Network compensation of cyclic GMP-dependent protein kinase II knockout in the hippocampus by Ca^{2+}-permeable AMPA receptors

Seonil Kim[a,1], Roseann F. Titcombe[a,1], Hong Zhang[b], Latika Khatri[a], Hiwot K. Girma[a], Franz Hofmann[c], Ottavio Arancio[b], and Edward B. Ziff[a,2]

[a]Department of Biochemistry and Molecular Pharmacology, New York University School of Medicine, New York, NY 10016; [b]Taub Institute for Research on Alzheimer's Disease and the Aging Brain, Columbia University, New York, NY 10032; and [c]Technical University of Munich, 80802 Munich, Germany

Edited by Michael Eldon Greenberg, Harvard Medical School, Boston, MA, and approved February 3, 2015 (received for review September 10, 2014)

Gene knockout (KO) does not always result in phenotypic changes, possibly due to mechanisms of functional compensation. We have studied mice lacking cGMP-dependent kinase II (cGKII), which phosphorylates GluA1, a subunit of AMPA receptors (AMPARs), and promotes hippocampal long-term potentiation (LTP) through AMPAR trafficking. Acute cGKII inhibition significantly reduces LTP, whereas cGKII KO mice show no LTP impairment. Significantly, the closely related kinase, cGKI, does not compensate for cGKII KO. Here, we describe a previously unidentified pathway in the KO hippocampus that provides functional compensation for the LTP impairment observed when cGKII is acutely inhibited. We found that in cultured cGKII KO hippocampal neurons, cGKII-dependent phosphorylation of inositol 1,4,5-trisphosphate receptors was decreased, reducing cytoplasmic Ca^{2+} signals. This led to a reduction of calcineurin activity, thereby stabilizing GluA1 phosphorylation and promoting synaptic expression of Ca^{2+}-permeable AMPARs, which in turn induced a previously unidentified form of LTP as a compensatory response in the KO hippocampus. Calcineurin-dependent Ca^{2+}-permeable AMPAR expression observed here is also used during activity-dependent homeostatic synaptic plasticity. Thus, a homeostatic mechanism used during activity reduction provides functional compensation for gene KO in the cGKII KO hippocampus.

LTP | Ca^{2+}-permeable AMPA receptors | gene knockout | calcineurin

Some gene deletions yield no phenotypic changes because of functional compensation by closely related or duplicate genes (1). However, such duplicate gene activity may not be the main compensatory mechanism in mouse (2), although this possibility is still controversial (3). A second mechanism of compensation is provided by alternative metabolic pathways or regulatory networks (4). Although such compensatory mechanisms have been extensively studied, especially in yeast and nematode (1), the roles of metabolic and network compensatory pathways are not well understood in mouse.

Long-term potentiation (LTP) and long-term depression (LTD) are long-lasting forms of synaptic plasticity that are thought to be the cellular basis for learning and memory and proper formation of neural circuits during development (5). NMDA receptor (NMDAR)-mediated synaptic plasticity is a generally agreed postsynaptic mechanism in the hippocampus (5). In particular, synaptic Ca^{2+} influx through NMDARs is critical for LTP and LTD through control of various protein kinases and phosphatases (6). LTP is in part dependent upon the activation of protein kinases, which phosphorylate target proteins (6). Several kinases are activated during the induction of LTP, including cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinases (cGKs) (6). In contrast, LTD results from activation of phosphatases that dephosphorylate target proteins (6), and calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase, is important for LTD expression (7). AMPA receptors (AMPARs) are postsynaptic glutamate receptors that mediate rapid excitatory transmission in the central nervous system (8). During LTP, activated kinases phosphorylate AMPARs, leading to synaptic trafficking of the receptors to increase synapse activity (5). For LTD, activation of postsynaptic phosphatases induces internalization of AMPARs from the synaptic membrane, thereby reducing synaptic strength (5). Therefore, both protein kinases and phosphatases control synaptic trafficking of AMPARs, underlying LTP and LTD.

AMPARs are tetrameric ligand-gated ion channels that consist of a combinatorial assembly of four subunits (GluA1–4) (9). Studies of GluA1 knockout (KO) mice show that GluA1 is critical for LTP in the CA1 region of the hippocampus (10). GluA1 homomers, like all GluA2-lacking/GluA1-containing receptors, are sensitive to polyamine block and are Ca^{2+}-permeable, whereas GluA2-containing AMPARs are Ca^{2+}-impermeable (9). Moreover, GluA1 is the major subunit that is trafficked from recycling endosomes to the synaptic membrane in response to neuronal activity (11). Phosphorylation of GluA1 within its intracellular carboxyl-terminal domain (CTD) can regulate AMPAR membrane trafficking (12). Several CTD phosphorylations regulate trafficking (6). In particular, PKA and cGKII both phosphorylate serine 845 of GluA1, increasing the level of extrasynaptic receptors (13, 14). Therefore, activation of PKA and cGKII during LTP induction increases GluA1 phosphorylation, which enhances AMPAR activity at synapses. On the other hand, calcineurin dephosphorylates serine 845 of

Significance

Deletion of genes in organisms does not always give rise to phenotypes because of the existence of compensation, even though phenotypes may be found when gene activity is blocked acutely. The cGMP-dependent kinase II knockout is a typical example of this apparent paradox. The knockout shows no evident impairment of LTP, whereas acute inhibition of kinase activity significantly decreases it. This paper describes a previously unidentified form of network-based compensation for cGMP-dependent kinase II gene knockout that is not dependent on expression of duplicate or paralogous genes. Furthermore, the compensation described here is mediated by a mechanism similar to that used by activity-dependent homeostatic synaptic plasticity, suggesting that neurons in complex brains may overcome an unexpected genetic lesion by using existing homeostatic mechanisms.

Author contributions: S.K., R.F.T., O.A., and E.B.Z. designed research; S.K., R.F.T., H.Z., L.K., and H.K.G. performed research; F.H. contributed new reagents/analytic tools; S.K., R.F.T., H.Z., L.K., O.A., and E.B.Z. analyzed data; and S.K., R.F.T., and E.B.Z. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]S.K. and R.F.T. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: edward.ziff@nyumc.org.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1417498112/-/DCSupplemental.

GluA1, which enables GluA1-containing AMPARs to be endocytosed from the plasma membrane during LTD (15, 16). This removes synaptic AMPARs, leading to reduction of receptor function during LTD. Taken together, the activity-dependent trafficking of synaptic GluA1 is regulated by the status of phosphorylation in the CTD, which provides a critical mechanism underlying LTP and LTD.

