Inositol polyphosphate multikinase mediates extinction of fear memory

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Inositol polyphosphate multikinase (IPMK), the key enzyme for the biosynthesis of higher inositol polyphosphates and phosphatidylinositol 3,4,5-trisphosphate, also acts as a versatile signaling player in regulating tissue growth and metabolism. To elucidate neurobehavioral functions of IPMK, we generated mice in which IPMK was deleted from the excitatory neurons of the postnatal forebrain. These mice showed no deficits in either novel object recognition or spatial memory. IPMK conditional knockout mice formed cued fear memory normally but displayed enhanced fear extinction. Signaling analyses revealed dysregulated expression of neural genes accompanied by selective activation of the mechanistic target of rapamycin (mTOR) regulatory enzyme p85 S6 kinase 1 (S6K1) in the amygdala following fear extinction. The IPMK mutants also manifested facilitated hippocampal long-term potentiation. These findings establish a signaling action of IPMK that mediates fear extinction.

inositol polyphosphate multikinase | memory | fear extinction | long-term potentiation

Inositol polyphosphate multikinase (IPMK) is a pleiotropic protein. As an enzyme, it catalyzes the production of water-soluble inositol polyphosphates (IPs), such as inositol 1,4,5,6-tetrakisphosphate [Ins(1,4,5,6)P_4] (1, 2) and lipid-bound phosphatidylinositol 3,4,5-triphosphates (3, 4). The catalytic products of IPMK directly influence downstream target proteins. For example, deletion of IPMK in mouse embryonic fibroblasts reduced Akt kinase signaling by interrupting the PI-3 kinase activity of IPMK (4). In addition to its catalytic role in inositol phosphate metabolism, IPMK noncatalytically regulates major signaling factors, including mechanistic target of rapamycin (mTOR), p53, serum response factor, and tumor necrosis factor receptor-associated factor 6 (5–8). In mammalian cells, therefore, IPMK coordinates the activity of diverse signaling networks involved in gene expression, growth, metabolism, and innate immunity (9–11). In a previous study, our group reported that the conditional *Ipmk* knockout during the early embryonic stage of neurodevelopment by using *Nestin-Cre* transgenic mice blunted the response of immediate early genes to electroconvulsive stimuli and exhibited a spatial memory deficit under the Barnes maze test (12). However, nothing is known of IPMK's physiologic impact on neural functions in the context of specific neuronal cell types.

Memory of fearful events forms quickly and is difficult to erase. In rodent models, fear can be created via Pavlovian conditioning paradigms, in which a biologically neutral stimulus (such as a tone) or context (conditioned stimulus, or CS) is paired with an aversive, unconditioned stimulus (US, such as a footshock). The memory of the CS–US association is robust and long-lasting, but if the CS is repeatedly presented in the absence of the US, the fear response is gradually reduced through a process known as extinction (13, 14). Extinction of fear memory is learned by the formation of new memories that suppress conditioning-generated fear memories (15, 16). Molecular mechanisms and neural systems that underlie the formation of fear memories are relatively well known, but the fundamental principles underlying fear extinction still remain unclear (17–19). Similar to fear conditioning, extinction appears to depend on reciprocal connections among the medial prefrontal cortex, the amygdala, and the hippocampus (20–22). Only a few signaling molecules, such as histone deacetylase (23, 24), protein kinase A (25, 26), calcineurin (27), and p85 S6 kinase 1 (S6K1) (28), have been linked to fear memory extinction. Despite their potential importance in the therapy of dysregulated fear responses [e.g., posttraumatic stress disorder (PTSD) and phobia], there has been limited understanding into molecular players mediating fear extinction.

In the present study, we have generated IPMK conditional knockout mice in which *Ipmk* was deleted from the excitatory neurons of the postnatal forebrain. These mice exhibited selective enhancement of fear extinction without any alteration of either fear conditioning or spatial memory. Following fear extinction, p85 S6K1 signaling was selectively elevated in the amygdala of IPMK conditional knockout mice. Measurements of synaptic strength

Significance

Inositol polyphosphate multikinase (IPMK) is a pleiotropic enzyme for the biosynthesis of inositol polyphosphates and phosphatidylinositol 3,4,5-trisphosphate, but the neural function of IPMK has remained largely elusive. By conditionally deleting IPMK from the excitatory neurons of the postnatal forebrain in mice, we found that IPMK knockout mice exhibited normal cued fear conditioning but showed enhanced fear extinction accompanied by selective activation of p85 S6 kinase 1 in the IPMK-null amygdala following fear extinction. Electrophysiological recording analyses further uncovered facilitated hippocampal long-term potentiation from IPMK knockout mice. Thus, our data propose IPMK as a molecular player in the control of fear extinction.

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Conflict of interest statement: S.K. and P.-G.S. are coauthors on a 2016 research article; they did not collaborate directly on the paper.

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in hippocampal slices revealed facilitated late long-term potentiation (L-LTP). Together, our results define IPMK as a regulator of fear extinction and synaptic plasticity.

