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ORIGINAL ARTICLE

Forebrain-specific ablation of phospholipase Cγ1 causes manic-like behavior

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Manic episodes are one of the major diagnostic symptoms in a spectrum of neuropsychiatric disorders that include schizophrenia, obsessive-compulsive disorder and bipolar disorder (BD). Despite a possible association between BD and the gene encoding phospholipase Cγ1 (*PLCG1*), its etiological basis remains unclear. Here, we report that mice lacking phospholipase Cγ1 (PLCγ1) in the forebrain (*Plcg1*$^{f/f}$*; CaMKII*) exhibit hyperactivity, decreased anxiety-like behavior, reduced depressive-related behavior, hyperhedonia, hyperphagia, impaired learning and memory and exaggerated startle responses. Inhibitory transmission in hippocampal pyramidal neurons and striatal dopamine receptor D1-expressing neurons of *Plcg1*-deficient mice was significantly reduced. The decrease in inhibitory transmission is likely due to a reduced number of γ-aminobutyric acid (GABA)-ergic boutons, which may result from impaired localization and/or stabilization of postsynaptic CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) at inhibitory synapses. Moreover, mutant mice display impaired brain-derived neurotrophic factor-tropomyosin receptor kinase B-dependent synaptic plasticity in the hippocampus, which could account for deficits of spatial memory. Lithium and valproate, the drugs presently used to treat mania associated with BD, rescued the hyperactive phenotypes of *Plcg1*$^{f/f}$*; CaMKII* mice. These findings provide evidence that PLCγ1 is critical for synaptic function and plasticity and that the loss of PLCγ1 from the forebrain results in manic-like behavior.

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INTRODUCTION

Synapse formation and function are tightly regulated by multiple signaling cascades, and disruption of such synaptic signaling pathways is implicated in various neuropsychiatric disorders.[1] Among those, impairment of the phospholipase Cγ1 (PLCγ1)-mediated pathway appears to be causally linked to neuropsychiatric disorders such as depression,[2] epilepsy[3] and bipolar disorder (BD).[4–6] PLCγ1 generates the second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol via the hydrolysis of membrane-bound phosphatidylinositol 4,5-bisphosphate when triggered by multiple neurotransmitters and neuromodulators. Multiple genome-wide association studies identified PLC signaling as a pathway that contributes to the risk for BD,[7] although the association was recently challenged.[8] A genome-wide linkage analysis study also identified a gene encoding phospholipase Cγ1 (*PLCG1*) as a susceptibility locus for BD.[9] Indeed, BD patients with a dinucleotide repeat in the *PLCG1* genomic region are good responders to lithium, suggesting involvement of the PLCγ1 signaling pathway in BD.[4] Interestingly, PLCγ1 controls recycling of IP_3, which is modulated by lithium.[10] Brain-derived neurotrophic factor (BDNF), a regulator of diverse synaptic functions, regulates PLCγ1 activity.[11] Moreover, a BDNF gene polymorphism has been identified as a potential risk allele for BD.[12,13] However, despite this, it remains unclear whether and how dysfunction of BDNF/PLCγ1 signaling contributes to the lithium-responsive symptoms of BD.

Here, we found that mice with forebrain-selective deletion of PLCγ1 (*Plcg1*$^{f/f}$*; CaMKII* mice) exhibit manic-like behavior, as well as deficits in inhibitory transmission and BDNF-dependent synaptic plasticity. We also discovered potential molecular mechanisms whereby the disruption of PLCγ1 signaling in the hippocampus leads to such dysfunctions of inhibitory synapses. Those synaptic deficits could contribute to at least some of the manic behavioral phenotypes displayed by mutant mice. Given the limited number of animal models that are able to fulfill the validity criteria for manic-like behavior,[14] *Plcg1*$^{f/f}$*; CaMKII* mice may be a reliable model for the manic phase of BD.

MATERIALS AND METHODS

Detailed descriptions of the Methods and materials are included in the Supplementary Methods section.

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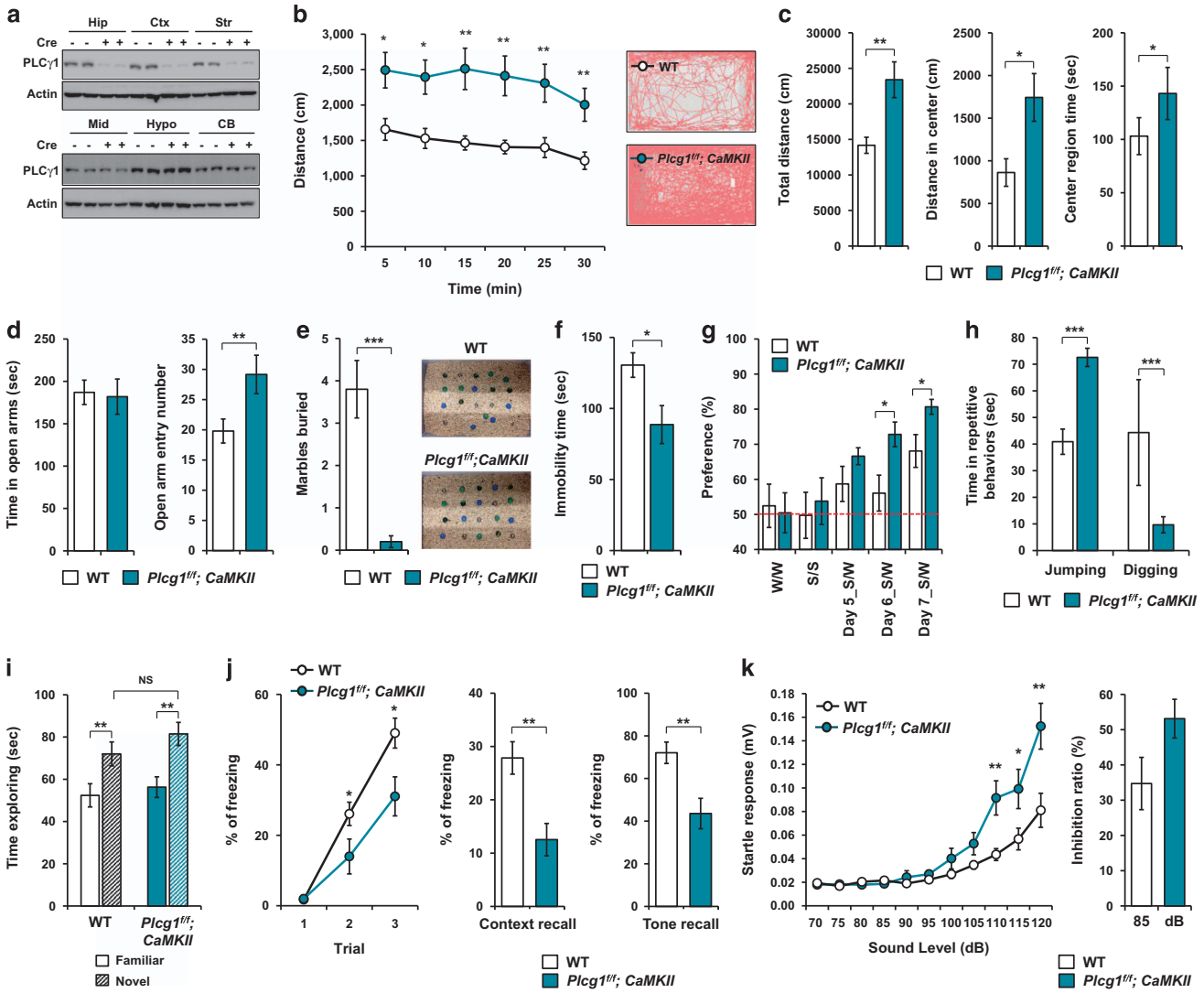