Several studies have shown that acute inhibition of cGKII impairs hippocampal LTP (13, 17, 18). However, cGKII KO animals show apparently normal LTP in the hippocampus (19), suggesting that a form of functional compensation takes place in the KO hippocampus. Here, we show that cGKII KO reduces Ca^{2+} signals by decreasing cGKII-dependent phosphorylation of inositol 1,4,5-trisphosphate receptors (IP3Rs), which in turn lowers calcineurin activity in hippocampal neurons, which stabilizes phosphorylation of GluA1 in homomeric, Ca^{2+}-permeable AMPARs (CPARs). This elevates CPARs at the synapse as a previously unidentified compensatory mechanism for hippocampal LTP in cGKII-deficient animals that is alternative to the form of LTP expressed in WT.

Results

Hippocampal LTP in the cGKII KO Is Not Impaired, and cGKI Does Not Provide Compensation. We first verified a function for cGKII in LTP by conducting electrophysiological recordings to measure field excitatory postsynaptic potentials (fEPSPs) of hippocampal slices from adult mice in the presence of 10 μM Rp-8–pCPT–cGMPS, inhibiting both cGKI and cGKII (20). LTP was evoked with theta-burst tetanus at Schaeffer collateral–CA1 synapses following 10 min of perfusion with 10 μM Rp-8–pCPT–cGMPS or vehicle (Fig. 1 *A* and *B*). As shown previously (13, 17), acute inhibition of cGKII in WT caused a robust decrease in LTP compared with vehicle-treated control (WT, 255.2 ± 13.1%, and WT + Rp-8–pCPT–cGMPS, 155.5 ± 22.2%, $P = 0.002$) (Fig. 1 *A* and *B*). We confirmed that hippocampal LTP in cGKII-deficient animals exhibited no obvious impairment, consistent with the previous finding (19) (Fig. 1 *A* and *B*). Input–output relation was measured in the both WT and KO hippocampus, showing KO animals exhibited normal synaptic transmission as shown previously (19) (Fig. 1*C*). Moreover, we treated KO hippocampal slices with 10 μM Rp-8–pCPT–cGMPS and found no significant differences between LTP in the absence and presence of the cGK inhibitor, suggesting that cGKI is not compensating for the absence of cGKII (Fig. 1 *A* and *B*), and consistent with the previous study showing that the double KO of cGKI and II has no LTP impairment in the hippocampus (19). Taken together, we confirm that acute inhibition of cGKII in WT hippocampal slices impairs LTP, whereas the cGKII KO hippocampus shows no apparent LTP defects and that compensation for cGKII KO is not provided by cGKI.

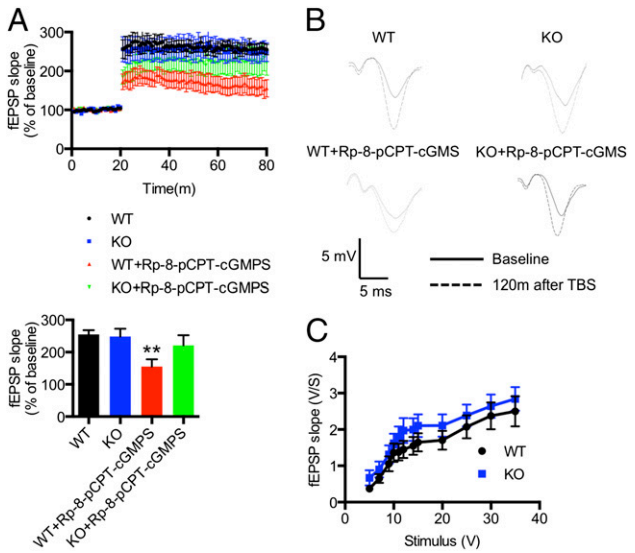

Fig. 1. No impairment of hippocampal LTP in the cGKII KO. (*A*) Inhibition of cGKs significantly decreases LTP induced by TBS in WT slices, but not in the KO. Summary graph shows mean fEPSP slopes measured 60 min after LTP induction (n = 10 KO, 8 KO + Rp-8–pCPT–cGMS, 9 WT, and 7 WT + Rp-8–pCPT–cGMS animals; $^{**}P < 0.01$, two-way ANOVA with Fisher's LSD test). (*B*) Representative raw traces of fEPSPs in each condition shown in *A*. Solid lines represent baseline braces before TBS, and dotted lines indicate traces 120 min after TBS. (*C*) Input–output relation shows no difference in WT and KO slices (n = 14 KO and 14 WT animals).

Synaptic Expression of CPARs Is Required for LTP in the cGKII KO Hippocampus. Because cGKII activity regulates synaptic strength through control of AMPAR trafficking during LTP expression (13), we studied the effect of the KO on spontaneous synaptic transmission by measuring miniature EPSCs (mEPSCs) in 14–17 d in vitro (DIV14–17) cultured mouse hippocampal neurons obtained from WT and KO animals (Fig. 2*A*). KO neurons exhibited increased average mEPSC amplitude (WT, 15.36 ± 0.43 pA, and KO, 18.17 ± 0.82 pA, $P = 0.0007$) (Fig. 2*B*), whereas mEPSC frequency was not changed (Fig. 2*C*). There was a significant decrease in mEPSC decay time (peak to 10%) in the KO (WT, 4.11 ± 0.30 pA, and KO, 2.79 ± 0.10 pA, $P < 0.0001$) (Fig. 2*D*). Because CPARs exhibit a shorter decay time (21–23), this also indicated the presence of CPARs at KO synapses. We next used 20 μM 1-naphthyl acetyl spermine (naspm), a blocker of CPARs, to determine if CPARs were responsible for an increase of the amplitude in KO neurons (Fig. 2*A*). Naspm treatment significantly reduced the increased amplitude in KO neurons (WT + naspm, 15.36 ± 0.43 pA, and KO + naspm, 13.81 ± 0.18 pA, $P < 0.0001$) (Fig. 2*B*), but frequency was not altered (Fig. 1*C*). Naspm also significantly increased decay time (WT + naspm, 4.19 ± 0.23 ms, and KO + naspm, 4.11 ± 0.16 ms, $P < 0.0001$) (Fig. 2*D*). The CPAR inhibitor had no effect on mEPSCs of WT neurons, suggesting that CPARs had no major functions under the basal WT condition (Fig. 2 *A*–*D*). Taken together, cGKII KO hippocampal neurons expressed CPARs at the basal synapse.