Results

Deletion of *Ipmk* from Forebrain Excitatory Neurons Does Not Affect Brain Morphology. Physiological roles of IPMK in mature neural circuits are poorly understood because the global deletion of *Ipmk* results in lethality as early as embryonic day 9.5 (29). To investigate the effect of IPMK loss on neural function in adult animals, we crossed floxed *Ipmk* mice ($Ipmk^{f/f}$) with *CaMKII-Cre* mice to generate mice in which IPMK was selectively deleted from the forebrain excitatory neurons (Fig. 1*A*). We confirmed markedly diminished IPMK expression in forebrain regions, such as the hippocampus and amygdala, but not in the cerebellum (Fig. 1*B*). $Ipmk^{f/f}$;*CaMKII-Cre* mice (n = 14) appeared healthy with no gross impairment, and at age 16 wk had similar body weights as littermate control $Ipmk^{f/f}$ mice (n = 11) (Fig. 1 *C–E*). Histological analysis using hematoxylin and eosin (H&E) staining and Nissl staining of $Ipmk^{f/f}$;*CaMKII-Cre* revealed normal brain architecture (Fig. 1 *F* and *G*). Immunostaining of the mature neuron marker NeuN and astrocyte marker GFAP showed no morphological defects in the brains of $Ipmk^{f/f}$;*CaMKII-Cre* mice (Fig. 1*H*). We further found no difference in the staining pattern or intensity of markers for excitatory (e.g., VGLUT1, Shank2) and inhibitory (VGAT) synapses in $Ipmk^{f/f}$;*CaMKII-Cre* and littermate control mice (*SI Appendix*, Fig. S1), indicating that the selective deletion of *Ipmk* in postnatal glutamatergic neurons does not influence neuronal complexity.

We next performed behavioral tests to assess basal motor functions. There was no significant difference between control and knockout mice in the rotarod test (Fig. 1 *I* and *J*). Thus, $Ipmk^{f/f}$;*CaMKII-Cre* mice have normal brain anatomical features and motor function.

$Ipmk^{f/f}$;*CaMKII-Cre* Mice Show Normal Cognition and Spatial Memory Behavior. We examined whether $Ipmk^{f/f}$;*CaMKII-Cre* mice exhibit impaired learning or memory. To evaluate the role of IPMK forebrain signaling in working memory, we analyzed spontaneous alternation in the Y-maze. $Ipmk^{f/f}$ and $Ipmk^{f/f}$;*CaMKII-Cre* mice engaged in similar numbers of alternations (*SI Appendix*, Fig. S2 *A* and *B*), suggesting that working memory of $Ipmk^{f/f}$;*CaMKII-Cre* mice is intact. To examine whether IPMK deletion affects recognition memory, we used the novel object recognition test. $Ipmk^{f/f}$;*CaMKII-Cre* and $Ipmk^{f/f}$ mice showed similar levels of preference for the novel object at the retention phase (*SI Appendix*, Fig. S2 *C–E*), which indicates that IPMK deletion does not affect recognition memory in mice. We next subjected $Ipmk^{f/f}$;*CaMKII-Cre* and control mice to the Morris water maze: mice were trained to find a platform hidden in cloudy water over the course of 7 d (two-way ANOVA, $F_{1,140} = 1.764$, $P \geq 0.05$); on day 8, the platform was removed, and mice were subjected to a 60-s probe trial (*SI Appendix*, Fig. S2 *F* and *G*). $Ipmk^{f/f}$;*CaMKII-Cre* and control $Ipmk^{f/f}$ mice did not differ in their acquisition of the platform location (two-way ANOVA, $F_{1,60} = 0.083$, $P \geq 0.05$) and exhibited a similar preference for the target quadrant during the probe trial (*SI Appendix*, Fig. S2 *H* and *I*). Therefore, we conclude that $Ipmk^{f/f}$;*CaMKII-Cre* mice have no defect in cognition as well as spatial memory.

$Ipmk^{f/f}$;*CaMKII-Cre* Mice Display Enhanced Fear Extinction. Next, we employed a classical fear-conditioning test to assess the cognitive responses of $Ipmk^{f/f}$;*CaMKII-Cre* mice to contextual cues (*SI Appendix*, Fig. S3). In this paradigm, an association between a footshock (unconditioned stimulus; US) and either the context or an audible tone (conditioned stimulus; CS) results in learned fear. At later time points, freezing responses are measured in