Figure 1. *Plcg1*$^{f/f}$*; CaMKII* mice harboring phospholipase Cγ1 (PLCγ1) deletion in the forebrain exhibit manic-like behavior. (**a**) Western blots showing loss of PLCγ1 from the forebrain of *Plcg1*$^{f/f}$*; CaMKII* mice (hippocampus, Hip; cortex, Ctx; striatum, Str) but not from the midbrain (Mid), hypothalamus (Hypo) or cerebellum (CB). (**b** and **c**) Locomotor activity of wild-type (WT) and *Plcg1*$^{f/f}$*; CaMKII* mice in the open field test ($n = 8$–10 per group), (**d**) the elevated plus maze test ($n = 10$–12 per group), (**e**) the marble burying test ($n = 11$–12 per group), (**f**) the forced swimming test ($n = 11$–12 per group), (**g**) the two-bottle sucrose preference test (WT, $n = 5$ vs *Plcg1*$^{f/f}$*; CaMKII*, $n = 9$) (two-bottle choice (W; water, S; sucrose)). (**h**) Jumping and digging behavior (WT, $n = 9$ vs *Plcg1*$^{f/f}$*; CaMKII*, $n = 6$). (**i**) The novel object recognition test ($n = 12$ per group). (**j**) Cued fear conditioning in WT and *Plcg1*$^{f/f}$*; CaMKII* mice (WT, $n = 11$ vs *Plcg1*$^{f/f}$*; CaMKII*, $n = 10$). (**k**) Startle response test (left graph) and prepulse inhibition (right graph) in WT and *Plcg1*$^{f/f}$*; CaMKII* mice ($n = 12$ per group). Error bars represent ± s.e.m. $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$. CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II.

Mice

CaMKII-Cre[15] and *Nestin-Cre*[16] mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and housed under a 12- h light–dark cycle and given access to food and water *ad libitum*. Only male mice were used for physiological and behavioral analyses. All procedures were approved by the Institutional Animal Care and Utilization Committee of POSTECH and conducted in accordance with the UNIST Guide for the Care and Use of Laboratory Animals (UNISTIACUC-14-005).

Generation of PLCγ1 conditional knockout mice

The *Plcg1*-targeting vector was designed to delete exons 3 − 5 by inserting loxP sites into introns 2 and 5. The Neo cassette flanked by FRT sites was inserted into intron 5 adjacent to the second loxP site. The *Plcg1*-targeting vector DNA construct was electroporated into mouse embryonic stem cells and single clones were microinjected into blastocysts. The F1 mice were then crossed with flippase transgenic mice to eliminate the FRT-Neo cassette. *Plcg1*-floxed mice, originally on a mixed 129 × C57BL/6 background (Supplementary Figure 1a), were backcrossed with C57BL/6 mice for at least eight generations before the experiments. The targeted allele was detected with 5′ and 3′ probes by Southern blotting analysis with *Eco*RI digestion (Supplementary Figure 1b). The *Plcg1*$^{+/+}$, *Plcg1*$^{f/+}$ and *Plcg1*$^{f/f}$ mice were genotyped using primer 1 (5′-GCACAGCAGACAGA CTTGGAC-3′) and primer 2 (5′-GTTGCTCAAGGTGAAGGCTCT-3′) (Supplementary Figure 1c). Deletion of exons 3–5 was achieved by crossing *Plcg1*$^{f/+}$ mice with *CaMKII-Cre* mice or *Nestin-Cre* mice to produce postnatal forebrain- and brain-specific *Plcg1*-knockout mice, respectively.

Statistical analysis

Statistical analyses were performed using SPSS (version 17.0) and the normality of the data distribution was assessed using the Kolmogorov–Smirnov test. Student's unpaired *T*-test, nonparametric Mann–Whitney *U*-test or analysis of variance followed by Tukey's *post hoc* test was

performed, as specified in the Supplementary Tables. All data are presented as mean ± s.e.m. Significance is indicated by $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

RESULTS

Hyperactive behavior and memory impairment in mice harboring forebrain-specific deletion of PLCγ1

PLCγ1 signaling contributes to neuronal survival, neural circuit development and synaptic plasticity.[11,17,18] Despite its potential importance, the pathophysiological role of PLCγ1 in mature neural circuits is poorly understood, partly because global deletion of the *Plcg1* gene results in lethality as early as embryonic day 9.[19] To explore the effect of PLCγ1 loss on the neural function and behavior of adult animals, we deleted PLCγ1 selectively from forebrain neurons by crossing floxed *Plcg1* mice with *CaMKII-Cre* mice[15] (Supplementary Figure 1). Immunoblotting indicated significant suppression of PLCγ1 expression in forebrain areas, including the cortex, the hippocampus and the striatum (Figure 1a). Nissl-stained sections of brain from *Plcg1$^{f/f}$; CaMKII* mice revealed intact anatomical features: typical neuronal organization of the hippocampal formation (Supplementary Figure 2a) and unaltered number of neurons and astrocytes in the cortex, CA1 and CA3 (cornu ammonis fields 1 and 3) regions (Supplementary Figure 2b).