Several studies suggest that LTP is increased when CPARs are present in the hippocampus compared with WT (16, 24–26). Because we found synaptic expression of CPARs in KO hippocampal neurons, we hypothesized that CPAR expression could compensate in the KO for LTP impairment found when cGKII is acutely inhibited. To test this, we measured fEPSPs of hippocampal slices from WT or cGKII KO mice in the presence of 20 μM naspm (Fig. 2*E*). When LTP was evoked with theta-burst stimulation (TBS) following perfusion with 20 μM naspm, we found that CPAR inhibition completely blocked LTP in KO hippocampal slices (WT + naspm, 224.1 ± 15.2%, and KO + naspm, 110.2 ± 9.8%, $P < 0.0001$) (Fig. 2*E*). Consistent with previous findings (27), inhibition of CPARs had no effect on LTP in WT slices (Fig. 2*E*). Thus, this suggests that hippocampal LTP in the KO has a previously unidentified dependence on CPARs.

Because phosphorylation of serine 845 in the CTD of GluA1 [pGluA1(S845)] is important for synaptic CPAR expression (23, 28), we measured levels of pGluA1(S845) and other AMPAR subunits in purified postsynaptic density (PSD) from the hippocampus of both WT and KO animals. There were significantly increased levels of pGluA1(S845) ($P = 0.04$) and total GluA1 ($P < 0.0001$) and decreased levels of GluA2/3 ($P = 0.002$) in the PSD of the KO hippocampus compared with the PSD of WT (Fig. 3*A*). We further determined that surface GluA1 levels were increased ($P = 0.0008$), whereas surface GluA2/3 was reduced ($P = 0.048$) in cultured KO hippocampal neurons compared with WT neurons (Fig. 3*B*). This biochemical analysis suggests that homomeric GluA1 CPARs are expressed in cGKII KO hippocampal neurons, mediating LTP in the KO hippocampus.

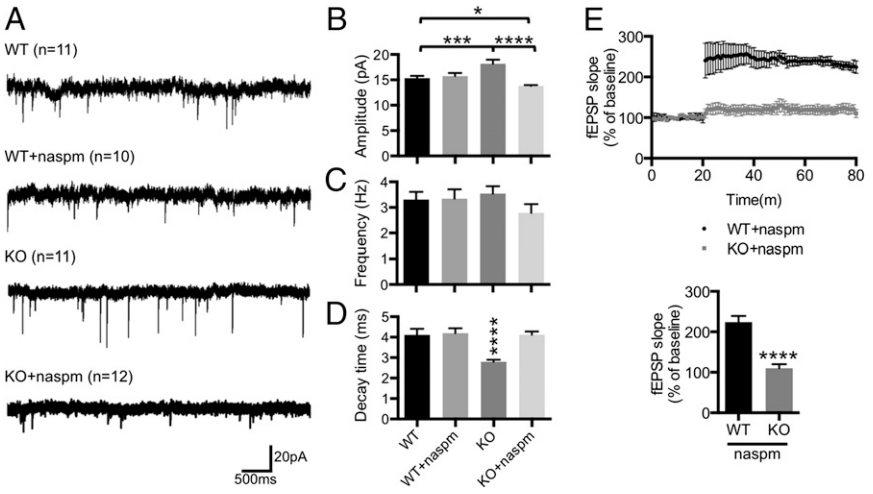

Fig. 2. Synaptic CPAR expression is required for hippocampal LTP in the KO. (*A*) Representative traces of mEPSC recordings in each condition (*n*, number of cells). (*B*) A CPAR-mediated increase in average mEPSC amplitude in the KO (*$P < 0.05$, ***$P < 0.001$, and ****$P < 0.0001$, one-way ANOVA with Fisher's LSD test). (*C*) No difference in average mEPSC frequency in each condition. (*D*) A CPAR-mediated decrease in average decay time (peak to 10%) in the KO (****$P < 0.0001$, one-way ANOVA with Fisher's LSD test). (*E*) Inhibition of CPAR blocks LTP in the KO but not in WT slices. Summary graph shows mean fEPSP slopes measured 60 min after LTP induction (n = 4 KO + naspm and 4 WT + naspm animals; ****$P < 0.0001$, two-way ANOVA with Fisher's LSD test).

CPAR-Dependent Hippocampal LTP in the KO Employs Distinct Synaptic Signaling. Given the similarity of PKA and cGKII functions, we considered the possibility that PKA activity was increased as a compensation for lack of cGKII, and thereby pGluA1(S845) levels were increased in the KO hippocampus. However, we found that there was no difference in PKA activity in KO and WT hippocampal neurons (Fig. S1). We also found that treatment of WT hippocampal slices with the PKA inhibitor, KT5720 (1 μM), resulted in a dramatic reduction in LTP compared with WT treated with vehicle (WT, 255.2 ± 13.1%, and WT + KT5720, 163.3 ± 15.8%, $P = 0.0002$) (Fig. 4*A*), consistent with the previously reported role of PKA in LTP (29). Interestingly, perfusion with KT5720 into KO slices had no significant effect on LTP (Fig. 4*A*), suggesting that a previously unidentified mechanism for LTP is used by the cGKII KO that is independent of cGKII and PKA but dependent on CPARs.

