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CaMKII phosphorylation of TARPγ-8 is a mediator of LTP and learning and memory

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Summary

Protein phosphorylation is an essential step for the expression of long-term potentiation (LTP), a long-lasting, activity-dependent strengthening of synaptic transmission widely regarded as a cellular mechanism underlying learning and memory. At the core of LTP is the synaptic insertion of AMPA receptors (AMPARs) triggered by the NMDA receptor-dependent activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). However, the CaMKII substrate that increases AMPAR-mediated transmission during LTP remains elusive. Here, we identify the hippocampus-enriched TARPγ-8, but not TARPγ-2/3/4, as a critical CaMKII substrate for LTP. We found that LTP induction increases TARPγ-8 phosphorylation, and that CaMKII-dependent enhancement of AMPAR-mediated transmission requires CaMKII phosphorylation sites of TARPγ-8. Moreover, LTP and memory formation, but not basal transmission, are significantly impaired in mice lacking CaMKII phosphorylation sites of TARPγ-8. Together, these findings demonstrate that TARPγ-8 is

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Author Contributions

S.T. conceived the project. S.T., J.P., and P.E.C. wrote the manuscript. J.P. performed all biochemical and histochemical studies. S.T., M.M. and K.S.K. generated gene-targeting mice and antibodies. P.E.C. supervised and A.E.C., S.L designed and performed all electrophysiological recordings. M.R.P. and Y.M. supervised and J.P. performed behavior analysis. All authors contributed to the final version of the manuscript.

a crucial mediator of CaMKII-dependent LTP and therefore a molecular target that controls synaptic plasticity and associated cognitive functions.

eTOC Blurb (In Brief)

Park et al. report hippocampus-enriched TARPγ-8 as a critical CaMKIIα substrate for LTP and learning and memory. LTP increases TARPγ-8 phosphorylation and this phosphorylation is required for CaMKII-dependent increase of AMPAR-mediated transmission, LTP and fear conditioning.

Introduction

Long-term potentiation (LTP) is an activity-dependent strengthening of synaptic transmission thought to be the cellular basis of learning and memory. Early work demonstrated that postsynaptic CaMKIIα activation by Ca^{2+} via N-methyl-D-aspartate receptors (NMDARs) is necessary and sufficient for the expression of NMDAR-dependent postsynaptic LTP of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-mediated transmission (Lisman et al., 2012; Malenka and Nicoll, 1999). While CaMKIIα is both necessary and sufficient for LTP induction, it is surprising that the specific target(s) of CaMKIIα that increases AMPAR transmission during LTP remain elusive.

AMPARs comprise a complex of the pore-forming AMPAR subunits with auxiliary transmembrane AMPAR regulatory protein (TARP) and cornichon-like (CNIH) subunits in the brain (Jackson and Nicoll, 2011; Yan and Tomita, 2012). Notably, GluA1 knockout (KO), TARPγ-8 KO, and CNIH2 KO mice show LTP impairment, highlighting the importance of these molecules in LTP (Herring et al., 2013; Rouach et al., 2005; Zamanillo et al., 1999). Given that these KO mice exhibit altered expression of the hippocampal AMPAR components (AMPARs and auxiliary subunits), the LTP impairment could be due to indirect effects on the hippocampal AMPAR complex.

The GluA1 subunit is phosphorylated at serine (S) 831 by CaMKIIα *in vitro* and during LTP (Barria et al., 1997; Lee et al., 2000; Mammen et al., 1997), and single-channel analysis has shown that homomeric channels with phosphorylated GluA1 have greater channel conductance than those with non-phosphorylated GluA1 or GluA1/GluA2 heteromers (Derkach et al., 1999; Oh and Derkach, 2005). However, GluA1.S831A knock-in (KI) mice have normal LTP (Lee et al., 2010), suggesting that GluA1.S831 is not required for LTP, or that there are compensatory mechanisms in GluA1.S831A KI mice. TARPγ-2 is phosphorylated in the brain, and can also be phosphorylated by CaMKIIα or protein kinase C *in vitro* (Tomita et al., 2005). Overexpression of phosphomimic TARPγ-2 (nine serine-to-aspartic acid mutations) in the hippocampus impairs LTP (Tomita et al., 2005), whereas hippocampal transmission is unaltered in TARPγ-2 mutant/*stargazer* mice (Hashimoto et al., 1999). Furthermore, overexpressed kainate receptors can be inserted into synapses during LTP in AMPAR-deficient neurons, suggesting that CaMKIIα phosphorylation of the native AMPAR complex may not be required for LTP (Granger et al., 2013). Because most of these studies have been conducted in non-physiological overexpression systems, the CaMKIIα substrate required for LTP under normal conditions remains unclear. Here, we report that

TARPγ-8 is a critical CaMKIIα substrate for hippocampal LTP and as a result, a crucial molecule for learning and memory.