Behaviorally, we did not detect any impairment in basic motor and visual functions in *Plcg1$^{f/f}$; CaMKII* mice (Supplementary Figures 3a and b). Importantly, *Plcg1$^{f/f}$; CaMKII* mice showed hyperactivity in the open field test (greater total distance traveled) and spent significantly more time in the center of the field than wild-type (WT) littermates, which is a sign of reduced anxiety (Figures 1b and c). Consistent with the reduction of anxiety levels, *Plcg1$^{f/f}$; CaMKII* mice entered the open arms of the elevated plus maze more frequently than WT littermates, although they spent a similar amount of time in the open arms (Figure 1d). In the marble burying test, another measure of anxiety-like behavior, *Plcg1$^{f/f}$; CaMKII* mice buried fewer marbles (Figure 1e). These results indicate that mutant mice are less anxious than WT littermates. Interestingly, *Plcg1$^{f/f}$; CaMKII* mice showed significantly less immobility than WT mice in the forced swimming test, which is a measure of depression-like behavior (Figure 1f), whereas the tail suspension test revealed no difference between the two genotypes (Supplementary Figure 3c). In the sucrose preference test, *Plcg1$^{f/f}$; CaMKII* mice consumed more sucrose than WT mice (Figure 1g), indicating that *Plcg1$^{f/f}$; CaMKII* mice tend to be hyperhedonic, as observed in human mania. Notably, the mutant mice spent more time repetitively jumping, but less time in digging in stranger-free home cages (Figure 1h). The weight of *Plcg1$^{f/f}$; CaMKII* mice was comparable with that of WT mice at 4 weeks, but was significantly lower at 8 weeks despite greater food intake (Supplementary Figures 3d and e), which might be related to increased locomotion.

BDNF-tropomyosin receptor kinase B (TrkB)-mediated PLCγ1 signaling is causally involved in learning and memory, as evidenced by the cognitive/behavioral deficits of *Trkb$^{PLC/PLC}$*-knock-in mice lacking TrkB-PLCγ1 docking sites.[20,21] *Plcg1$^{f/f}$; CaMKII* and WT mice showed a comparable ability to recognize novel objects (Figure 1i). Next, we examined whether *Plcg1$^{f/f}$; CaMKII* mice also show impaired learning and memory. To this end, we compared associative memory between the two genotypes using the Pavlovian fear conditioning paradigms. In the auditory fear conditioning test, *Plcg1$^{f/f}$; CaMKII* mice exhibited a significantly lower level of freezing response (Figure 1j). We also observed reduced freezing in the contextual fear (Supplementary Figure 3f). However, *Plcg1$^{f/f}$; CaMKII* and WT mice showed similar sensitivity to pain (Supplementary Figure 3g), suggesting that the reduced freezing of *Plcg1$^{f/f}$; CaMKII* mice was not due to alteration of pain sensitivity. Unexpectedly, *Plcg1$^{f/f}$; CaMKII* mice exhibited an exaggerated startle response to high-decibel tones when compared with WT littermates, but prepulse inhibition did not differ from that of WT mice (Figure 1k). Thus, the deficit in acquisition and expression of fear memory did not appear to be simply attributable to decreased responses toward conditioning stimuli. Taken together, these results indicate that PLCγ1 expression in the forebrain is required for normal hippocampus-dependent memory. Importantly, *Plcg1$^{f/f}$; CaMKII* mice showed manic-like behavior, including hyperactivity, hyperphagia, decreased anxiety, hyperhedonia and impaired cognitive ability, as well as increased startle responses.

BD patients are emotionally unstable and an episode of mania or depression could be induced by imposed stresses.[22] Accordingly, BD mouse models exhibit altered behavioral states in response to stresses. For example, *Dbp$^{-/-}$* mice display a shift from a depressive state to a manic-like behavior when under stress, whereas the emotional state of *Ank3$^{+/-}$* mice switches toward depression-like behavior upon exposure to chronic stress.[23,24] To test whether the manic-like behavior of *Plcg1$^{f/f}$; CaMKII* mice can be reversed or affected by stress, we assessed the effect of different type of stress or sleep deprivation on behavior. We found that the manic-like behavior of *Plcg1$^{f/f}$; CaMKII* mice was not affected by acute restraint stress (Supplementary Figures 4a–e), chronic social isolation stress (Supplementary Figure 4f–j) or sleep deprivation (Supplementary Figure 5). Unlike *Ank3$^{+/-}$*- and *Dbp*-knockout mice, *Plcg1$^{f/f}$; CaMKII* mice show hyperactivity at baseline. The behavioral differences between *Plcg1$^{f/f}$; CaMKII* and BD-like mice, particularly at baseline, rendered a direct comparison of the stress-induced conversion of behavioral states difficult in this analysis.

Reduced inhibitory transmission in *Plcg1$^{f/f}$; CaMKII* mice

Manic-like behavior and memory impairment in *Plcg1$^{f/f}$; CaMKII* mice prompted us to examine the possible impact of PLCγ1 deletion on synaptic transmission and synaptic plasticity. First, we assessed glutamatergic transmission by eliciting field excitatory postsynaptic potentials from the hippocampal CA1 area while stimulating the Schaffer collateral (SC) pathway, but failed to detect any difference in input–output curves between *Plcg1$^{f/f}$; CaMKII* mice and their WT littermates (Figure 2a). In the whole-cell patch recording of CA1 neurons, we measured the ratios of *N*-methyl-D-aspartic acid (NMDA) receptor (NMDAR)-mediated excitatory postsynaptic currents to α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor-mediated EPSCs (AMPAR-EPSCs), but again found no difference (Figure 2b). Thus, the input–output relationship and NMDAR/AMPAR current ratios represent intact excitatory transmission in *Plcg1$^{f/f}$; CaMKII* mice. Consistent with the physiological data, western blotting of crude synaptosomal fractions isolated from *Plcg1$^{f/f}$; CaMKII* and WT mice revealed no difference in the expression levels of various postsynaptic proteins, including AMPAR subunits (GluA1 − 3), NMDAR subunits (GluN1, 2A and 2B), neuroligins (NL1 − 3) and postsynaptic density protein-95 (Figure 2c).