CPAR-mediated hippocampal LTP has been found in several conditions, including in the GluA2 KO (24, 25, 30) and following calcineurin inhibition (16, 23) and acute stress (26). The CPAR-mediated LTP is functionally distinct from classical NMDAR-mediated LTP (24–26, 30). Indeed, LTP in the KO was not dependent on PKA (Fig. 4*A*), consistent with LTP in GluA2 KO mice (30), in which all AMPARs are Ca^{2+}-permeable. CPAR-dependent LTP is completely inhibited by treating slices with a Ca^{2+} chelator, indicating that Ca^{2+} is required (30). Thus, we hypothesize that Ca^{2+} influx via CPARs in addition to via NMDARs in the cGKII KO induces an alternative synaptic signaling pathway that expresses a form of LTP that is distinct from WT. To test whether CPAR-mediated LTP in the cGKII KO was dependent on NMDARs, we treated slices with 100 μM (2*R*)-amino-5-phosphonopentanoate (APV), a blocker of NMDARs, and measured fEPSPs (Fig. 4*B*). APV treatment was sufficient to inhibit both WT and KO LTP (WT, 210.4 ± 18.3%, and WT + APV, 107.3 ± 9.5%, $P < 0.0001$; and KO, 176.4 ± 21.9%, and KO+APV, 100.9 ± 8.3%, $P = 0.0001$) (Fig. 4*B*), suggesting that CPAR-mediated LTP in the cGKII KO required NMDARs, consistent with the previous role of NMDARs in CPAR-mediated LTP in mice expressing GluA2 (16, 26). Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) also phosphorylates GluA1 during LTP induction, on serine 831, but is not required for CPAR-mediated LTP in the GluA2 KO hippocampus (30). The CaMKII inhibitor, KN-93 (10 μM), significantly reduced LTP in WT but not in KO slices (WT, 221.9 ± 18.1%, and WT + KN-93, 160.4 ± 16.1%, $P = 0.017$) (Fig. 4*C*), indicating that LTP in the KO is not dependent on CaMKII. PKC can phosphorylate GluA1 serine 818, and PKC-driven synaptic incorporation of GluA1 is important for LTP expression (31). In accordance with the established importance of PKC in LTP, fEPSPs showed a robust decrease in both WT and KO LTP upon treatment with the PKC inhibitor, chelerythrine (5 μM) (WT, 221.9 ± 18.1%, and WT + chelerythrine, 152.0 ± 7.9%, $P = 0.0002$; and KO, 239.4 ± 9.1%, and KO + chelerythrine, 177.0 ± 8.3%, $P = 0.0002$) (Fig. 4*D*). Taken together, although hippocampal LTP in the KO appears to be normal, it is PKA and CaMKII-independent but NMDAR and PKC-dependent, suggesting that CPAR-mediated LTP in the KO is significantly different from LTP mediated by the classical WT pathway.

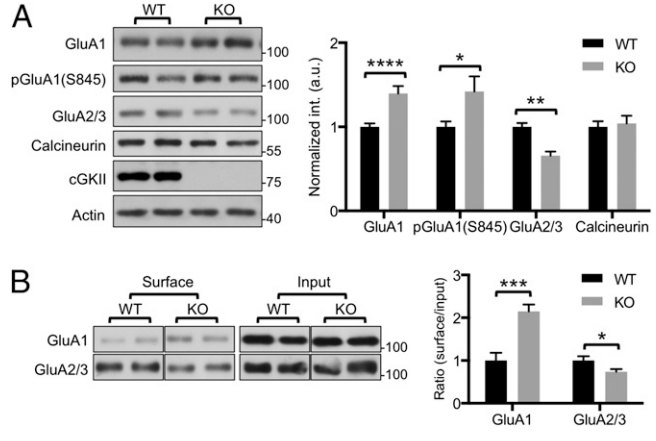

Fig. 3. Synaptic elevation of GluA1 and reduction of GluA2/3 in hippocampal KO neurons. (*A*) Representative immunoblots and quantitative analysis of PSD from the hippocampus of WT and KO mice showing GluA1 levels are increased whereas GluA2/3 levels are reduced in the KO (n = 3 WT and 3 KO animals; *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$, unpaired two-tailed Student's t tests). (*B*) Representative immunoblots of surface biotinylation and a summary graph in the WT and KO neurons showing surface GluA1 levels are elevated, but surface GluA2/3 is decreased in the KO (n = 3 experiments; *$P < 0.05$ and ***$P < 0.001$, unpaired two-tailed Student's t tests).

Calcineurin Activity Is Reduced in cGKII KO Hippocampal Neurons. Increasing the level of pGluA1(S845) can be achieved by decreasing phosphatase activity (23). To measure in vivo calcineurin activity directly, we used a fluorescence resonance energy transfer (FRET)-based calcineurin activity sensor (23, 32). Although we found that calcineurin protein levels were not changed in PSD of the KO hippocampus (Fig. 3*A*), KO neurons showed a significant reduction in calcineurin activity compared with WT cells ($P = 0.0006$) (Fig. 5). Taken together, calcineurin activity is significantly decreased in cGKII-deficient neurons, a change that stabilizes pGluA1(S845), and thereby expresses synaptic CPARs as a compensatory response. Notably, this or a

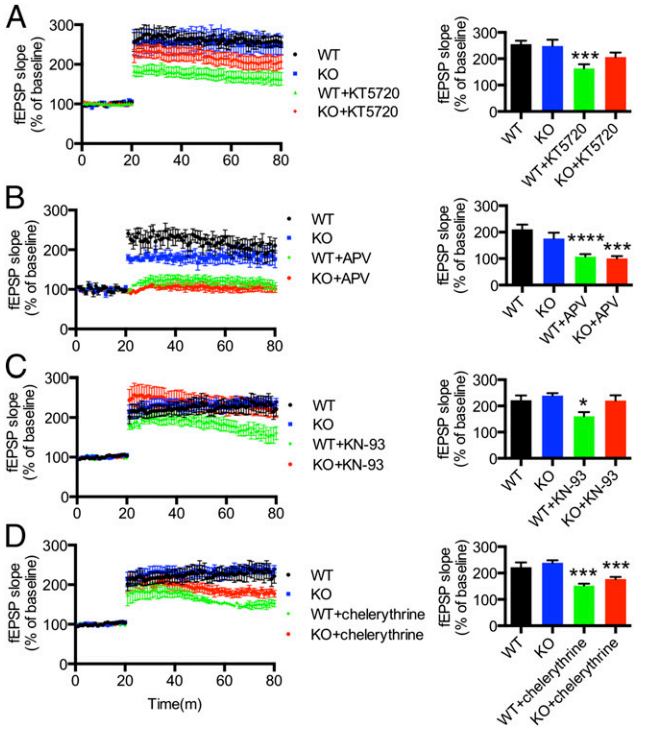

Fig. 4. PKA and CaMKII-independent but NMDAR and PKC-dependent LTP in the KO. (*A*) Inhibition of PKA activity significantly reduces hippocampal LTP in WT but not in KO slices (n = 13 KO + KT5720, 9 WT, 10 KO, and 11 WT + KT5720 animals; ***P < 0.001, two-way ANOVA with Fisher's LSD test). (*B*) NMDARs are required for LTP in both WT and KO hippocampus (n = 4 KO, 3 KO + APV, 4 WT, and 4 WT + APV animals; ***P < 0.001 and ****P < 0.0001, two-way ANOVA with Fisher's LSD test). (*C*) Inhibition of CaMKII activity significantly decreases hippocampal LTP in WT but not in KO slices (n = 7 KO, 11 KO + KN-93, 6 WT, and 8 WT + KN-93 animals; *P < 0.05, two-way ANOVA with Fisher's LSD test). (*D*) Inhibition of PKC significantly impairs LTP in both WT and KO hippocampus (n = 7 KO, 6 KO + chelerythrine, 6 WT, and 5 WT + chelerythrine animals; ***P < 0.001, two-way ANOVA with Fisher's LSD test). All summary graphs show mean fEPSP slopes measured 60 min after LTP induction.

closely related pathway is also used during activity deprivation-induced homeostatic synaptic plasticity (23).