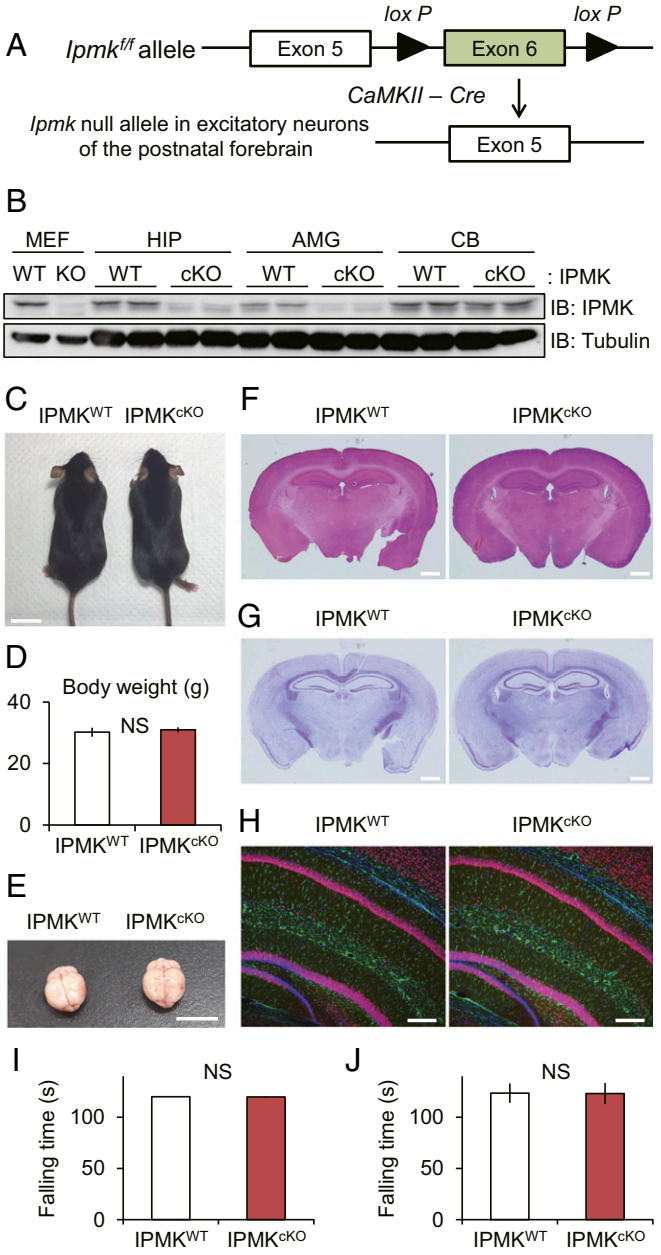

Fig. 1. Generation and characterization of $Ipmk^{f/f}$;*CaMKII-Cre* mice. (*A*) Diagram depicting the structure of *Ipmk*, with emphasis on exons 5 and 6 and the lox P-insertion site near exon 6. We used floxed *Ipmk* mice without Cre expression as controls ($Ipmk^{f/f}$; $IPMK^{WT}$) in our experiments. (*B*) IPMK protein levels were reduced in the hippocampus and amygdala of $Ipmk^{f/f}$;*CaMKII-Cre* ($IPMK^{cKO}$) mice. Samples of cerebellum, which does not express the *CaMKII-Cre* gene, showed similar IPMK levels between of $IPMK^{cKO}$ and $IPMK^{WT}$ mice. (*C*) Representative images of normal bodies and weights of $IPMK^{WT}$ and $IPMK^{cKO}$ mice of 4 mo of age. (Scale bar, 2 cm.) (*D*) Normal body weights of $IPMK^{WT}$ and $IPMK^{cKO}$ mice of same age (Student's t test, NS, $P \geq 0.05$). (*E*) The normal brain size of the $IPMK^{cKO}$ mice. (Scale bar, 10 mm.) (*F* and *G*) H&E staining (*F*) and cresyl violet staining (*G*) showed normal brain structures in adult $IPMK^{cKO}$ mice. (Scale bars, 1 mm.) (*H*) Representative confocal images of hippocampal sections from $IPMK^{WT}$ and $IPMK^{cKO}$ mice, which were triply labeled for the neuronal marker, NeuN (red), the glial marker, GFAP (green), and DAPI (blue). (Scale bars, 200 μm.) (*I* and *J*) Rotarod test for motor function; (*I*) The average time spent on the rod in the fixed-speed test (Student's t test, NS, $P \geq 0.05$), while (*J*) The average falling time spent on the rod in the accelerating-speed test (Student's t test, NS, $P \geq 0.05$). In all experiments, $IPMK^{WT}$ littermates served as controls for $IPMK^{cKO}$ mice; n = 11 ($IPMK^{WT}$) and 14 ($IPMK^{cKO}$). AMG, amygdala; CB, cerebellum; HIP, hippocampus; MEF, mouse embryonic fibroblast. Data are presented as the mean ± SE.