To further examine the physiological roles of PLCγ1 in basal synaptic transmission, we monitored miniature EPSCs and miniature inhibitory postsynaptic currents (mIPSCs). The frequency and the amplitude of miniature EPSCs were largely unaffected in *Plcg1$^{f/f}$; CaMKII* CA1 pyramidal neurons (Supplementary Figures 6a and b). Consistent with miniature EPSC data, dendritic spine densities were indistinguishable between *Plcg1$^{f/f}$; CaMKII* and WT mice (Supplementary Figures 6c and d). In contrast with miniature EPSCs, the frequency (but not the amplitude) of mIPSCs was markedly reduced in hippocampal slices from *Plcg1$^{f/f}$; CaMKII* mice compared with those from WT mice (Figures 2d and e). Given both the reduced levels of PLCγ1 in the striatum and the hyperactive locomotion of *Plcg1$^{f/f}$; CaMKII* mice, we started to examine changes in basal synaptic transmission in the dorsal striatum.

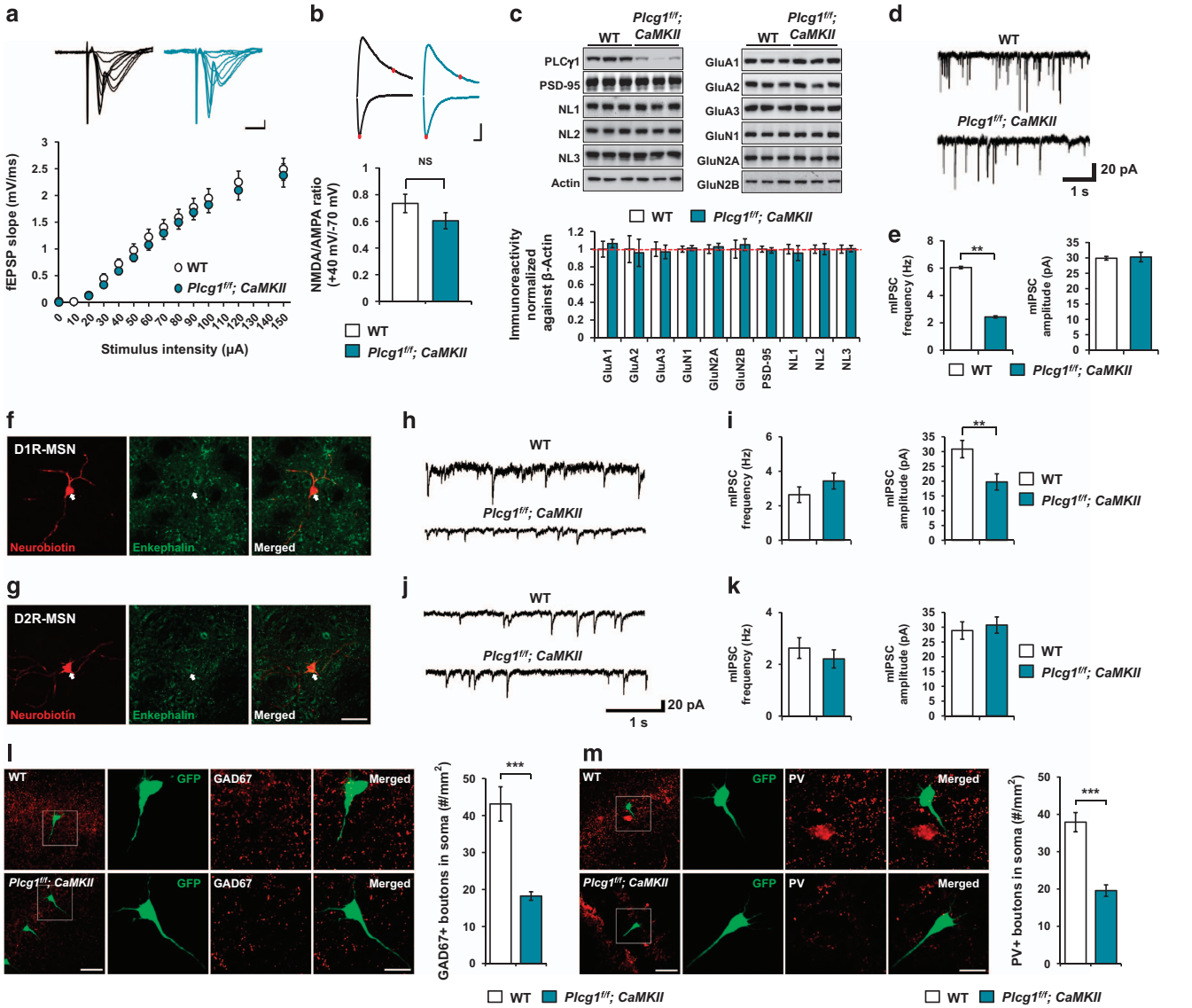

Figure 2. Phospholipase Cγ1 (PLCγ1) deletion reduces inhibitory but not excitatory synaptic transmission. (**a**) Stimulus intensity vs field excitatory postsynaptic potentials (fEPSPs) slope (input – output relation) at the hippocampal Schaffer collateral (SC)-CA1 synapses of wild-type (WT) and *Plcg1*$^{f/f}$*; CaMKII* mice ($n = 20$ per group). Scale bars: 1 mV and 10 ms. (**b**) The ratios of *N*-methyl-D-aspartic acid receptor/α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor-excitatory postsynaptic current (NMDAR/AMPAR-EPSCs) at CA1 pyramidal neurons from WT ($n = 20$) and *Plcg1*$^{f/f}$*; CaMKII* ($n = 14$) mice. Scale bars: 50 pA and 10 ms. (**c**) Immunoblots (upper) and densitometric quantification (lower) of the synaptosomal fractions of hippocampal lysates from WT and *Plcg1*$^{f/f}$*; CaMKII* mice for the following postsynaptic proteins: AMPA receptor subunits (GluA1 – 3), NMDA receptor subunits (GluN1, 2A, 2B), postsynaptic density protein-95 (PSD-95) and neuroligin 1–3 (NL1–3). (**d**) Representative miniature inhibitory postsynaptic current (mIPSC) traces from WT and *Plcg1*$^{f/f}$*; CaMKII* hippocampal pyramidal CA1 neurons. (**e**) Average values for mIPSC frequency and amplitude (WT, $n = 6$ vs *Plcg1*$^{f/f}$*; CaMKII*, $n = 8$). (**f** and **g**) Representative images of immunostained neurobiotin-injected neurons. The recorded striatal neurons were identified either as putative dopamine receptor D1-medium spiny neurons (D1R-MSNs) (**f**) or as dopamine receptor D2 (D2R)-MSNs (**g**) by immunostaining for the D2R-MSN marker, enkephalin. Scale bar = 50 μm. (**h** and **j**) Representative mIPSC traces from putative D1R-MSN (**h**) or D2R-MSN (**j**) of WT and *Plcg1*$^{f/f}$*; CaMKII* mice (D1: WT, $n = 10$ vs *Plcg1*$^{f/f}$*; CaMKII*, $n = 7$; D2: WT, $n = 8$, vs *Plcg1*$^{f/f}$*; CaMKII*, $n = 10$). (**i** and **k**) Bar graphs for D1-MSN (**i**) or D2-MSN (**k**) mIPSC frequency and amplitude. (**l** and **m**) Distribution of glutamic acid decarboxylase 67 (GAD67)-positive (**l**) or parvalbumin (PV)-positive (**m**) boutons (red) contacting the somata of WT and *Plcg1*$^{f/f}$*; CaMKII* hippocampal CA1 pyramidal neurons infected with lentivirus expressing GFP. (**l**) GAD67-positive boutons on WT ($n = 19$) and *Plcg1*$^{f/f}$*; CaMKII* ($n = 63$) somata. (**m**) PV-positive boutons on WT and *Plcg1*$^{f/f}$*; CaMKII* somata ($n = 18 – 37$ per group). Scale bars: 50 μm for left images and 20 μm for magnified views. Error bars represent ± s.e.m. ***$P < 0.001$ and **$P < 0.01$. CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II.