Results

CaMKIIα phosphorylates TARPγ-8 directly

As a potential CaMKIIα substrate for driving AMPARs into synapses, we focused on TARPγ-8, a major component of hippocampal AMPAR complex (Tomita et al., 2003). If TARPγ-8 is a CaMKIIα substrate, it should be phosphorylated directly by CaMKIIα. To test this, we examined *in vitro* CaMKIIα phosphorylation of the cytoplasmic domain of TARPγ-8 (γ-8cyto) produced in a thioredoxin fusion-protein expression system employing a phosphorus-32 (^{32}P) probe, [γ-^{32}P]ATP. Mutant γ-8cyto with nine serine-to-alanine substitutions between R270 and G293 (9A) was phosphorylated at only 3.2% of the level of observed for wild-type (WT) γ-8cyto, as demonstrated by ^{32}P incorporation (Figures 1A and 1B). Radio-Edman sequencing of a TARPγ-8 mimetic synthetic peptide (residues 270–293) phosphorylated by CaMKIIα *in vitro* revealed that serine residues at position 277 (S277) and 281 (S281) were major CaMKIIα phosphorylation sites, whereas the serine residue at position 275 (S275) was a weak CaMKIIα phosphorylation site (Figure 1C).

Next, we examined changes in the phosphorylation status of TARPγ-8 at S277 upon LTP induction with an antibody against phosphorylated-S277 (pS277). To facilitate biochemical detection of LTP-associated changes, we employed chemically induced LTP (chemLTP) (Kopec et al., 2006). Our anti-γ-8.pS277 antibody recognized a specific band in forebrain lysate from WT and TARPγ-2 mutant/*stargazer* mice, but not in those from TARPγ-8 KO mice, or in WT lysate treated with lambda phosphatase (Figure 1D). Following chemLTP induction (see Experimental Procedures), immunoblot assay of synaptosome fractions from the acute hippocampal slices revealed a significant increase in phosphorylation at S277 in TARPγ-8, with no change in TARPγ-8, GluA1, or PSD-95 protein levels (Figures 1E and 1F). Furthermore, this increase was blocked by the NMDAR antagonist d-APV (100 μM) (Figure 1E) and the CaMKII inhibitor KN93 (20 μM) (Figure 1F), indicating that CaMKIIα directly phosphorylates TARPγ-8, and that TARPγ-8 phosphorylation is increased following the induction of NMDAR- and CaMKII-dependent chemLTP.

LTP requires CaMKIIα phosphorylation of TARPγ-8

To test whether TARPγ-8 is a CaMKIIα substrate in synaptic plasticity *in vivo*, we generated a TARPγ-8 KI mouse (γ-8^{Cm}) in which S277 and S281 residues were substituted by alanine residues using standard gene-targeting techniques (Figures S1A–S1C). The anti-TARPγ-8 antibody recognized a specific band in hippocampal lysate from both WT and γ-8^{Cm} KI mice, whereas anti-γ-8.pS277 antibody recognized a band only in hippocampal WT lysate (Figures S1D and S1E). In addition, the anti-γ-8.pS277 antibody recognized thioredoxin fused γ-8cyto only when phosphorylated by recombinant CaMKIIα or β *in vitro* (Figure S1F). In γ-8^{Cm} KI mice, LTP at the Schaffer collateral to CA1 pyramidal cell synapse (Sch-CA1) was reduced by ~60 % compared to WT mice (Figures 2A–2C). Notably, this reduction is similar to the 75% reduction observed in TARPγ-8 KO mice

(Rouach et al., 2005), suggesting that two γ-8 phosphorylation sites mediate most of the TARPγ-8-dependent LTP.

A reduction in AMPAR expression, as seen in TARPγ-8 KO mice (Rouach et al., 2005), could account for the LTP deficit in γ-8^{Cm} KI mice. We therefore examined protein levels in total, postsynaptic density (PSD), and extrasynaptic (i.e. octyl glucoside-solubilized synaptosome) hippocampal fractions. As previously reported (Rouach et al., 2005), expression of AMPAR GluA1 and GluA2/3 subunits was substantially reduced in total hippocampal lysate from the TARPγ-8 KO mice, without a change in PSD-enriched protein PSD-95 expression (Figure 2D). In contrast, protein levels in the total lysate, PSD, and extrasynaptic fractions were unaltered in hippocampi from γ-8^{Cm} KI mice (Figures 2D–2F). In addition, the AMPA/NMDA ratio at Sch-CA1 synapses remained unchanged in γ-8^{Cm} KI mice relative to WT control littermates (Figure 2G). Both the input-output function and paired-pulse ratio were also normal in slices from γ-8^{Cm} KI mice (Figures 2H and 2I), suggesting that the more subtle γ-8^{Cm} KI genetic manipulation does not alter AMPAR-mediated basal synaptic transmission as previously observed in CaMKIIα KO and CaMKIIα KI mice substituting autophosphorylated threonine to alanine (T286A) (Giese et al., 1998; Silva et al., 1992). We also examined the potential contribution to LTP of three other type I TARP isoforms (TARPγ-2/3/4) that shared the phosphorylation sites (Tomita et al, 2005) using the non-phosphorylatable TARPγ-2 KI mice in which nine phosphorylated serine residues are replaced with alanine (TARPγ-2^{SA}) as well as γ-3, and γ-4 KO mice (Letts et al., 1998; Menuz et al., 2009; Sumioka et al., 2010). Unlike γ-8^{Cm} KI mice (Figures 2A–2C), both TARPγ-2^{SA} KI mice and triple mutant γ-2^{SA} KI/γ-3 KO/γ-4 KO mice showed normal LTP (Figures 2J–2L and S2A). Consistent with the notion that synaptic AMPARs arise from an extrasynaptic pool of receptors, γ-8 as well as the AMPAR GluA1 and GluA2/3 subunits localized preferentially in the extrasynaptic fraction compared to other type I TARP (Figure S2B). Together, our results suggest that the major CaMKII phosphorylation sites disrupted in the γ-8^{Cm} KI mice mediate LTP directly.