either the same test chamber that was used for the conditioning for contextual fear or a modified chamber where the audible tone is played for cued fear. We did not observe any significant difference in the freezing levels of *Ipmk*$^{f/f}$ (n = 22) and *Ipmk*$^{f/f}$;*CaMKII-Cre* mice (n = 24) for either CS (two-way ANOVA, $F_{1,135} = 3.616$, $P \geq 0.05$) (Fig. 2*A*). Similar to the Morris water maze results, *Ipmk*$^{f/f}$;*CaMKII-Cre* mice showed normal acquisition and memory in both contextual (Student's t test, $P \geq 0.05$) (Fig. 2*B*) and cued fear-conditioning paradigms (Fig. 2*C*). To further investigate the effects of IPMK deletion on the extinction of cued fear memory, mice at 24 h postconditioning were reexposed to the audible tone without the footshock. During this cued extinction learning paradigm, *Ipmk*$^{f/f}$;*CaMKII-Cre* mice displayed a significantly lower freezing response than *Ipmk*$^{f/f}$ littermate control mice, suggesting that IPMK deletion was associated with enhanced fear extinction (day 1, two-way ANOVA, $F_{1,400} = 38.547$, $^{***}P < 0.001$) (Fig. 2*D*). We retested the mice at 24 h after the extinction learning paradigm by reexposing them to the same tone. Under this condition, the *Ipmk*$^{f/f}$;*CaMKII-Cre* mice continued to exhibit a reduced freezing response (day 2, two-way ANOVA, $F_{1,400} = 9.127$, $^{**}P < 0.01$) (Fig. 2*D*). These results suggest that IPMK in excitatory neurons plays a selective role in constraining fear extinction. We further examined a cue-dependent freezing response 48 h after conditioning (*SI Appendix*, Fig. S4*A*). *Ipmk*$^{f/f}$;*CaMKII-Cre* mice apparently exhibited normal freezing (*SI Appendix*, Fig. S4*B*), thus suggesting that the enhanced extinction found from the *Ipmk*$^{f/f}$;*CaMKII-Cre* mice shown in Fig. 2*D* does not reflect the progressive loss of memory over time. We also performed the cued extinction test 1 h postconditioning (*SI Appendix*, Fig. S5*A*). Under this immediate extinction training, we failed to detect any difference in the freezing response between *Ipmk*$^{f/f}$;*CaMKII-Cre* and *Ipmk*$^{f/f}$ mice (*SI Appendix*, Fig. S5*B*). This indicates that consolidation of fear extinction memory is required for IPMK-mediated processes.

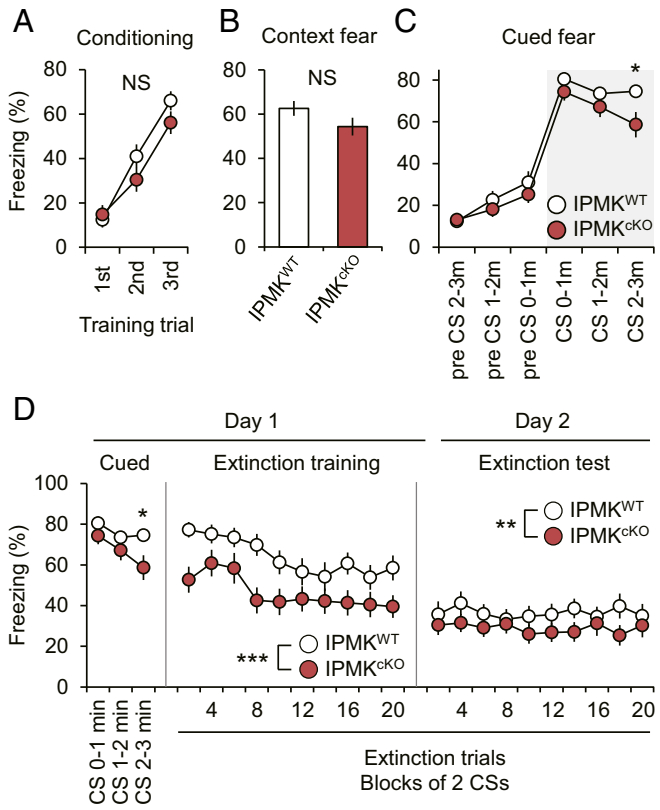

Fig. 2. *Ipmk*$^{f/f}$;*CaMKII-Cre* mice display enhanced fear extinction but normal fear acquisition and memory. (*A*–*C*) Context fear and cued fear memory are normal in *Ipmk*$^{f/f}$;*CaMKII-Cre* (IPMK^{cKO}) mice (3–4 mo). The rate of freezing response was quantified during fear training (*A*; $F_{1,135} = 3.616$, $P \geq 0.05$), the contextual fear-conditioning test (*B*; Student's t test, $P \geq 0.05$), and the cued fear-conditioning test (*C*; Student's t test, $^{*}P < 0.05$). (*D*) Tone-dependent freezing rates of *Ipmk*$^{f/f}$ (IPMK^{WT}) and IPMK^{cKO} mice at extinction day 1 and extinction day 2. Extinction performance is expressed as percentage of freezing during 10 blocks (2 tones) for each day (day 1, two-way ANOVA, $F_{1,400} = 38.547$, $^{***}P < 0.001$; day 2, two-way ANOVA, $F_{1,400} = 9.127$, $^{**}P < 0.01$; NS, $P \geq 0.05$). In all experiments, IPMK^{WT} littermates served as controls for IPMK^{cKO} mice; n = 22 (IPMK^{WT}) and 24 (IPMK^{cKO}). Data are presented as the mean ± SE.