Reduction of cGKII-Dependent IP3R Phosphorylation and Ca^{2+} Signals in cGKII KO Neurons Mediates Synaptic CPAR Expression. cGKs can regulate intracellular Ca^{2+} signaling in neurons, although the molecular mechanism is not yet understood (33). Because calcineurin activity is regulated by intracellular Ca^{2+} (34), we hypothesize that cGKII affects Ca^{2+} signaling in neurons. IP3Rs in the ER are responsible for intracellular Ca^{2+} release (35). Phosphorylation of serine 1756 in neuronal IP3Rs [pIP3R(S1756)] is mainly mediated by PKA and cGKs, and phosphorylation can increase Ca^{2+} currents in neuronal tissues (36). We tested whether neuronal IP3R phosphorylation was altered in the KO. Because both PKA and cGKs are able to phosphorylate serine 1756 in IP3Rs, we treated both WT and KO neurons with 1 μM KT5720 for 12 h to determine the role of cGKII in phosphorylation of serine 1756 selectively. We observed that pIP3R(S1756) levels were significantly decreased in KO neurons when PKA was inhibited (P = 0.0012) (Fig. 6*A*). Because phosphorylation of this site increases Ca^{2+} efflux from the ER (36), we next recorded spontaneous Ca^{2+} currents using GCaMP5 and measured total Ca^{2+} signals, as was done previously (23) (Fig. 6*B* and Fig. S2). WT neurons had high Ca^{2+} signals in cell bodies, whereas Ca^{2+} transients in KO cells were significantly decreased (P = 0.0005) (Fig. 6*B*), consistent with decreased cGKII-dependent IP3R (S1756) phosphorylation (Fig. 6*A*). To determine the effects of PKA or cGKs on Ca^{2+} transients in WT neurons, we added 1 μM KT5720 or 10 μM Rp-8–pCPT–cGMPS at the time of imaging and found that acute inhibition of either PKA (P = 0.0068) or cGKs (P = 0.0015) each blocked half of total Ca^{2+} signals (Fig. 6*B*). Treatment with both inhibitors together further decreased Ca^{2+} signals (P < 0.0001) (Fig. 6*B*), suggesting that both PKA and cGKs are important for GCaMP5 signals in WT neurons. Similar experiments were performed in KO neurons, and we found that PKA inhibition in KO neurons completely inhibited Ca^{2+} currents (P = 0.0009) (Fig. 6*B*), suggesting that PKA-mediated phosphorylation is responsible for Ca^{2+} signals found in KO cells. Conversely, 10 μM Rp-8–pCPT–cGMPS treatment in KO neurons had no effect on Ca^{2+} signals, in contrast to WT (Fig. 6*B*). Because 10 μM Rp-8–pCPT–cGMPS can inhibit both cGKI and cGKII activity (20), these results confirmed that cGKII, but not cGKI, had an important role in regulating Ca^{2+} signals via neuronal IP3R phosphorylation in the hippocampus. Moreover, 50 μM 2-Aminoethoxydiphenyl borate (2APB), a blocker of IP3Rs, significantly lowered Ca^{2+} signals in both WT (P < 0.0001) and KO neurons (P = 0.0012) (Fig. 6*B*), suggesting that the Ca^{2+} transient we observed here was dependent on IP3R-mediated ER Ca^{2+} efflux, which is in turn regulated by PKA and cGKII-dependent neuronal IP3R phosphorylation in hippocampal neurons. Taken together, both PKA and cGKII are able to phosphorylate IP3Rs, which increases Ca^{2+} currents from the ER in cultured hippocampal neurons. When cGKII is eliminated by the KO, cGKII-mediated pIP3R(S1756) levels are reduced, and thereby Ca^{2+} signals are lowered in KO hippocampal neurons, followed by a reduction of calcineurin activity that underlies the CPAR-dependent mechanism for LTP compensation in the cGKII KO.

Because IP3R-mediated Ca^{2+} currents were significantly affected in the KO (Fig. 6 *A* and *B*), we next examined a role of ER-released Ca^{2+} via IP3Rs in synaptic CPAR expression. We added the IP3R agonist, D-*myo*-IP3, hexapotassium salt (1 μM), to the internal electrode solution and measured mEPSCs in KO hippocampal neurons (Fig. 6*C*). Consistent with our findings, the activation of IP3Rs was sufficient to decrease the mEPSC amplitude (KO, 18.15 ± 0.79 pA, and KO + IP3, 14.73 ± 0.42 pA, P = 0.001) and increase the decay time (peak to 10%) (KO, 3.13 ± 0.25 ms, and KO + IP3, 3.95 ± 0.16 ms, P = 0.005) in the KO compared with neurons that were not treated with the agonist, whereas the mEPSC frequency was not altered in either condition (Fig. 6*C*). Ryanodine receptors (RyRs) are another type of Ca^{2+} channel in the ER that conducts Ca^{2+} release (35). The RyR activator, caffeine (1 mM), was applied to the internal electrode solution, and a role of RyR-mediated Ca^{2+} release in synaptic CPAR expression was assayed by measuring mEPSCs (Fig. 6*C*). Unlike the IP3R agonist, caffeine had no effect on mEPSCs in KO hippocampal neurons (Fig. 6*C*). This suggests that selective reduction of IP3R-mediated ER Ca^{2+} release

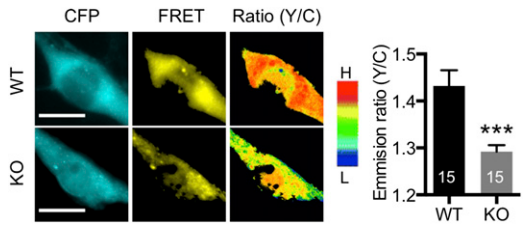

Fig. 5. In vivo calcineurin activity is reduced in hippocampal KO neurons. Shown are representative images of CFP channel, FRET channel, and pseudocolored emission ratio (Y/C) in each condition [blue (L), low emission ratio, and red (H), high emission ratio]. (Scale bar, 10 μm.) A summary graph shows a decrease in average of the emission ratio (Y/C) in the KO (n, number of cells; ***P < 0.001, unpaired two-tailed Student's t tests).