CaMKIIα increases AMPAR-mediated transmission through TARPγ-8 phosphorylation

CaMKIIα activation downstream of NMDARs is required for LTP and is also sufficient to drive AMPARs into synapses (Hayashi et al., 2000; Lledo et al., 1995; Pettit et al., 1994; Silva et al., 1992). To test whether CaMKIIα-mediated potentiation requires TARPγ-8 phosphorylation, we measured the effect of CaMKIIα mutants on AMPAR-mediated synaptic transmission in γ-8^{Cm} KI mice. The activity of these mutants was validated using a heterologous expression system. Lysate from HEK cells expressing GFP-tagged CaMKIIα with a threonine-to-aspartic acid substitution at residue 286 (T286D) showed Ca^{2+}/calmodulin-independent constitutive CaMKIIα activity higher than lysate expressing GFP alone, whereas HEK cell lysate expressing CaMKIIα WT and kinase-dead CaMKIIα (K42M) did not (Figure 3A).

We next generated an adeno-associated virus (AAV) carrying GFP-CaMKIIα, injected it into hippocampi of 30–35-day-old mice, and assayed GFP-labeled protein levels in their hippocampi 5 days later (Figures 3B and 3C). Following injection of GFP-CaMKIIα.T286D, TARPγ-8 phosphorylation at S277 was significantly increased, while

total TARPγ-8 levels remained unchanged (Figure 3C). Moreover, neurons expressing GFP-CaMKIIα.T286D showed significantly larger AMPA/NMDA ratios in WT mice than in γ-8^{Cm} KI mice (Figure 3D). Compared to the robust enhancement in AMPA/NMDA ratio (Figure 3D), changes in phosphorylation levels of γ-8.S277 upon GFP-CaMKIIα.T286D expression were mild (Figure 3C). This observation could be due to basal phosphorylation at TARPγ-8 S277 in the PSD fraction, which is consistent with high phosphorylation of synaptic TARPγ-8, including S277 (Figure S3A). Together, these findings indicate that CaMKIIα increases AMPAR-mediated transmission through CaMKIIα phosphorylation of TARPγ-8.

To further examine whether CaMKIIα phosphorylation of TARPγ-8 sufficiently enhances AMPAR transmission, we mutated the CaMKIIα phosphorylation sites of TARPγ-8 S277 and S281 to phosphomimic aspartic acid (DD) or non-phospho mimic alanine (AA). We generated AAVs carrying the γ-8 mutants fused with the self-cleaving 2A peptide and GFP as a reporter. To avoid potential contribution of other seven phosphorylatable serine residues between R270 and G293 (Figure 1A), we mutated all seven sites to either alanine or aspartic acid (Figure S3B). Protein expressions of these mutants and GFP were confirmed by western blotting in AAV-infected primary cultured neurons (Figure 3E). GFP signal was confirmed in acute hippocampal slices from WT mouse brains injected with AAV 4–6 days in advance (Figure 3F). Whole cell recording from GFP positive neurons revealed significantly higher AMPA/NMDA ratios in neurons expressing phosphomimic γ-8 at the CaMKIIα phosphorylation sites (DD) than those in neurons expressing non-phosphomimic γ-8 (AA) (Figure 3G). These results indicate that CaMKIIα phosphorylation of TARPγ-8 is sufficient to enhance AMPAR transmission.

TARPγ-8 phosphorylation is critical for learning and memory

Finally, we evaluated contextual memory and cued fear memory in γ-8^{Cm} KI mice. Following training with foot shock(s) (0.5 mA or 1.0 mA delivered for the last 2 s of a 20-s tone), we measured freezing in response to a shock-paired context or in a novel context in response to a shock-paired cue (Figures 4A and S4). We used three conditions to determine whether the threshold for fear learning was shifted in the γ-8^{Cm} KI mice. While the initial baseline between γ-8^{Cm} KI and WT was the same, during training with a mild stimuli (1 or 3 presentations of a 0.5-mA shock), the γ-8^{Cm} KI mice spent less time freezing than WT mice both during tone presentation and during the inter-trial intervals, suggesting that within-session learning was impaired. When a stronger conditioning stimulus (6 presentations of a 1.0-mA shock) was employed, the group difference in freezing time was diminished (Figures 4B and 4C). Both contextual memory and cued memory were significantly impaired following training with 0.5 mA shock presentations (Figures 4D and 4E), suggesting that TARPγ-8 phosphorylation is important for setting the threshold for learning both contextual and cue associations in the fear conditioning paradigm.

Discussion

The findings of the present study indicate that TARPγ-8 is a crucial CaMKIIα substrate involved in hippocampal LTP and in learning fear associations. We demonstrated that

CaMKIIα phosphorylates TARPγ-8 *in vitro* and that CaMKIIα phosphorylation of TARPγ-8 is increased upon induction of chemLTP. Moreover, we found that LTP and CaMKIIα-induced increase in AMPAR-mediated transmission requires phosphorylation of TARPγ-8 by CaMKIIα. Lastly, disruption of CaMKIIα phosphorylation sites of TARPγ-8 also impaired fear learning. The discovery of a critical downstream target of CaMKIIα in LTP fills in a long-standing knowledge gap in our understanding of the molecular basis of LTP and its contribution to learning and memory.