***Ipmk* Deletion Selectively Up-Regulates the p85 S6K1 Signaling in the Amygdala.** Given the pleiotropic role of IPMK as an intracellular signaling hub, we hypothesized that the loss of IPMK might alter major cellular signaling events. To test this hypothesis, we analyzed the phosphorylation of various signaling factors. The amygdala (but not hippocampus) of *Ipmk*$^{f/f}$;*CaMKII-Cre* mice that underwent cued fear extinction exhibited up-regulation of phosphorylated p85 S6K1 threonine-412, which is a direct substrate of mTORC1 (Fig. 3 *A*–*C*). However, levels of phosphorylated Akt, ERK, and mTOR were similar in the brain samples prepared from *Ipmk*$^{f/f}$;*CaMKII-Cre* and *Ipmk*$^{f/f}$ mice that experienced fear conditioning (*SI Appendix*, Fig. S6*A*). We further failed to detect any alteration of other major neural signaling events (e.g., JNK, PKC, STAT) in naive *Ipmk*$^{f/f}$;*CaMKII-Cre* mice or those exposed to fear conditioning and fear extinction (*SI Appendix*, Fig. S6*B*). These results suggest that the loss of IPMK from forebrain excitatory neurons does not alter the basal phosphorylation state of the interconnected signaling pathways but selectively activates p85 S6K1 after cued fear extinction in the amygdala.

***Ipmk* Deletion Enhances the Late Phase of Hippocampal LTP.** To determine whether genetic deletion of *Ipmk* in the excitatory neurons could influence synaptic plasticity, we monitored long-term potentiation (LTP) of the hippocampus. We delivered two trains of high-frequency stimulation (HFS) to Schaffer collateral inputs and recorded the field excitatory postsynaptic potential (fEPSP) in the stratum radiatum of hippocampal area, CA1 (RM-ANOVA, $^{*}P < 0.05$) (Fig. 4). Late LTP of *Ipmk*$^{f/f}$;*CaMKII-Cre* mice was enhanced compared with that of wild-type littermates (L-LTP; Fig. 4 *A* and *B*). We also analyzed early LTP (E-LTP; Fig. 4*C*), which is a transient form of LTP that does not require gene transcription or mRNA translation. However, we found no significant difference between control and *Ipmk*$^{f/f}$;*CaMKII-Cre* mice in this regard. Together, these results indicate that IPMK is a key regulator that is required specifically for the induction and maintenance of L-LTP. Since Ins(1,4,5,6)P_4 was recently discovered as a cofactor essential for class I histone deacetylase (HDAC) which regulates LTP and memory processing (23, 24, 30–32), we further checked levels of class I HDAC and histone acetylation in the hippocampal tissues. We observed no apparent change between control and *Ipmk*$^{f/f}$;*CaMKII-Cre* mice (*SI Appendix*, Fig. S7), suggesting that IPMK-mediated LTP phenotype is not related to HDAC regulation.

Discussion

It is challenging to uncover signaling proteins that are critical for learning and memory and even more difficult to link these molecules to cognitive behaviors and related diseases, such as PTSD (33, 34). Many genetic factors have been associated with the fine control of memory acquisition, consolidation, and retrieval. However, only a few molecular targets (e.g., histone deacetylase, L-type voltage-gated calcium channel, protein kinase A) have been causally linked to memory extinction (23–28). A pleiotropic protein, IPMK mediates numerous biological processes (e.g., growth)