Intriguingly, the amplitude of mIPSCs was significantly and selectively reduced in the dopamine receptor D1 (D1R)-expressing striatal neurons, but not in the dopamine receptor D2-expressing striatal neurons of *Plcg1*$^{f/f}$*; CaMKII* mice, whereas the frequency of mIPSCs remained unaffected (Figures 2f–k). As activation of D1R-expressing striatal neurons is sufficient to promote locomotion,[25] reduced inhibitory input into D1R-expressing neurons is most likely to increase locomotor activity, which could contribute to the hyperactivity observed in *Plcg1*$^{f/f}$*; CaMKII* mice. Taken together, these physiological data provide evidence that the abnormal behavior of *Plcg1*$^{f/f}$*; CaMKII* mice results from defective inhibitory inputs in the hippocampus and the striatum.

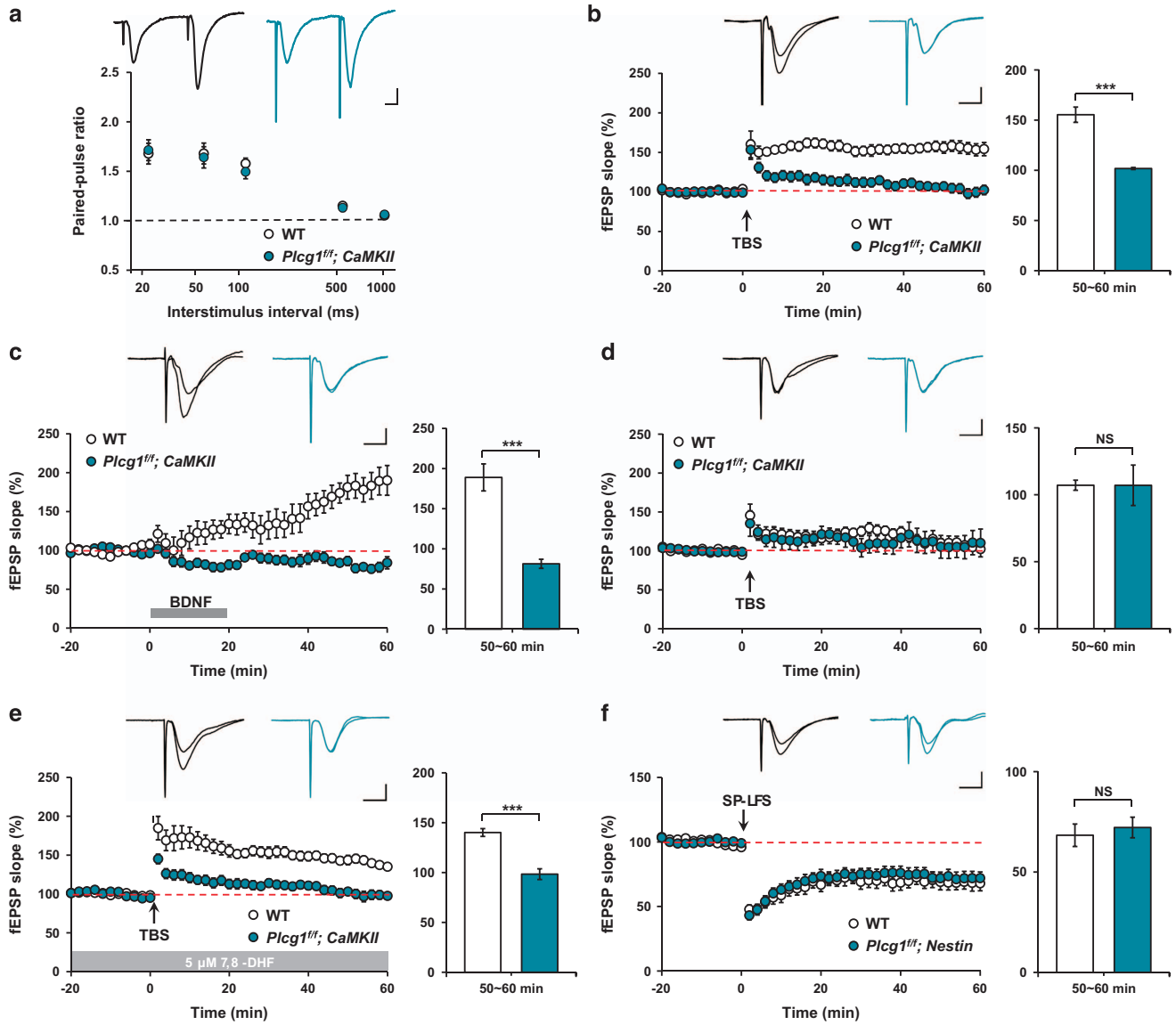

Figure 3. Deficit in brain-derived neurotrophic factor (BDNF)-TrkB-dependent plasticity at Schaffer collateral (SC)-CA1 synapses of phospholipase Cγ1 (PLCγ1)-deficient mice. (**a**) Representative paired-pulsed field excitatory postsynaptic potentials (fEPSPs) (50 ms interval) and paired-pulse ratios (PPRs). (**b**) θ-Burst stimulation-long-term potentiation (TBS-LTP) (wild-type (WT), $n = 10$ vs *Plcg1*$^{f/f}$*; CaMKII*, $n = 14$). Inserts above: Superimposed sample baselines and post-TBS fEPSPs. (**c**) BDNF-induced synaptic enhancement (BDNF-LTP) (WT, $n = 7$ vs *Plcg1*$^{f/f}$*; CaMKII*, $n = 7$). (**d**) Occlusion of TBS-LTP induction by BDNF-LTP. TBS-LTP induction after BDNF-LTP in WT and *Plcg1*$^{f/f}$*; CaMKII* ($n = 7$/group). (**e**) Effect of the TrkB agonist, 7, 8-dihydroxyflavone (7, 8-DHF) (5 μM), on TBS-LTP ($n = 9$ per group). (**f**) Synaptically evoked *N*-methyl-D-aspartic acid receptor (NMDAR)-LTD (1 Hz at 900 pulses) of the SC-CA1 pathway (WT, $n = 10$ vs *Plcg1*$^{f/f}$*; Nestin*, $n = 18$). Scale bars: 1 mV and 10 ms. Error bars represent ± s.e.m. ***$P < 0.001$. NS, not significant. CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II.

Impaired formation of inhibitory synapses in PLCγ1-deficient pyramidal neurons

Given our physiological data and the fact that the density of glutamic acid decarboxylase 65 (GAD65), GAD67 and parvalbumin (PV)-containing cells in the hippocampi of BD patients is reduced,[26] we hypothesized that γ-aminobutyric acid (GABA)-ergic connections would be defective in *Plcg1*$^{f/f}$*; CaMKII* mice. Therefore, we immunostained hippocampi prepared from either *Plcg1*$^{f/f}$*; CaMKII* mice or WT littermates for GAD67, a major GABA-producing enzyme expressed in the presynaptic terminals of GABAergic synapses. We detected a significant decrease in GAD67 staining on the somata of the CA1 pyramidal neurons of *Plcg1*$^{f/f}$*; CaMKII* mice compared with those of WT mice, indicating a reduced number of GABAergic boutons (Figure 2l). As PV-expressing GABAergic interneurons causally regulate neuronal excitability and the generation of γ-oscillations,[27] we also examined changes in GABAergic synapses from PV-positive neurons to pyramidal neurons. There was also a significant reduction in the number of PV-positive puncta on the somata of pyramidal neurons in *Plcg1*$^{f/f}$*; CaMKII* mice (Figure 2m). These findings indicate that a decrease in inhibitory transmission is attributable to a reduced number of synaptic inputs from PV-expressing GABAergic neurons.

PLCγ1 deletion affects BDNF-TrkB-dependent synaptic plasticity

PLCγ1 is involved in BDNF-TrkB signaling-dependent long-term potentiation (LTP).[21,28,29] To investigate the possible effects of PLCγ1 deletion on BDNF-dependent or -independent forms of