Previous studies have attempted to identify CaMKII downstream signaling cascades involved in LTP (Lisman et al., 2012). However, thus far no obvious targets of CaMKIIα phosphorylation that drive synaptic insertion of AMPARs have emerged (Herring and Nicoll, 2016b). Our findings implicate TARPγ-8 as a key substrate of NMDAR-dependent LTP in the hippocampus. We found that CaMKIIα activity-dependent increase in AMPAR-mediated transmission was significantly impaired when the CaMKIIα phosphorylation sites of TARPγ-8 were disrupted, supporting the notion that TARPγ-8 is a CaMKIIα substrate for AMPAR delivery to synapses during LTP. TARPγ–8 phosphorylation may increase the stability or capture of AMPARs in the postsynaptic density. AMPARs interact with PSD-95-like MAGUKs through the PDZ binding motif of TARPs and by this means are stabilized at synapses under basal transmission (Nicoll et al., 2006). An increase in TARP interaction with PSD-95 during LTP was proposed based on the occlusion of LTP by PSD-95 overexpression (Ehrlich et al., 2007; Stein et al., 2003) and the requirement of TARP PDZ binding for CaMKII-mediated diffusional trap of AMPARs (Opazo et al., 2010). However, despite substantial LTP reduction in γ-8 KO mice (Rouach et al., 2005), deletion of the PDZ binding domain (γ-8Δ4) in γ-8Δ4 KI mice showed normal LTP (Sumioka et al., 2011). Furthermore, PSD-95 KO mice showed enhanced LTP (Beique et al., 2006; Carlisle et al., 2008a; Migaud et al., 1998) with reduction in basal transmission (Beique et al., 2006; Carlisle et al., 2008a), suggesting that the PDZ binding of TARPγ-8 to PSD-95 is not necessary for LTP. Stabilization or capture of AMPARs during LTP may be mediated by PDZ-independent mechanisms. Phosphorylation-related electrostatic changes of the TARPγ-2 cytoplasmic domain can affect TARP interaction with negatively-charged plasma membrane lipids, and dissociation of the TARPγ-2 cytoplasmic domain from lipids upon its phosphorylation is required for its interaction with PSD-95 (Hafner et al., 2015; Sumioka et al., 2010). While TARPγ-8 dissociation from the plasma membrane could be sufficient to trigger AMPAR/TARP complex driving into synapses through lateral diffusion, delivery from an intracellular pool could also contribute (Ahmad et al., 2012; Jurado et al., 2013; Park et al., 2004).

We observed a residual LTP (~40%) in γ-8^{Cm} KI mice that carry disruption of two CaMKIIα phosphorylation sites in TARPγ-8 (i.e. S277 and S281) (Figures 2A–2C). Likewise, a previous study also showed some residual LTP (~26%) in γ-8 KO mice (Rouach et al., 2005). While the 14% difference between γ-8 ^{Cm} KI and γ-8 KO mice could be due to compensation by remaining intact sites, such as S275 (Figure 1C), these results also suggest TARPγ-8 independent mechanisms of LTP. An obvious redundant LTP target is other type I TARP isoforms (γ-2/3/4) that shared phosphorylation sites with γ-8. However, using gene-targeting mice we found unique roles of TARPγ-8 as LTP substrate among other type I TARPs (Figure 2 and S2). Given that phosphorylation sites are not conserved in the

type II TARPγ-5/7 (Kato et al., 2008; Tomita et al., 2005), our results support an additional TARP-independent mechanism underlying the residual LTP observed in TARPγ-8 mutant mice.

There are probably multiple CaMKIIα substrates that are important for the induction and/or expression of LTP. Indeed, recent evidence suggests that, besides components of native AMPAR complex (Granger et al., 2013), SynGAP and Kalirin/Trio family proteins are other potential CaMKII substrates (Araki et al., 2015; Herring and Nicoll, 2016a). Multiple CaMKII phosphorylation sites were identified in both SynGAP (Araki et al., 2015; Carlisle et al., 2008b; Oh et al., 2004) and Kalirin/Tiam1/Trio family proteins (Fleming et al., 1999; Tolias et al., 2005; Xie et al., 2007). Molecular replacement of WT protein with SynGAP mutant proteins carrying mutations in CaMKII phosphorylation sites showed impairments in structural plasticity and chemical LTP in primary neurons (Araki et al., 2015) and in LTP in organotypic slice cultures manipulated with Trio/Kalirin (Herring and Nicoll, 2016a). Although multiple CaMKII substrates for LTP may exist, the present study demonstrates that disruption of only TARPγ-8 phosphorylation impairs LTP and performance in a fear-conditioning task. The selective effect of our TARPγ-8 manipulation on plasticity, but not basal transmission, is reminiscent of previous observations in which CaMKIIα was knocked out or its autophosphorylation site (T286A) was mutated (Giese et al., 1998; Silva et al., 1992). Our finding that disrupting major CaMKIIα phosphorylation sites of TARPγ-8 phenocopies CaMKIIα-deficient mice strongly supports the notion that an NMDAR activation/CaMKIIα activation/TARPγ-8 phosphorylation sequence is essential for normal plasticity. Finally, our behavioral results show that TARPγ-8-dependent LTP is particularly important for setting the threshold for fear learning, thus providing additional evidence for LTP as a crucial cellular mechanism underlying learning and memory.

