (**e**) are much reduced in CA1 of *rictor fb-KO* mice. Normalized data (**b**;  $n=4$  per group,  $t=4.042$ ,  $**p<0.01$ ; **d** left;  $n=4$  per group,  $t=2.762$ ,  $*p<0.05$ ; **d** right;  $n=4$  per group,  $t=0.519$ ,  $p=0.623$ ; **f**; p-PAK  $n=4$  per group,  $t=9.054$ ,  $***p<0.001$ ; p-Cofilin  $n=4$  per group,  $t=4.486$ ,  $**p<0.01$ ). **g**) We hypothesized that mTORC2 regulates Rac1-GTPase activity (and signaling) through the recruitment of a specific Rac1-GTPase GEF. To test this hypothesis, we co-transfected HEK293T cells with myc-tagged Rictor and flag-tagged GEFs or GAPs, and then performed co-immunoprecipitation (IP) experiments. myc-Rictor selectively pulled-down flag-Tiam1 (T-cell-lymphoma invasion and metastasis-1), a specific Rac1-GEF that is highly enriched in neurons<sup>62</sup>. Flag-tagged -Tiam1, -ephexin, -Abr and -Bcr were coexpressed in HEK293T cells with myc-Rictor and anti-flag immunoprecipitates were analyzed by anti-flag (top) and anti-myc (middle) immunoblotting. **h**) Diagram of the Tiam1 constructs (FL=full length, DH=point mutations in the DH domain; PDZ=deletion mutant encoding only the PDZ domain). Flag-tagged FL, DH or PDZ were co-expressed with myc-tagged Rictor in HEK293T cells. **i**) Anti-flag immunoprecipitates were analyzed by anti-myc (top) and anti-flag (middle) immunoblots whereas anti-myc immunoprecipitates (**j**) were analyzed by anti-flag (top) and anti-myc immunoblots (middle). **k**) Endogenous Tiam1 interacts with endogenous Rictor. Immunoprecipitates of anti-Tiam1 or anti-IgG (control) were prepared from adult hippocampal extracts and analyzed by Rictor (top) and Tiam1 (bottom) immunoblots. Arrows point to the interaction between Tiam1 and Rictor. **l**) Golgi-impregnation shows that spine density of apical CA1 pyramidal neurons is reduced in *rictor fb-KO* mice [scale bar 5  $\mu\text{m}$ ;  $n=70$  (20-25 neurons/mouse; 3 mice per group),  $t=2.791$ ,  $**p<0.01$ ].