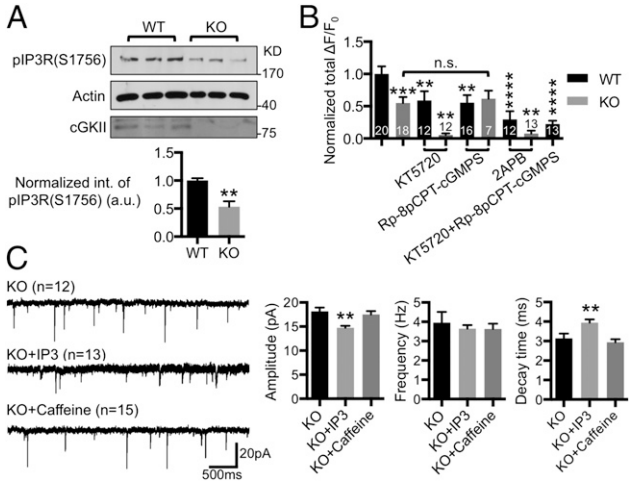

Fig. 6. Reduction of cGKII-dependent pIP3R(S1756) and Ca^{2+} signals in hippocampal KO neurons mediates synaptic CPAR expression. (*A*) Representative immunoblots and quantitative analysis of pIP3R(S1756) levels in WT and KO neurons in the presence of the PKA inhibitor showing that pIP3R(S1756) is reduced in the KO ($n = 3$ experiments; $^{**}P < 0.01$, unpaired two-tailed Student's *t* tests). (*B*) Normalized average of total Ca^{2+} signals in each condition reveals that reduced IP3R mediated Ca^{2+} signals in the KO (*n*, number of neurons; $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$, one-way ANOVA with Fisher's LSD test). (*C*) Representative traces of mEPSC recordings in each condition (*n*, number of cells), average mEPSC amplitude, frequency, and decay time (peak to 10%) showing selective IP3R-dependent synaptic CPAR expression in the KO ($^{**}P < 0.01$, one-way ANOVA with Fisher's LSD test).

provides a cellular basis for synaptic CPAR expression possibly via a decrease in calcineurin activity.

Tetrodotoxin-Induced Homeostatic Synaptic Plasticity Is Occluded in the cGKII KO. Notably, the Ca^{2+} and calcineurin-mediated pathway used for compensation in the KO identified here is similar to the one that provides homeostatic synaptic plasticity following activity reduction by tetrodotoxin (TTX) (23). If the cGKII KO and TTX treatments use the same pathway, it is possible that TTX will be unable to induce homeostatic synaptic plasticity in KO neurons. To test this, we treated KO hippocampal neurons with 2 μM TTX for 48 h and measured mEPSCs (Fig. S3*A*). We found that TTX treatment had no effect on mEPSCs in KO neurons (Fig. S3*A*), suggesting that the pathway mediated by reduction of Ca^{2+} signaling and calcineurin activity in KO hippocampal neurons occludes TTX-induced homeostatic plasticity. Previous studies of homeostatic synaptic plasticity in cultured cortical neurons showed that all synapses on a neuron globally change their strength by the same factor, making the mechanism multiplicative (37). In contrast, in cultured hippocampal neurons, homeostatic changes differentially alter synapses, implying that homeostatic changes in these neurons do not follow a strictly multiplicative mechanism (22). Moreover, experience-induced homeostatic plasticity in visual cortex in adult mice does not occur via a global multiplicative mechanism (38). Cumulative probability distribution of the mEPSC amplitude was significantly increased in cGKII KO hippocampal neurons (Fig. S3*B*). Furthermore, distribution of the KO scaled down fitted to WT; thus, our compensatory mechanism in the KO followed a multiplicative process (Fig. S3*B*). This suggests that synaptic compensation in cGKII KO hippocampal neurons operates via the Ca^{2+} and calcineurin-dependent mechanism but is multiplicative homeostatic synaptic plasticity.

Discussion

The effects of genotypic changes on phenotype may be influenced by complex gene interactions (39). For example, deletion of genes in organisms does not always give rise to phenotypes because of the existence of compensatory pathways, even though a phenotype may be observed if gene activity is blocked acutely in vitro (1). The cGKII KO is a typical example of this apparent paradox, as the KO shows no impairment of hippocampal LTP, whereas acute in vitro inhibition of kinase activity blocks LTP (13, 17–19). Here, we describe a previously unidentified alternative pathway that compensates for cGKII deletion. In the WT hippocampus, TBS leads to the influx of Ca^{2+} through NMDARs and an increase in synaptic Ca^{2+}, a trigger for LTP (5). This can activate several kinases, including PKA and cGKII, that phosphorylate GluA1 to increase synaptic expression of AMPARs during LTP expression (6). Moreover, PKA and cGKII can phosphorylate IP3Rs to increase Ca^{2+} currents from the ER, which activates calcineurin. Activated calcineurin provides a negative feedback loop that regulates IP3R and Ca^{2+} signaling via dephosphorylation of IP3Rs (40). Calcineurin also dephosphorylates GluA1, promoting endocytosis of GluA1. This indicates that kinases and calcineurin act antagonistically on both IP3R-dependent Ca^{2+} signaling and GluA1 trafficking during LTP expression. Conversely, in the cGKII KO hippocampus, cGKII-dependent phosphorylation of neuronal IP3Rs is decreased, leading to a reduction of Ca^{2+} signals. This lowers calcineurin activity, which stabilizes pGluA1(S845) and promotes synaptic expression of CPARs. We further show that synaptic CPARs provide a previously unidentified PKA and CaMKII-independent but NMDAR and PKC-dependent form of LTP as a part of a compensatory process in the cGKII KO hippocampus. Interestingly, requirement of NMDARs for CPAR-mediated hippocampal LTP is still controversial. Notably, CPAR-dependent LTP in GluA2 KO mice is NMDAR-independent (24–26, 30), whereas other conditions that express synaptic CPARs in the presence of endogenous GluA2 show NMDAR-dependent LTP (16, 26). Our cGKII KO model, which expresses GluA2, also requires NMDARs for CPAR-dependent LTP (Fig. 4*B*). This suggests that GluA2 expression may contribute to this difference. Taken together, although hippocampal LTP in the cGKII KO appears to be normal, a distinct pathway is used. Indeed, our recent findings that cGKII KO mice display behavioral abnormalities (41, 42) suggest that the compensatory mechanism is imperfect and does not fully restore cGKII-dependent function.