Experimental Procedures

Full Experimental Procedures are available in the Supplemental Experimental Procedures. All data are given as mean ± s.e.m. Statistical significance between means was calculated using unpaired Student’s t test or one-way ANOVA with posthoc Tukey’s test. All animal handling was in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Yale University and the Albert Einstein College of Medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- CaMKIIα phosphorylates TARPγ-8 directly at S277 and S281
- TARPγ-8 phosphorylation at CaMKIIα sites is enhanced during chemical LTP
- CaMKIIα enhances AMPAR-mediated transmission via TARPγ-8 phosphorylation sites
- CaMKIIα phosphorylation of TARPγ-8 is required for LTP and learning and memory

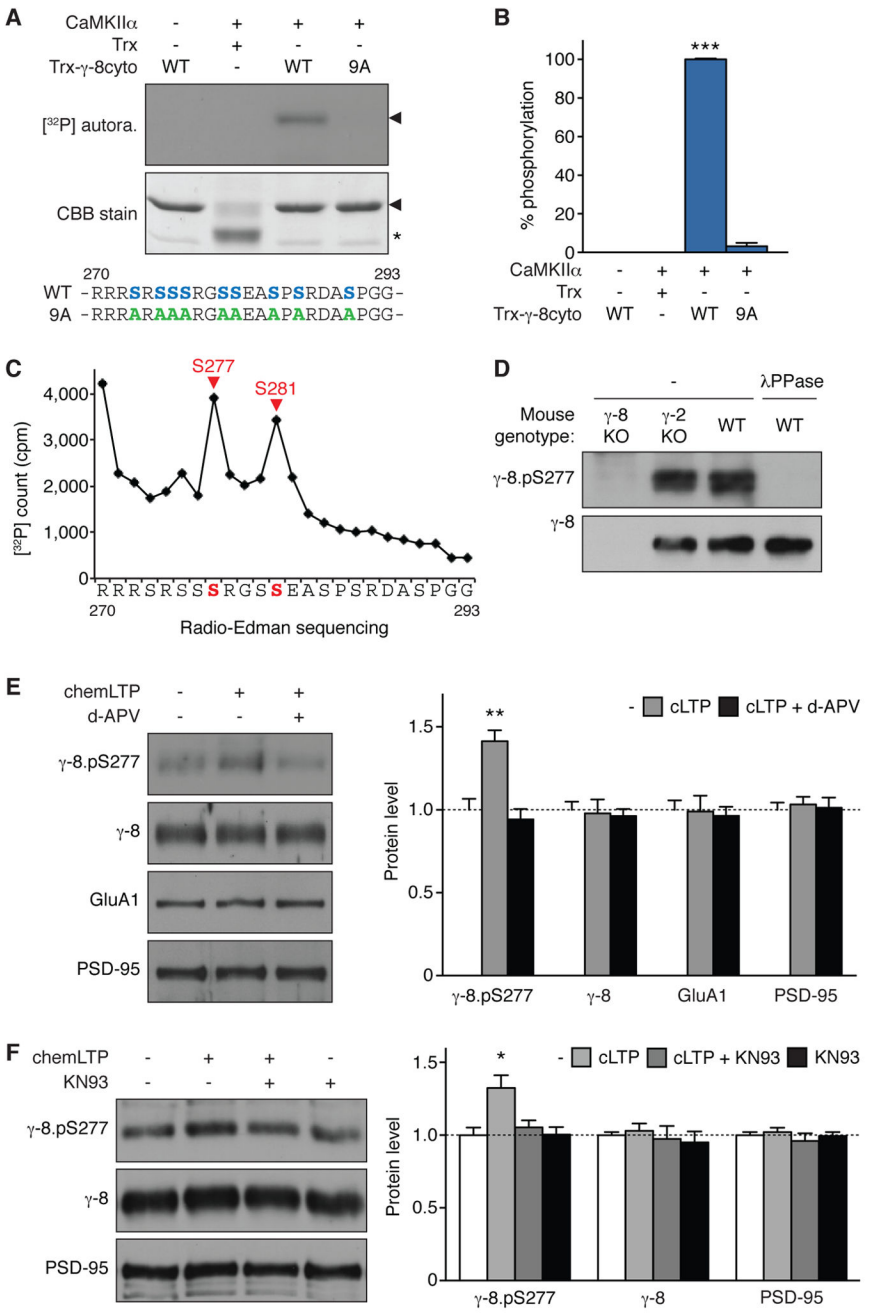

Figure 1. TARPγ-8 phosphorylation at a CaMKIIα site is enhanced during NMDAR-dependent chemLTP

(A) *In vitro* CaMKIIα phosphorylation sites in TARPγ-8. CaMKIIα phosphorylates WT γ-8cyto (fused with thioredoxin [Trx]), but not a mutant γ-8cyto with nine serine-to-alanine substitutions between R270 and G293 (9A). (B) Quantitative analysis of γ-8cyto phosphorylation by CaMKIIα ($n = 4$). (C) Radio-Edman sequencing ($n = 3$) revealed two major CaMKIIα phosphorylation sites (S277 and S281). (D) Anti-γ-8.pS277 antibody recognized a specific band in hippocampal lysate from WT and TARPγ-2 mutant mice, but not in lysate from TARPγ-8 KO mice or in WT lysate treated with λ phosphatase. (E and F)

ChemLTP in acute hippocampal slices increased TARPγ-8 phosphorylation at S277 in an NMDAR and CaMKII-dependent manner, without changes in the total protein levels of TARPγ-8, GluA1, or PSD-95. The NMDAR antagonist d-APV (E, 100 μM, $n = 5$) and the CaMKII inhibitor KN93 (F, 20 μM, $n = 4$) blocked TARPγ-8 phosphorylation at S277 upon chemLTP induction. Data are shown as mean ± s.e.m.; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, one-way ANOVA followed by post-hoc Tukey's multiple comparisons.