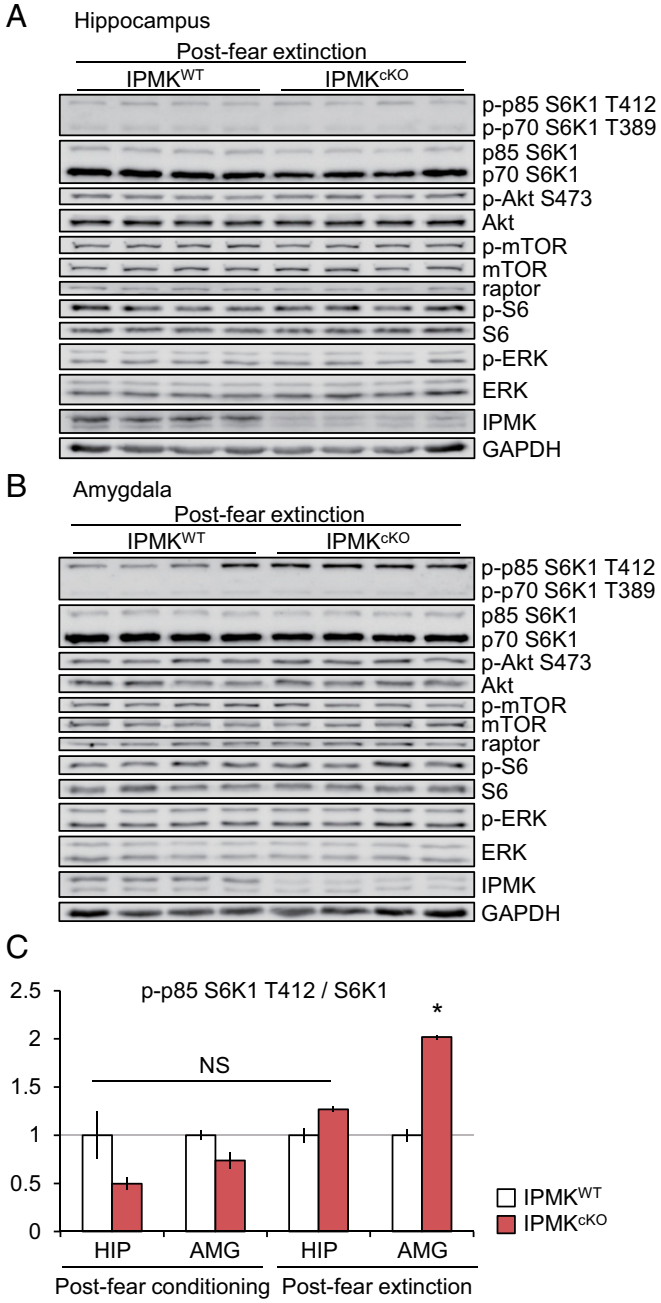

Fig. 3. *Ipmk*$^{f/f}$;*CaMKII-Cre* mice exhibit up-regulation of p85 S6K1 T412 in the amygdala. (*A* and *B*) Analysis of various signaling factors related to Akt and mTOR signaling in the hippocampus (*A*) and amygdala (*B*) of IPMK^{WT} and IPMK^{cKO} mice. Protein lysates of hippocampus and amygdala were prepared from four mice of each genotype at 30 min after the fear extinction test and subjected to immunoblotting. (*C*) Levels of p85 S6K1 T412 in IPMK^{cKO} mice were quantified and normalized to those in IPMK^{WT} control animals. Protein samples were prepared from mice that underwent fear conditioning or fear extinction session. In all experiments, IPMK^{WT} littermates served as controls for IPMK^{cKO} mice. HIP, hippocampus; AMG, amygdala. All graphs are plotted as the mean ± SE. Student's *t* test, $*P < 0.05$; NS, $P \geq 0.05$.

enzymatically via the biosynthesis of inositol polyphosphates and phosphatidylinositol 3,4,5-trisphosphates, or noncatalytically to control key signaling factors (10, 11, 35). Previously, we conditionally deleted *Ipmk* from neuroprogenitor cells by crossing *Ipmk*$^{f/f}$ mice with *Nestin-Cre* transgenic mice and found that IPMK knockout lowers the induction of immediate early genes, such as *c-jun*, *c-fos*, and *arc*, and a defect in long-term memory as assessed by the Barnes maze, a test in which mice use visual cues to identify a hidden escape box (12). However, these studies did not address the functional requirement for IPMK in mature neural circuits or roles in specific behaviors and/or molecular changes.

Here, we generated *Ipmk*$^{f/f}$;*CaMKII-Cre* mice, in which Cre expression commences 2.5 wk after birth and is restricted to the excitatory neurons of the forebrain (Fig. 1). *Ipmk*$^{f/f}$;*CaMKII-Cre* mice exhibited intact learning in contextual fear conditioning and spatial memory tests (*SI Appendix*, Fig. S2), but showed a selective enhancement in fear extinction, as evidenced by a significantly enhanced decline in freezing when mice were subject to extinction without footshock 24 h after fear conditioning (Fig. 2). Together, our results imply that IPMK in forebrain excitatory neurons during extinction promotes conditioned responses that had been learned during fear conditioning. It is also interesting to note that *Ipmk*$^{f/f}$;*CaMKII-Cre* mice showed normal reversal learning in the Morris water maze (*SI Appendix*, Fig. S2*I*), which further suggests that improved behavioral flexibility by the loss of