**Fig. 5. Restoring actin polymerization rescues the impaired L-LTP and contextual LTM caused by mTORC2 deficiency**

**a)** Western blots show that jasplakinolide (JPK; 50 nM) increased the low F-actin/G-actin ratio in CA1 slices from *rictor* fb-KO mice ( $n=4$  per group,  $t=3.821$ ,  $*p<0.05$ ) but not in control slices ( $n=4$  per group,  $t=0.253$ ,  $p=0.157$ ). **b)** The same concentration of JPK restored L-LTP in *rictor* fb-KO slices ( $n=7$  per group, LTP at 30 min, vehicle  $67 \pm 7.7\%$ , JPK  $73 \pm 11.3\%$ ,  $H=0.0667$ , ANOVA on Ranks,  $p=0.852$ ; LTP at 220 min, vehicle  $23 \pm 4.9\%$ , JPK  $73 \pm 12.5\%$ ,  $F_{(1,12)}=9.81$ ,  $p<0.01$ ) but had no effect on L-LTP in WT slices (**c**;  $n=7$  per group, LTP at 30 min: vehicle  $74 \pm 9.4\%$ , JPK  $72 \pm 11.4\%$ ,  $F_{(1,12)}=0.989$ ,  $p=0.784$ ; LTP at 220 min: vehicle  $64 \pm 8.7\%$ , JPK  $68 \pm 14.2\%$ ;  $F_{(1,12)}=0.010$ ,  $p=0.921$ ). The actin polymerization inhibitor Cytochalasin-D (Cyt-D; 100 nM) blocked L-LTP in WT slices (**c**; at 220 min  $26 \pm 7.8\%$ ,  $F_{(1,12)}=9.215$ ,  $p<0.01$ ) but had no effect in *rictor* fb-KO slices (**b**, at 220 min  $21 \pm 7.9\%$ ,  $F_{(1,13)}=0.163$ ,  $p=0.694$ ). Insets in **b** and **c** are superimposed traces recorded before and 220 min after tetani. Calibration: 5 ms, 2 mV. **d-e)** Bilateral infusion of JPK (50 ng) into the dorsal hippocampus of *rictor* fb-KO mice ( $n=8$  per group), immediately after a strong training protocol (two pairings of a tone with a 0.7 mA foot-shock, 2s), boosted contextual LTM (**d**;  $F_{(1,14)}=4.827$ ,  $*p<0.05$ ) but not auditory LTM (**e**;  $F_{(1,14)}=0.0407$ ,  $p=0.843$ ). Freezing was assessed 24 hr after training, as described in Fig. 2. **f)** In WT mice ( $n=8$  per group) JPK bilateral infusion (50 ng) had no effect on contextual LTM ( $F_{(1,14)}=0.129$ ,  $p=0.726$ ). **g)** A single tetanic train elicits only E-LTP in vehicle-treated slices but a sustained L-LTP in JPK-treated slices ( $n=7$  for vehicle,  $n=8$  for JPK; LTP at 180 min: vehicle  $19 \pm 5.2\%$ , JPK  $81 \pm 14.5\%$ , ANOVA on Ranks  $H=10.59$ ;  $p<0.001$ ). Inset in **g** are superimposed single traces recorded before and 180 min after tetani. Calibration: 5 ms, 2 mV. **h)** Intra-hippocampal infusion of JPK immediately after a weak training (a single pairing of a tone with a 1s, 0.7 mA foot-shock) enhanced contextual fear LTM ( $n=15$  for vehicle and  $n=16$

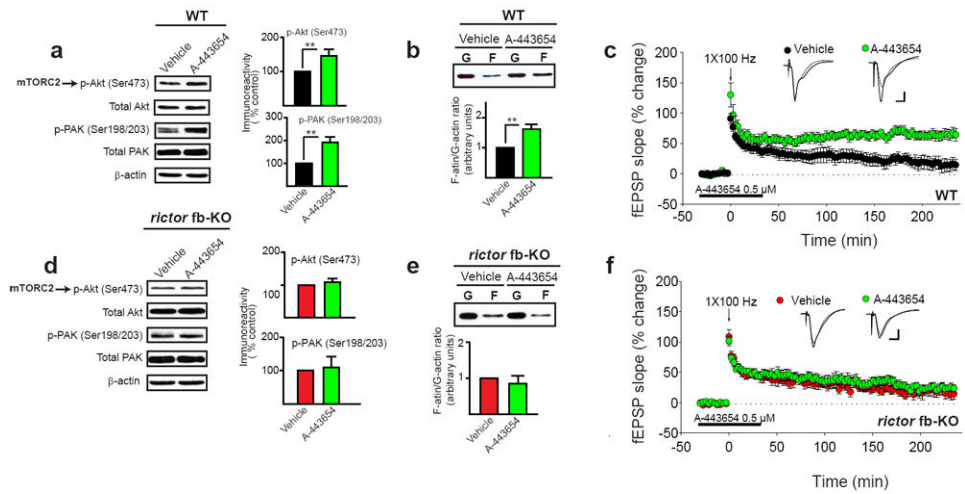
for JPK;  $F_{(1, 29)}=4.320$ ,  $*p<0.05$ ) but not contextual fear STM (i,  $n=9$  for vehicle and  $n=10$  for JPK,  $H=0.00167$ , ANOVA on Ranks,  $p=0.967$ ).