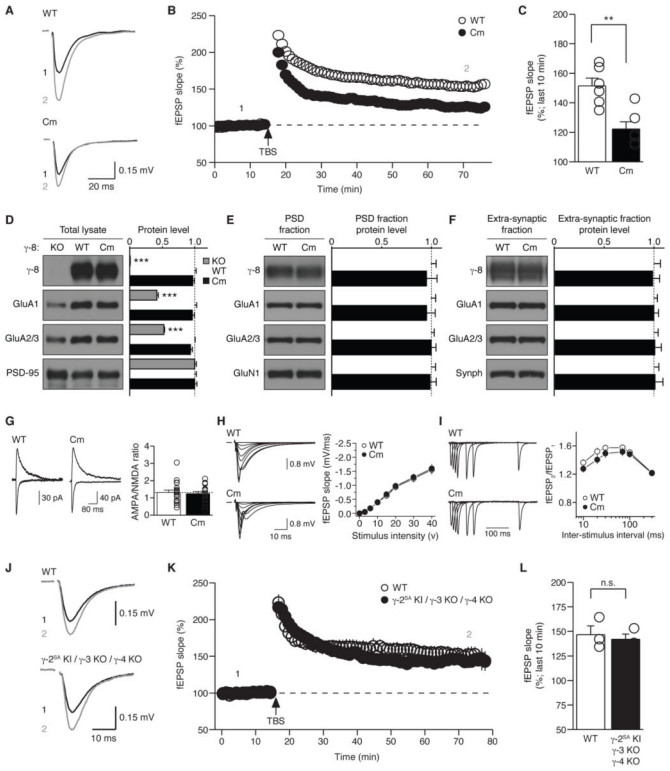

Figure 2. CaMKIIα phosphorylation sites of TARPγ-8 are required for hippocampal LTP

(A and B) Average traces (A) and summary plots (B) showing that LTP induced by theta-burst stimulation was impaired in mice with disrupted CaMKIIα phosphorylation sites in TARPγ-8 (Cm, γ-8^{Cm} KI mice) relative to WT littermates (WT). Sample traces were taken at times indicated by the numbers on the summary plot. (C) Reduced LTP in *Cm/Cm* mice versus WT littermates (mean ± s.e.m.; last 10 min WT: 151.5 ± 5.24, 6 animals/38 slices *vs.* Cm: 122.2 ± 4.89, 6 animals/36 slices; $^{**}P < 0.01$). (D) Total protein expression of AMPARs is unaltered in γ-8^{Cm} KI mice, but reduced in TARPγ-8 KO mice; $^{***}P < 0.001$ ($n = 4$), one-way ANOVA followed by post-hoc Tukey's multiple comparisons. (E and F) Normal TARPγ-8 and AMPAR expression in the PSD (E) and extrasynaptic (F) fractions ($n = 6$) from γ-8^{Cm} KI mice. (G) AMPAR- and NMDAR-mediated EPSCs ratio do not differ between WT (1.31 ± 0.14, $n = 19$ slices from 4 animals) and γ-8^{Cm} KI mice (1.24 ± 0.12, $n = 17$ slices from 4 animals; P=0.73). Representative averaged EPSCs (left) and summary data (right) are shown. (H and I) Disrupted CaMKIIα phosphorylation sites in TARPγ-8 (Cm) did not alter input-output function (H) or paired-pulse ratios (I) measured at 10-ms, 20-ms, 30-ms, 70-ms, 100-ms, and 300-ms inter-stimulus intervals, relative to WT littermates. (J–L) Representative traces (J) and summary plots (K) showing that LTP was normal in the triple mutant mice of γ-2^{SA} KI/γ-3 KO/γ-4 KO compared to WT control littermates. Sample traces were taken at times indicated by the numbers on the summary plot. (L) Normal LTP (WT: 146.68 ± 9.04, 3 animals/12 slices *vs.* γ-2^{SA} KI/γ-3KO/γ-4KO: 141.91 ± 5.49, 3 animals/15 slices; P= 0.36146). Data are shown as mean ± s.e.m.