In primary sensory neurons of the dorsal root ganglia, cGKI also mediates potentiation of IP3R activity through phosphorylation of the receptor, leading to increased Ca^{2+} release from the ER, which is functionally linked to synaptic LTP (43). IP3R-associated cGK substrate (IRAG) is required for cGKI-mediated phosphorylation of IP3Rs (44). Notably, IRAG is not expressed in the hippocampus of mouse brains (45). Therefore, cGKI may not be a major regulator of IP3R-mediated Ca^{2+} release in the hippocampus, although it is important for controlling Ca^{2+} currents through IP3Rs in other tissues. In contrast, because cGKII can be associated with ER membranes (13), it may directly phosphorylate neuronal IP3Rs to increase Ca^{2+} release upon nitric oxide signaling activation in the hippocampus.

Phosphorylation of Dopamine- and cAMP-regulated phosphoprotein 32 (DARPP32), a substrate for cGKs (46), is significantly decreased in the cGKII KO striatum (Fig. S4*A*). Moreover, neuronal nitric oxide synthase (nNOS) is highly expressed in the striatum (47). This suggests that cGK-mediated NO signaling plays critical roles in the striatum. We also have shown previously that levels of pGluA1(S845) are reduced in the PSD of the prefrontal cortex (PFC) in the KO relative to WT (42). Thus, unlike the hippocampus, there are kinase-deficiency phenotypes in the PFC and striatum, indicating that the compensatory mechanism we have demonstrated here may not act globally in the KO. In particular, significantly higher cGKII protein levels are found in these areas in WT, whereas the hippocampus contains lower levels of cGKII (Fig. S4*B*). This indicates that cGKII function in the PFC and striatum may not be replaced or compensated in the KO, unlike the hippocampus, where compensation is evident.

Deletion of the cGKII gene cannot be compensated simply by the activity of cGKI in hippocampal LTP, suggesting the existence of an alternative, nonparalogous gene-dependent compensatory pathway. The existence of such a pathway is confirmed here, and the pathway is shown to be CPAR-dependent. The current work provides an example of how a complex brain system may adjust to the effects of gene deletion to recover function through compensation achieved by a mechanism of synaptic homeostasis. This finding extends the scope of calcineurin and CPAR-dependent homeostatic synaptic plasticity first described for activity deprivation (23) to compensation for gene KO. Such forms of network compensation may operate more generally to compensate for genetic lesions of the brain.

Materials and Methods

Mouse Hippocampal Neuron Cultures. All animal studies were performed with an approved protocol from New York University Langone Medical Center's Institutional Animal Care and Use Committee. Hippocampal primary neurons were prepared by a modification of the previously described method (23).

Slice Electrophysiology. Slice electrophysiology was performed as described previously (13).

mEPSC Recording. mEPSCs were measured in hippocampal neurons cultured from WT and KO animals (23).

Synaptosome Purification and Surface Biotinylation. PSD fractions from the WT and KO hippocampus were prepared as described previously (48).

Statistics. All statistical comparisons were analyzed with the GraphPad Prism6 software. Unpaired two-tailed Student's t tests were used in single comparisons. For multiple comparisons except LTP analysis, we used one-way ANOVA followed by Fisher's Least Significant Difference (LSD) test to determine statistical significance. For LTP, two-way ANOVA followed by Fisher's LSD test was used to determine the difference. The Kolmogorov–Smirnov test was used for comparisons of cumulative probabilities. Results were represented as a mean ± SEM, and a P value < 0.05 was considered statistically significant.

Details of the materials and methods are given in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank members of the E.B.Z. and O.A. laboratories for helpful discussions, Dr. Loren Looger (Janelia Farm) for providing the GCaMP5 construct, and Jin Zhang (Johns Hopkins University) for providing the FRET-based calcineurin biosensor. FRET assay was done in the Microscopy Core of New York University Langone Medical Center. This work was supported by a Blas Frangione postdoctoral fellowship (to S.K.), NIH Grant 5T32GM07308 (to R.F.T.), and NIH Grant 5R01MH067229 (to E.B.Z. and O.A.).