synaptic plasticity, we induced LTP in hippocampal slices from *Plcg1*$^{f/f}$*; CaMKII* or WT mice. While neither the basal SC-CA1 excitatory transmission nor the paired-pulse ratios were affected (Figures 2a and 3a), high-frequency stimulation-induced LTP was successfully and similarly induced in WT and *Plcg1*-deficient hippocampal slices (Supplementary Figure 7a). However, LTP induced by θ-burst stimulation (TBS), a form of synaptic plasticity dependent on BDNF-TrkB,[30,31] was disrupted in *Plcg1*$^{f/f}$*; CaMKII* slices when compared with that in WT slices (Figure 3b). TBS-LTP in *Plcg1*$^{f/f}$*; CaMKII* mice was not rescued by application of picrotoxin, a $GABA_A$ receptor ($GABA_AR$) blocker (Supplementary Figure 7b), indicating that the impairment was indifferent to any possible impact of PLCγ1 loss on fast inhibitory transmission.

Because BDNF perfusion itself can enhance excitatory transmission and lead to LTP,[30,32] we next tested whether PLCγ1 loss could also affect BDNF-induced LTP. LTP was induced by application of BDNF at the SC-CA1 pathway of WT mice, but not of *Plcg1*$^{f/f}$*; CaMKII* mice (Figure 3c). As the BDNF effect shares the same mechanisms as TBS when facilitating LTP,[30] we further examined whether BDNF pretreatment occludes TBS-LTP. After treatment of BDNF, TBS did not induce additional LTP in *Plcg1*$^{f/f}$*; CaMKII* slices, similar to observations in WT slices (Figure 3d). Moreover, a selective TrkB agonist, 7, 8-dihydroxyflavone[33] was unable to restore TBS-LTP in *Plcg1*$^{f/f}$*; CaMKII* slices (Figure 3e), which suggests that the underlying deficit seems to occur downstream from BDNF-TrkB. In addition, long-term depression induced by low-frequency stimulation or by application of the mGluR agonist 3, 5-dihydroxyphenylglycine did not differ between WT and *Plcg1*-deficient hippocampal slices (Figure 3f and Supplementary Figure 8). These results indicate that PLCγ1 deficiency in hippocampal CA1 pyramidal neurons impairs BDNF-TrkB-dependent LTP, and, as a result, this deficit may underlie the deficits in spatial learning and memory observed in *Plcg1*$^{f/f}$*; CaMKII* mice (Figure 1j).

Impairment of BDNF-mediated Ca^{2+} signaling and localization of CaMKII to inhibitory synapses of PLCγ1-deficient neurons

As BDNF triggers increased $[Ca^{2+}]_i$ in hippocampal neurons,[34] we examined the possible effects of PLCγ1 deletion on BDNF-induced $[Ca^{2+}]_i$ elevation. To faithfully delete PLCγ1 from cultured neurons, we took advantage of *Plcg1*$^{f/f}$*; Nestin* mice, which express Cre recombinase at an early stage of development.[35] BDNF-mediated $[Ca^{2+}]_i$ elevation was markedly attenuated in hippocampal neurons isolated from *Plcg1*$^{f/f}$*; Nestin* mice when compared with that in WT neurons (Figure 4a). Transient receptor potential cation channels (TRPCs) mediate BDNF-mediated $[Ca^{2+}]_i$ elevation,[17] and expression and activity of TRPC3 are regulated by PLCγ1.[36] Inconsistent with this possibility, however, the expression levels of both TRPC3 and 6 were indistinguishable between WT and *Plcg1*$^{f/f}$*; CaMKII* mice (Supplementary Figure 9). Furthermore, application of a TRPC6 activator, hyperforin, increased $[Ca^{2+}]_i$ to a similar extent in hippocampal neurons prepared from WT and *Plcg1*$^{f/f}$*; Nestin* mice, which implicates PLCγ1 as one of the upstream regulators of TRPCs (Supplementary Figure 10). Although it remains unclear how Ca^{2+} elevation could be affected by the loss of PLCγ1, disruption of Ca^{2+} signaling may account for impairments in TBS-LTP- and hippocampus-dependent spatial memory.