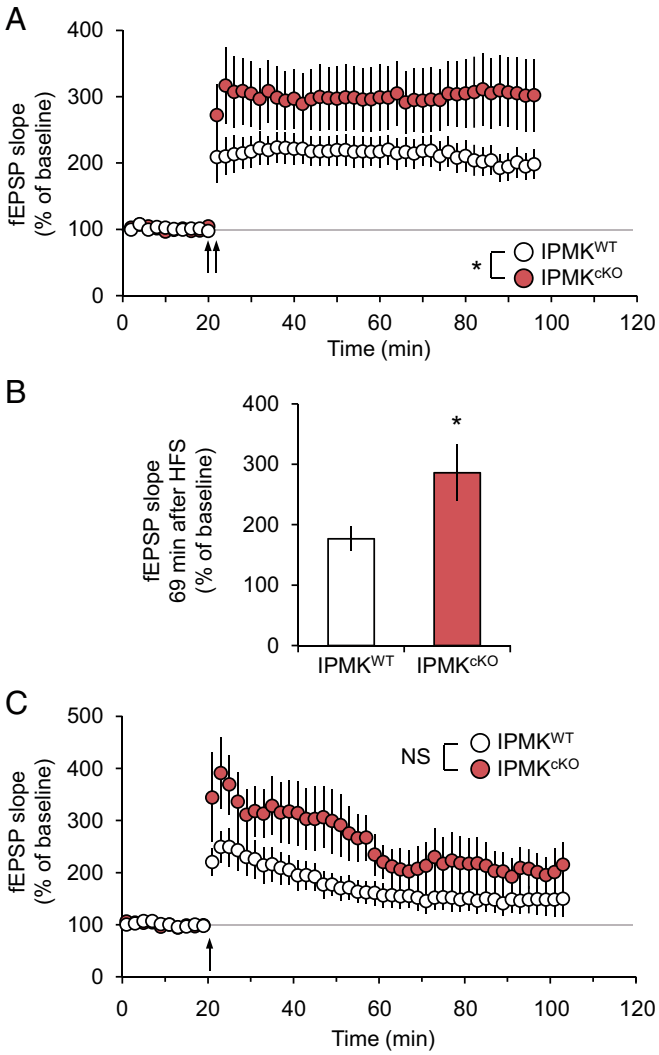

Fig. 4. *Ipmk*$^{f/f}$;*CaMKII-Cre* mice show enhanced L-LTP in the hippocampal area, CA1. (*A* and *B*) Enhanced late LTP (L-LTP) induced as two trains of high-frequency stimulation (HFS) at IPMK^{cKO} CA1 synapses. (*C*) Normal early LTP (E-LTP) in CA1 synapses of IPMK^{cKO} mice. Time courses of the effects of one train of frequency stimulation on the fEPSP initial slope. Data are presented as the mean ± SE. $*P < 0.05$; NS, $P \geq 0.05$; RM-ANOVA (*A*–*C*).

IPMK in excitatory neural circuits appears specific to fear extinction but not reversal spatial learning.

Because IPMK was previously shown to mediate Akt and mTOR signaling in nonneuronal cell culture settings, we examined these signaling pathways in the brain. We observed a selective increase of p85 S6K1 phosphorylation at the mTORC1-sensitive Thr412 in the amygdala of *Ipmk*$^{f/f}$;*CaMKII-Cre* mice (Fig. 3). This result is consistent with a previous report demonstrating that p70 S6K1 is selectively activated in the basolateral nucleus of the amygdala during extinction (28). Since regulation of S6K1 activities has been implicated in memory processing and a variety of psychiatric diseases (36–38), it is needed to precisely dissect the molecular link between neuronal IPMK and selective activation of p85 S6K1 within the different subregions of the amygdala.

In our electrophysiological recordings, *Ipmk*$^{f/f}$;*CaMKII-Cre* mice exhibited normal E-LTP, but facilitated L-LTP in hippocampal slices (Fig. 4). Curiously, the *Ipmk*$^{f/f}$;*CaMKII-Cre* mice displayed no defect in hippocampus-dependent learning as measured with the Morris water maze test and fear conditioning, but exhibited selective enhancement of cued fear extinction (Fig. 2 and *SI Appendix*, Fig. S2). One possibility for these different behavioral phenotypes is that deletion of IPMK within excitatory glutamatergic neurons in infralimbic medial prefrontal cortex (IL/mPFC) and basolateral amygdala (BLA) selectively facilitates the activation of intercalated (ITC) neurons which enhance inhibition of central nuclei of the amygdala (CeA), thereby preventing conditioned fear responses. It remains also possible to speculate robust potentiation of the glutamatergic synaptic input from IPMK-expressing "extinction neurons" in BLA to ITC. Undoubtedly, further work will be required to investigate synaptic properties of reciprocal excitatory inputs between IL/mPFC and BLA as well as changes in excitatory/inhibitory balance from BLA to CeA. It would also be the key to pinpoint biochemical pathways involving IPMK in the fine control of synaptic transmission. Importantly, precise roles of IPMK-dependent catalytic products [e.g., Ins(1,4,5,6)P_4, phosphatidylinositol 3,4,5-triphosphates] or IPMK-interacting signaling targets in fear extinction remain to be elucidated.

In conclusion, our study provides a loss-of-function investigation of the specific role of inositol polyphosphate kinase family by employing postnatal knockout in glutamatergic neural circuits, focusing on IPMK in hippocampal synaptic transmission and cognitive behavior. In summary, our study affords insights to the molecular signals underlying fear extinction. Future studies may identify in greater detail specific regions impacted by IPMK deletion. The medial prefrontal cortex, basolateral/lateral amygdala, and hippocampus are widely appreciated for their involvement in controlling fear extinction (19–21, 39, 40). Accordingly studies of these areas may clarify how IPMK regulates the fear memory circuit. Our discovery that IPMK controls fear extinction emphasizes the need to define in depth roles of IPMK in extinction-triggered mTORC1 signaling in the amygdala. Neural IPMK might be fruitfully targeted as a molecular constraint on fear extinction, with the goal of precisely controlling traumatic memories in psychiatric diseases, including PTSD.

Materials and Methods

Mice. Forebrain excitatory neuron-specific IPMK knockout mice were generated by crossing floxed *Ipmk* mice (*Ipmk*$^{f/f}$) with *CaMKII-Cre* mice. The floxed (f) allele of *Ipmk* was generated previously (5), and the *CaMKII-Cre* transgenic mice (41) were obtained from EUCOMM. These mice were maintained on a mixed 129SvEv; C57BL/6J background. In all experiments, IPMK^{WT} (*Ipmk*$^{f/f}$) littermates served as controls for IPMK^{cKO} (*Ipmk*$^{f/f}$;*CaMKII-Cre*) mice. Male mice were used for all behavioral experiments at 12–16 wk of age. All mice were bred and housed under specific pathogen-free conditions in a 12-h light–dark schedule. Food and water were provided ad libitum. Animal protocols, materials, and methods were performed in accordance with guidelines approved by the Institutional Review Board of the Korea Advanced Institute of Science and Technology Animal Care and Use Committee.