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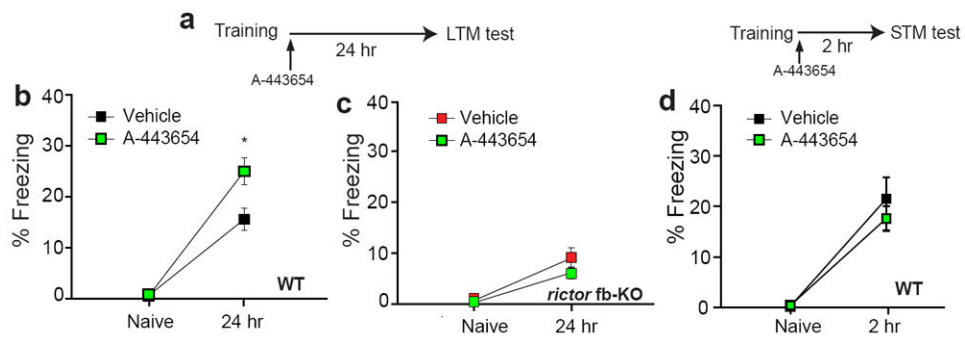
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**Fig. 6. A-443654 promotes mTORC2 activity, actin polymerization and facilitates L-LTP in WT mice but not in mTORC2-deficient mice**

**a-b)** Treating WT hippocampal slices for 30 min with A-443654 (0.5  $\mu$ M) increased the activity of both mTORC2 and PAK (**a**;  $n=5$ ,  $U=0.00$ ,  $**p<0.01$ ;  $n=4$ ,  $U=0.00$ ,  $**p<0.01$ ), as well as the F-actin/G-actin ratio (**b**;  $n=5$ ,  $t=3.995$ ,  $**p<0.01$ ). **c)** A-443654 (0.5  $\mu$ M) converted E-LTP elicited by single tetanic train into a sustained L-LTP in WT slices ( $n=6$  for vehicle,  $n=8$  for A-443654; LTP at 180 min: vehicle  $17 \pm 10.6\%$ , A-443654  $69 \pm 7.1\%$ ,  $F_{(1, 12)}=17.870$ ,  $p<0.001$ ). **d-e)** A-443654 (0.5  $\mu$ M) had no effect on mTORC2 and PAK activities (**d**;  $n=4$ ,  $t=0.830$ ,  $p=0.438$ ;  $n=4$ ;  $t=0.290$ ,  $p=0.777$ ) or on actin polymerization in *rictor* fb-KO slices (**e**;  $n=3$ ,  $t=0.680$ ,  $p=0.534$ ). **f)** A-443654 failed to induce L-LTP in *rictor* fb-KO slices ( $n=6$  for vehicle,  $n=7$  for A-443654; LTP at 180 min: vehicle  $24 \pm 8.2\%$ , A-443654  $28 \pm 6.2\%$ ,  $F_{(1, 11)}=2.25$ ,  $p=0.167$ ). Insets in **c** and **f** are superimposed traces recorded before and 180 min after tetani. Calibration: 5 ms, 2 mV.





**Fig. 7. A-443654 selectively enhances LTM in WT mice but not in mTORC2-deficient mice**  
**a)** Diagram of the experimental protocol. **b)** A single A-443654-injection (2.5 mg/kg i.p.) immediately after a *weak* training (a single pairing of a tone with a 1s, 0.7 mA foot-shock) enhanced contextual fear LTM in WT mice ( $n=18$  per group;  $F_{(1, 34)}=6.007$ ,  $*p<0.05$ ) but not in *rictor fb-KO* mice (**c**,  $n=10$  per group;  $F_{(1, 18)}=1.648$ ,  $p=0.22$ ). **d)** Similar freezing at 2 hr reflects normal contextual fear STM in vehicle-injected and A-443654-injected WT mice ( $n=9$  for vehicle and  $n=10$  for A-443654;  $F_{(1, 17)}=0.6$ ,  $p=0.449$ ). Freezing was assessed 2hr (**d**) or 24 hr (**a-c**) after training, as described in Fig. 2.