1. Hanada K, et al. (2009) Evolutionary persistence of functional compensation by duplicate genes in Arabidopsis. *Genome Biol Evol* 1:409–414.
2. Liao BY, Zhang J (2007) Mouse duplicate genes are as essential as singletons. *Trends Genet* 23(8):378–381.
3. Su Z, Wang J, Gu X (2014) Effect of duplicate genes on mouse genetic robustness: An update. *BioMed Research International* 2014:758672.
4. Gu Z, et al. (2003) Role of duplicate genes in genetic robustness against null mutations. *Nature* 421(6918):63–66.
5. Malenka RC, Bear MF (2004) LTP and LTD: An embarrassment of riches. *Neuron* 44(1):5–21.
6. Lee HK (2006) Synaptic plasticity and phosphorylation. *Pharmacol Ther* 112(3): 810–832.
7. Mulkey RM, Endo S, Shenolikar S, Malenka RC (1994) Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. *Nature* 369(6480):486–488.
8. Barry MF, Ziff EB (2002) Receptor trafficking and the plasticity of excitatory synapses. *Curr Opin Neurobiol* 12(3):279–286.
9. Cull-Candy S, Kelly L, Farrant M (2006) Regulation of Ca2+-permeable AMPA receptors: Synaptic plasticity and beyond. *Curr Opin Neurobiol* 16(3):288–297.
10. Zamanillo D, et al. (1999) Importance of AMPA receptors for hippocampal synaptic plasticity but not for spatial learning. *Science* 284(5421):1805–1811.
11. Ehlers MD (2000) Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. *Neuron* 28(2):511–525.
12. Derkach VA, Oh MC, Guire ES, Soderling TR (2007) Regulatory mechanisms of AMPA receptors in synaptic plasticity. *Nat Rev Neurosci* 8(2):101–113.
13. Serulle Y, et al. (2007) A GluR1-cGKII interaction regulates AMPA receptor trafficking. *Neuron* 56(4):670–688.
14. Roche KW, O'Brien RJ, Mammen AL, Bernhardt J, Huganir RL (1996) Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. *Neuron* 16(6): 1179–1188.
15. Lee HK, Kameyama K, Huganir RL, Bear MF (1998) NMDA induces long-term synaptic depression and dephosphorylation of the GluR1 subunit of AMPA receptors in hippocampus. *Neuron* 21(5):1151–1162.
16. Sanderson JL, et al. (2012) AKAP150-anchored calcineurin regulates synaptic plasticity by limiting synaptic incorporation of Ca2+-permeable AMPA receptors. *J Neurosci* 32(43):15036–15052.
17. Lu YF, Kandel ER, Hawkins RD (1999) Nitric oxide signaling contributes to late-phase LTP and CREB phosphorylation in the hippocampus. *J Neurosci* 19(23):10250–10261.
18. Zhuo M, Hu Y, Schultz C, Kandel ER, Hawkins RD (1994) Role of guanylyl cyclase and cGMP-dependent protein kinase in long-term potentiation. *Nature* 368(6472): 635–639.
19. Kleppisch T, et al. (1999) Long-term potentiation in the hippocampal CA1 region of mice lacking cGMP-dependent kinases is normal and susceptible to inhibition of nitric oxide synthase. *J Neurosci* 19(1):48–55.
20. Gamm DM, Francis SH, Angelotti TP, Corbin JD, Uhler MD (1995) The type II isoform of cGMP-dependent protein kinase is dimeric and possesses regulatory and catalytic properties distinct from the type I isoforms. *J Biol Chem* 270(45):27380–27388.
21. Mosbacher J, et al. (1994) A molecular determinant for submillisecond desensitization in glutamate receptors. *Science* 266(5187):1059–1062.
22. Thiagarajan TC, Lindskog M, Tsien RW (2005) Adaptation to synaptic inactivity in hippocampal neurons. *Neuron* 47(5):725–737.
23. Kim S, Ziff EB (2014) Calcineurin mediates synaptic scaling via synaptic trafficking of Ca2+-permeable AMPA receptors. *PLoS Biol* 12(7):e1001900.
24. Jia Z, et al. (1996) Enhanced LTP in mice deficient in the AMPA receptor GluR2. *Neuron* 17(5):945–956.
25. Wiltgen BJ, et al. (2010) A role for calcium-permeable AMPA receptors in synaptic plasticity and learning. *PLoS ONE* 5(9):e12818.
26. Whitehead G, et al. (2013) Acute stress causes rapid synaptic insertion of Ca2+-permeable AMPA receptors to facilitate long-term potentiation in the hippocampus. *Brain* 136(Pt 12):3753–3765.
27. Gray EE, Fink AE, Sariñana J, Vissel B, O'Dell TJ (2007) Long-term potentiation in the hippocampal CA1 region does not require insertion and activation of GluR2-lacking AMPA receptors. *J Neurophysiol* 98(4):2488–2492.
28. He K, et al. (2009) Stabilization of Ca2+-permeable AMPA receptors at perisynaptic sites by GluR1-S845 phosphorylation. *Proc Natl Acad Sci USA* 106(47):20033–20038.
29. Frey U, Huang YY, Kandel ER (1993) Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. *Science* 260(5114):1661–1664.
30. Asrar S, Zhou Z, Ren W, Jia Z (2009) Ca(2+) permeable AMPA receptor induced long-term potentiation requires PI3/MAP kinases but not Ca/CaM-dependent kinase II. *PLoS ONE* 4(2):e4339.
31. Boehm J, et al. (2006) Synaptic incorporation of AMPA receptors during LTP is controlled by a PKC phosphorylation site on GluR1. *Neuron* 51(2):213–225.
32. Newman RH, Zhang J (2008) Visualization of phosphatase activity in living cells with a FRET-based calcineurin activity sensor. *Mol Biosyst* 4(6):496–501.
33. Wang X, Robinson PJ (1997) Cyclic GMP-dependent protein kinase and cellular signaling in the nervous system. *J Neurochem* 68(2):443–456.
34. Mehta S, Zhang J (2014) Using a genetically encoded FRET-based reporter to visualize calcineurin phosphatase activity in living cells. *Methods Mol Biol* 1071:139–149.
35. Bading H (2013) Nuclear calcium signalling in the regulation of brain function. *Nat Rev Neurosci* 14(9):593–608.
36. Wagner LE, 2nd, Li WH, Yule DI (2003) Phosphorylation of type-1 inositol 1,4,5-trisphosphate receptors by cyclic nucleotide-dependent protein kinases: A mutational analysis of the functionally important sites in the S2+ and S2- splice variants. *J Biol Chem* 278(46):45811–45817.
37. Turrigiano GG, Nelson SB (2004) Homeostatic plasticity in the developing nervous system. *Nat Rev Neurosci* 5(2):97–107.
38. Goel A, Lee HK (2007) Persistence of experience-induced homeostatic synaptic plasticity through adulthood in superficial layers of mouse visual cortex. *J Neurosci* 27(25): 6692–6700.
39. Hartman JL, 4th, Garvik B, Hartwell L (2001) Principles for the buffering of genetic variation. *Science* 291(5506):1001–1004.
40. Heindorff K, Baumann O (2014) Calcineurin is part of a negative feedback loop in the InsP3/Ca^{2+} signalling pathway in blowfly salivary glands. *Cell Calcium* 56(3):215–224.
41. Wincott CM, et al. (2014) cGMP-dependent protein kinase type II knockout mice exhibit working memory impairments, decreased repetitive behavior, and increased anxiety-like traits. *Neurobiol Learn Mem* 114:32–39.
42. Wincott CM, et al. (2013) Spatial memory deficits and motor coordination facilitation in cGMP-dependent protein kinase type II-deficient mice. *Neurobiol Learn Mem* 99: 32–37.
43. Luo C, et al. (2012) Presynaptically localized cyclic GMP-dependent protein kinase 1 is a key determinant of spinal synaptic potentiation and pain hypersensitivity. *PLoS Biol* 10(3):e1001283.
44. Schlossmann J, et al. (2000) Regulation of intracellular calcium by a signalling complex of IRAG, IP3 receptor and cGMP kinase Ibeta. *Nature* 404(6774):197–201.
45. Geiselhöringer A, Gaisa M, Hofmann F, Schlossmann J (2004) Distribution of IRAG and cGKI-isoforms in murine tissues. *FEBS Lett* 575(1-3):19–22.
46. Tsou K, Snyder GL, Greengard P (1993) Nitric oxide/cGMP pathway stimulates phosphorylation of DARPP-32, a dopamine- and cAMP-regulated phosphoprotein, in the substantia nigra. *Proc Natl Acad Sci USA* 90(8):3462–3465.
47. Bredt DS, Hwang PM, Snyder SH (1990) Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* 347(6295):768–770.
48. Restituito S, et al. (2011) Synaptic autoregulation by metalloproteases and γ-secretase. *J Neurosci* 31(34):12083–12093.