TrkB defines a variety of synaptic features by activating various signaling molecules, including PLCγ1, phosphoinositide 3-kinase, extracellular signal-regulated kinase (ERK) and CaMKIIα.[11] Thus, we tested whether disrupting PLCγ1 affects the activity of those BDNF-induced singling pathways. BDNF-induced phosphoactivation of ERK, CaMKII and cAMP-response element-binding protein was significantly reduced in PLCγ1-deficient neurons, whereas phosphorylation of thymoma viral proto-oncogene (AKT) and mechanistic target of rapamycin remained unaltered (Figure 4b). Localization of phosphorylated CaMKII to inhibitory synapses is a prerequisite for appropriate development of GABAergic currents because CaMKII-mediated phosphorylation of $GABA_AR$ subunits is necessary for stable insertion into the postsynaptic membrane.[37] Accordingly, we reasoned that a reduction in mIPSC frequency and possible decreases in the number of functional inhibitory synapses onto PLCγ1-deficient neurons would be attributable to disruption of the postsynaptic localization of phosphorylated CaMKIIα. To address this hypothesis, we monitored the distribution of Thr286-phosphorylated CaMKIIα (pCaMKIIα) at inhibitory synaptic connections. Indeed, colocalization of pCaMKIIα with gephyrin was reduced in both the somata and neurites of PLCγ1-deficient neurons compared with WT neurons (Figures 4c, e and f). Interestingly, BDNF treatment significantly increased the colocalization of gephyrin and pCaMKIIα in WT neurons, but not in PLCγ1-deficient neurons (Figures 4d–f). As $GABA_AR$ α1 is a substrate of pCaMKIIα,[37] it is possible that loss of PLCγ1 decreases surface expression of the $GABA_AR$ α1 subunit on the somata of hippocampal neurons because this subunit is preferentially expressed over β2/3 at synapses innervated from PV-positive inhibitory neurons.[38] We compared the surface expression of $GABA_AR$ α1 subunit between WT and *Plcg1*$^{f/f}$*; Nestin* neurons in the absence of membrane permeabilization. We detected a significant decrease in $GABA_AR$ α1 staining in PLCγ1-deficient hippocampal neurons (Figure 4g). To corroborate the function of PLCγ1 in the synaptic localization of pCaMKIIα and the ensuing phosphorylation/translocation of $GABA_AR$ subunits, we also analyzed the surface expression of the $GABA_AR$ δ subunit, a predominant isoform of extrasynaptic $GABA_AR$, which is not a substrate of CaMKIIα.[37,39] Supporting the selective effect of CaMKIIα, surface expression of $GABA_AR$ δ was comparable between WT and PLCγ1-deficient neurons (Figure 4h). Collectively, these findings indicate that PLCγ1 is necessary for the formation and/or maintenance of functional inhibitory synapses containing $GABA_AR$ α1 by modulating the postsynaptic localization of pCaMKII.

Reversal of hyperactive behavior of *Plcg1*$^{f/f}$*; CaMKII* mice

To examine the predictive validity of *Plcg1*$^{f/f}$*; CaMKII* mice as an animal model of mania, we investigated the effects of the mood stabilizers normally used to treat BD patients (lithium and valproic acid (VPA) on *Plcg1*$^{f/f}$*; CaMKII* mice. To examine possible reversal of hyperactive behavior, mice were given lithium chloride (LiCl) in the drinking water (300 mg l^{-1}) for 14 days. We found that consumption of lithium decreased the locomotor activity of *Plcg1*$^{f/f}$*; CaMKII* mice in the open field test to a level comparable with that of WT littermates (Figures 5a and b). Interestingly, the levels of anxiety and depression in *Plcg1*$^{f/f}$*; CaMKII* mice were also normalized by lithium treatment (Figures 5c and d). Moreover, acute injection of LiCl (100 mg kg^{-1}) reduced the open field activity of *Plcg1*$^{f/f}$*; CaMKII* mice to the level of vehicle-treated WT mice, while having no significant effect in WT mice (Supplementary Figures 11a and b). Thus, lithium restored the abnormal behavior of *Plcg1*$^{f/f}$*; CaMKII* mice to normal levels. Similarly, VPA (200 mg kg^{-1}), reduced the locomotor activity of *Plcg1*$^{f/f}$*; CaMKII* mice to level observed in vehicle-treated WT littermates without any effect on the locomotion of WT mice (Figure 5e).

As a possible means of ameliorating the hyperactivity of *Plcg1*$^{f/f}$*; CaMKII* mice, we also attempted to use hyperforin, a TRPC6 channel activator that stimulates downstream effectors of the PLCγ1 pathway (such as CaMKII and cAMP-response element-binding protein).[17,40] When mice were injected intraperitoneally with hyperforin (1 mg kg^{-1} body weight), the hyperlocomotor activity of *Plcg1*$^{f/f}$*; CaMKII* mice was significantly reduced, although there was no effect on WT mice (Supplementary Figures 11c and d). Given the evidence that activation of TRPC6 elevates $[Ca^{2+}]_i$ in *Plcg1*-deficient neurons as effectively as in WT neurons (Supplementary Figure 10), restoration of Ca^{2+} signaling by TRPC6