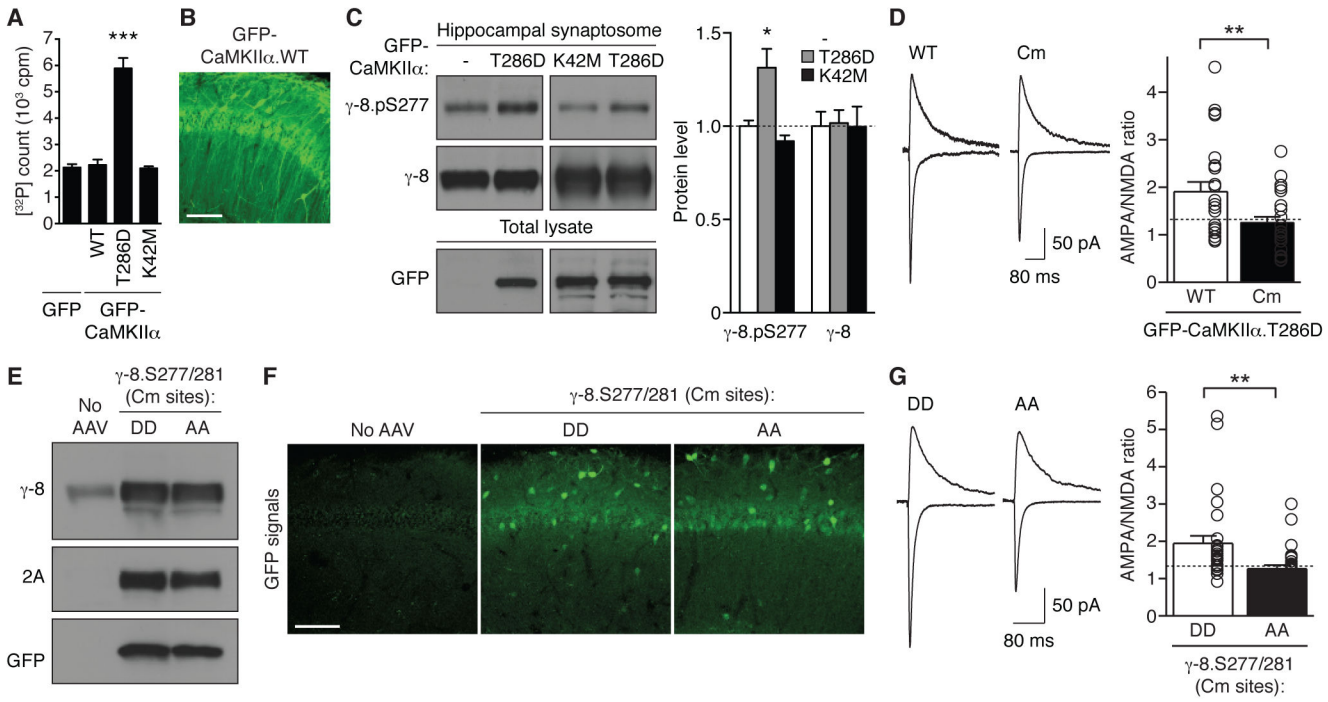

Figure 3. CaMKIIα induces synaptic insertion of AMPARs through TARPγ-8 phosphorylation

(A) CaMKIIα activity assay without calcium and calmodulin shows constitutive activity in CaMKIIα.T286D mutants compared to WT and kinase-inactive K42M mutants ($n = 4$). (B) Representative confocal image of hippocampal CA1 region infected with AAV carrying GFP-CaMKIIα.WT. Scale bar, 100 μm. (C) Stereotactic intra-hippocampal injection of adeno-associated virus expressing GFP-CaMKIIα.T286D increased TARPγ-8 phosphorylation at S277 significantly compared to both non-injected ($n = 4$) and K42M ($n = 3$) controls. No changes in TARPγ-8 protein levels were observed. GFP-CaMKIIα expression was confirmed in total lysates. (D) AAV-mediated expression of GFP-CaMKIIα.T286D induced AMPAR insertion into hippocampal CA1 synapses of WT mice, but not TARPγ-8 Cm mice. Representative averaged EPSCs (left) and summary data (right) showing that AMPA/NMDA ratios significantly reduced in TARPγ-8 Cm mice relative to WT controls (WT, 1.91 ± 0.2, 22 cells from 4 animals vs Cm, 1.26 ± 0.1, 22 cells from 4 animals; $P = 0.008$). (E) Two CaMKIIα phosphorylation sites of TARPγ-8 (S277 and S281) were mutated to phosphomimic aspartic acid (DD) or non-phosphomimic alanine (AA) and fused with the 2A peptide and GFP at their C-terminus (see Figure S3B). Protein expression of TARPγ-8 mutants was confirmed at DIV10 in primary hippocampal neurons upon AAV infection at DIV6. (F) Representative confocal images of hippocampal CA1 region infected with AAV carrying TARPγ-8 mutants-P2A-GFP. Specific GFP signal was observed upon AAV infection. Scale bar, 100 μm. (G) AAV-mediated expression of γ-8.S277/281DD, but not γ-8.S277/281AA, induces AMPAR insertion into hippocampal CA1 synapses of WT mice. Representative averaged EPSCs (left) and summary data (right) showing that AMPA/NMDA ratios significantly increased in γ-8.DD compared to γ-8.AA-expressing CA1 neurons (γ-8.DD, 1.94 ± 0.20, 29 cells from 8 animals; *vs.* γ-8.AA, 1.26 ± 0.10, 29 cells from 9 animals, $P = 0.0036$). Dashed lines in the graphs (D and G) indicate the AMPA/

NMDA ratio from non-injected WT mice in Figure 2D. Data are shown as mean ± s.e.m.; *P < 0.05, **P < 0.01 and ***P < 0.001. One-way ANOVA followed by post-hoc Tukey's multiple comparisons (A and C) and unpaired Student's t test (D and G).