Fear Conditioning and Extinction Test. Fear-conditioning chamber (Coulbourn Instruments) was used for fear conditioning. On day 1, each mouse was placed and allowed to explore the fear-conditioning chamber (chamber A) for 3 min. Subsequently, a conditional stimulus tone (CS, 4 kHz, 80 dB) was presented for 30 s, and during the last 1 s of CS presentation, scrambled footshock (US, 1 mA) was presented. Two more CS-US pairings were presented with 2 min trial interval. Mice were kept in the chamber for an additional 2 min after last shock. On day 2, contextual fear-conditioning tests were tested in the same chamber for 5 min without footshock and analyzed. For cued fear-conditioning tests, mouse was placed in white acrylic cylindrical chamber (chamber B). The baseline freezing level was measured for 3 min, before the onset of CS tone, which was presented for 3 min. Right after cued fear-conditioning tests, mice were submitted to fear extinction test in same chamber. Mice received repeated 20 CS presentations for 20 s, with 40-s no-stimulus interval. On day 3, fear extinction retrieval tests were performed in context B, with the same method as for the extinction test. All freezing behavior was recorded and determined automatically by FreezeFrame3 software (Coulbourn Instruments).

Western Blot Analysis. For Western blotting, mice were euthanized by decapitation and brains were gently extracted on ice. The hippocampi, amygdalae, cortices, and cerebella were dissected out and then homogenized in a lysis buffer [1% Nonidet P-40, 137 mM NaCl, 20 mM Tris·HCl (pH 8.0), 2 mM EDTA, 10% glycerol, 20 mM $NaVO_4$, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 20 mM PMSF] containing protease inhibitor mixture (Roche). The total protein concentrations were determined by the Bradford protein assay (Bio-Rad), and proteins were boiled at 95 °C for 5 min with SDS sample loading buffer (60 mM Tris·HCl, 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, and 0.1% bromophenol blue). Twenty micrograms of protein were electrophoresed on 10% SDS/PAGE gels, and separated proteins were transferred onto a nitrocellulose membrane. After blocking with 5% skim milk in Tris-buffered saline (TBST, 0.1% Tween-20) for 1 h at room temperature, membranes were incubated with primary antibodies overnight at 4 °C. Then, the membranes were treated with an HRP-conjugated secondary antibody for 1 h at room temperature. After each step, the membranes were rinsed three times for 10 min with TBST. The HRP signals were visualized with Clarity ECL substrate (Bio-Rad) and measured using ChemiDoc (Bio-Rad) program.

Antibodies. The primary antibodies were as follows: JNK1/2 (554285; BD Pharmingen); GAPDH (sc-32233; Santa Cruz Biotechnology), NeuN (MAB377), and GFAP (AB5804) (Millipore); VGLUT1 (135303), VGAT (131003), and Shank2 (162204) (Synaptic Systems); and acetyl-H4K5 (ab51997; Abcam). The following were purchased from Cell Signaling Technologies: phospho-STAT3 (9131), STAT3 (9139), phospho-JNK (4668), phospho-IκB (2859), IκB (4814), phospho-PKCα/β (9375), PKCα (2056), phospho-S6K1 (9205), S6K1 (9202), phospho-Akt S473 (9271), Akt (9272), phospho-mTOR (5536), mTOR (2972), raptor (2280), phospho-S6 (4856), S6 (2217), phospho-ERK (4370), ERK (4696), HDAC1 (34589), HDAC2 (57156), HDAC3 (85057), and acetyl-H3K9 (9649). The anti-IPMK antibody used was a custom rabbit polyclonal antibody. It was raised against a mouse IPMK peptide corresponding to amino acids 295–311 (SKAYSTHTKLYAKKHQS; Covance) containing an added N-terminal cysteine. HRP-conjugated secondary antibodies (NCL1460KR) were purchased from Thermo Fisher Scientific.

Hippocampal Slice Preparation. Brains from IPMK^{cKO} mice and their IPMK^{WT} littermates (3–4 mo of age) were quickly removed, and transverse hippocampal slices (400 μm) were prepared with a VT1200 Vibratome (Leica) in the following ice-cold cutting solution: 110 mM sucrose, 60 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 28 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 5 mM glucose, and 0.6 mM ascorbate. Slices were allowed to recover for 20 min at room temperature in a 50:50 solution of aCSF solutions. The artificial cerebrospinal fluid (aCSF) solution contained the following: 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 25 mM D-glucose, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. This was followed by additional recovery for 30 min in room temperature aCSF before being transferred for the electrophysiology recordings.

Electrophysiology. Electrophysiology was performed according to standard techniques, as described previously (42). Briefly, bipolar stimulating electrodes (92:8 Pt:Y) were placed at the border of hippocampal areas CA3 and CA1 along the Schaffer collateral pathway. aCSF-filled glass recording electrodes (1–3 MΩ) were placed in stratum radiatum of area CA1. Stable baseline synaptic transmission was established for at least 40 min. LTP was induced with two trains of high-frequency stimulation (HFS; 100 Hz for 1 s, with a 20 s interval).

Statistical Analysis. Differences between averages were analyzed using a two-tailed Student's *t* test or two-way ANOVA followed by Tukey's posttest. All data are expressed as means ± SE. Statistical significance was set at $P < 0.05$.

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