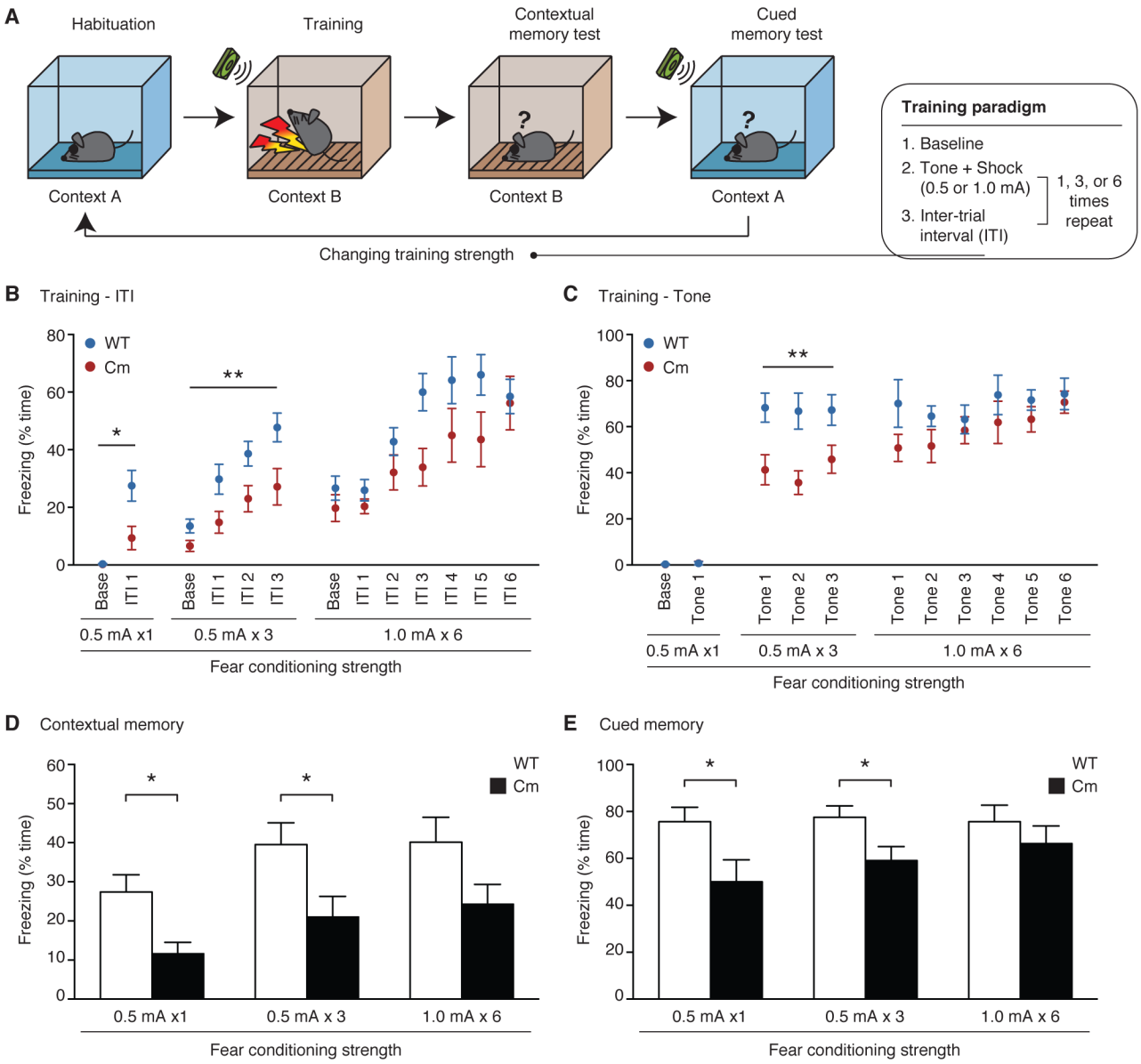

Figure 4. CaMKIIα phosphorylation of TARPγ-8 supports learning and memory

(A) Schematic representation of the contextual and cued fear conditioning paradigm employed. The illustrated series of sessions (habituation, training, contextual memory test, and cued memory test) was repeated three times with varying training intensity as indicated in the 'Training paradigm' box. (B and C) Learning curves for conditioning were shifted down significantly in TARPγ-8 Cm mice. All training sessions are plotted in separate graphs, dissociating baseline and inter-trial interval (ITI) periods in (B) and tone periods in (C). Both groups showed more freezing behavior with the accumulation of further training, but freezing levels induced by relatively mild shock intensity training (0.5 mA, 1–3 times) were significantly lower in TARP γ-8 Cm mice ($n = 7$) than in WT ($n = 8$) mice. Data are shown as mean ± s.e.m.; $*P < 0.05$, $**P < 0.01$, two-way repeated measures ANOVA. (D and E) Fear conditioning is impaired in TARP γ-8 Cm mice. Both contextual (D) and cued

(E) fear conditioning strength, as reflected by freezing behavior, were reduced in TARPγ-8 Cm mice ($n = 7$) relative to that in WT mice ($n = 8$). TARP γ-8 Cm mice were able to be conditioned up to the level of WT mice with extensive training. Data are shown as mean ± s.e.m.; $*P < 0.05$, two-tailed Student's t-tests.