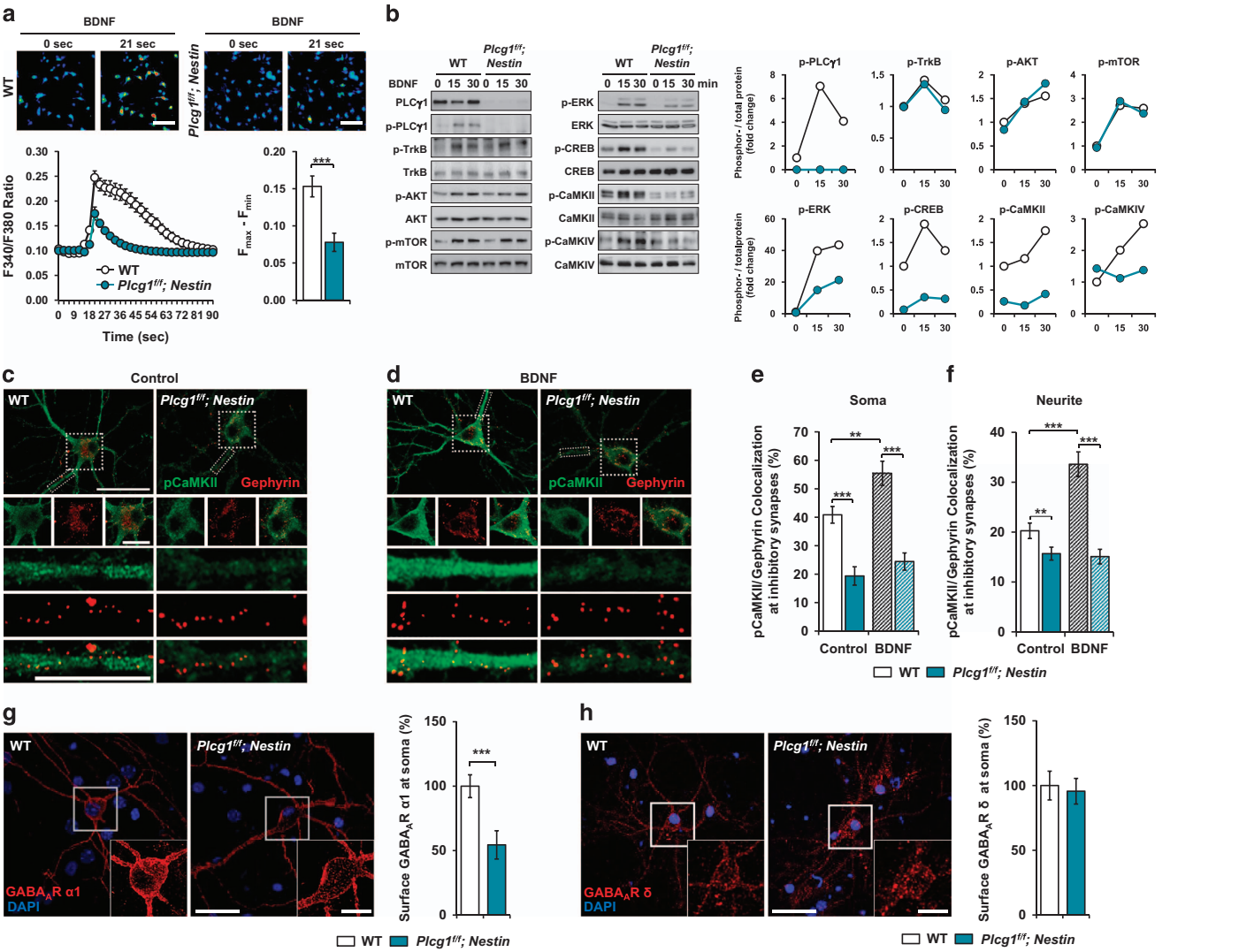

Figure 4. Impaired brain-derived neurotrophic factor (BDNF)-mediated Ca^{2+} signaling and altered distribution of activated Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in phospholipase Cγ1 (PLCγ1)-deleted neurons. (**a**) Representative 380 nm excitation images (upper); 30–50 neurons in each group. The Fura-2 fluorescence ratio (340/380 nm, F/F_0) (lower left) and the maximum change in $F_{340/380}$ (lower right) as a measure of $[Ca^{2+}]_i$ after BDNF treatment of cultured wild-type (WT) or PLCγ1-deficient hippocampal neurons is shown. Scale bar = 50 μm. (**b**) Cultured WT ($n = 21$) and PLCγ1-deficient hippocampal neurons from *Plcg1*$^{f/f}$*; Nestin* mice ($n = 22$) were stimulated with BDNF (100 ng ml^{-1}) for the indicated times and subjected to immunoblotting (left). Quantification of phosphoprotein levels (right). (**c** and **d**) Representative images of cultured WT and *Plcg1*$^{f/f}$*; Nestin* neurons after treatment with or without BDNF (100 ng ml^{-1}) for 30 min at days *in vitro* 20–21, followed by immunostaining for phosphorylated CaMKIIα (pCaMKII) (green) and gephyrin (red). (**e** and **f**) Quantification of pCaMKII – gephyrin colocalization at inhibitory postsynaptic somata ($n = 35 – 40$ per group) (**e**) and neurites ($n = 81 – 97$ per group) (**f**) from four independent cultures. (**g** and **h**) Representative images of hippocampal neurons and quantification of immunofluorescence signals in WT and *Plcg1*$^{f/f}$*; Nestin* mice ($n = 30 – 39$) after immunostaining (red) for surface $GABA_A$ receptor ($GABA_AR$) α1 (**g**) or $GABA_AR$ δ (**h**). Scale bars: 50 μm for full-scale images and 20 μm for insets. Error bars represent ± s.e.m. ***$P < 0.001$ and **$P < 0.01$.

may contribute to restoration of locomotion in *Plcg1*$^{f/f}$*; CaMKII* mice.

BD is a chronic and long-term illness that is often misdiagnosed as attention deficit hyperactivity disorder (ADHD).[41] However, ADHD symptoms tend to disappear with age[42] and ADHD mouse models also show reduced hyperactivity with age.[43] *Plcg1*$^{f/f}$*; CaMKII* mice continue to display hyperlocomotor activity when compared with age-matched WT mice, even at 8 months of age (Supplementary Figure 12). We also examined the behavioral response to amphetamine (Amp), a psychostimulant normally used to treat ADHD, but induces manic episodes in BD patients.[44] Amp administration increases locomotion in various manic-like models such as *GluR6-*, *Erk-*, *Clock*-knockout, and *Shank3*-overexpressing mice.[45–48] Supporting a manic-like, rather than an ADHD-like, behavioral phenotype, acute injection of Amp aggravated the hyperactivity of *Plcg1*$^{f/f}$*; CaMKII* mice (Figure 5f). Therefore, *Plcg1*$^{f/f}$*; CaMKII* does not appear to be directly related to ADHD; however, these mice may be a potential model of manic episodes associated with BD.

DISCUSSION

Aberrant synaptic functions and plasticity are believed to underlie neuropsychiatric disorders such as schizophrenia, ADHD and BD.[49,50] Here we show that PLCγ1 deletion affects inhibitory inputs and BDNF-dependent forms of synaptic plasticity (Supplementary

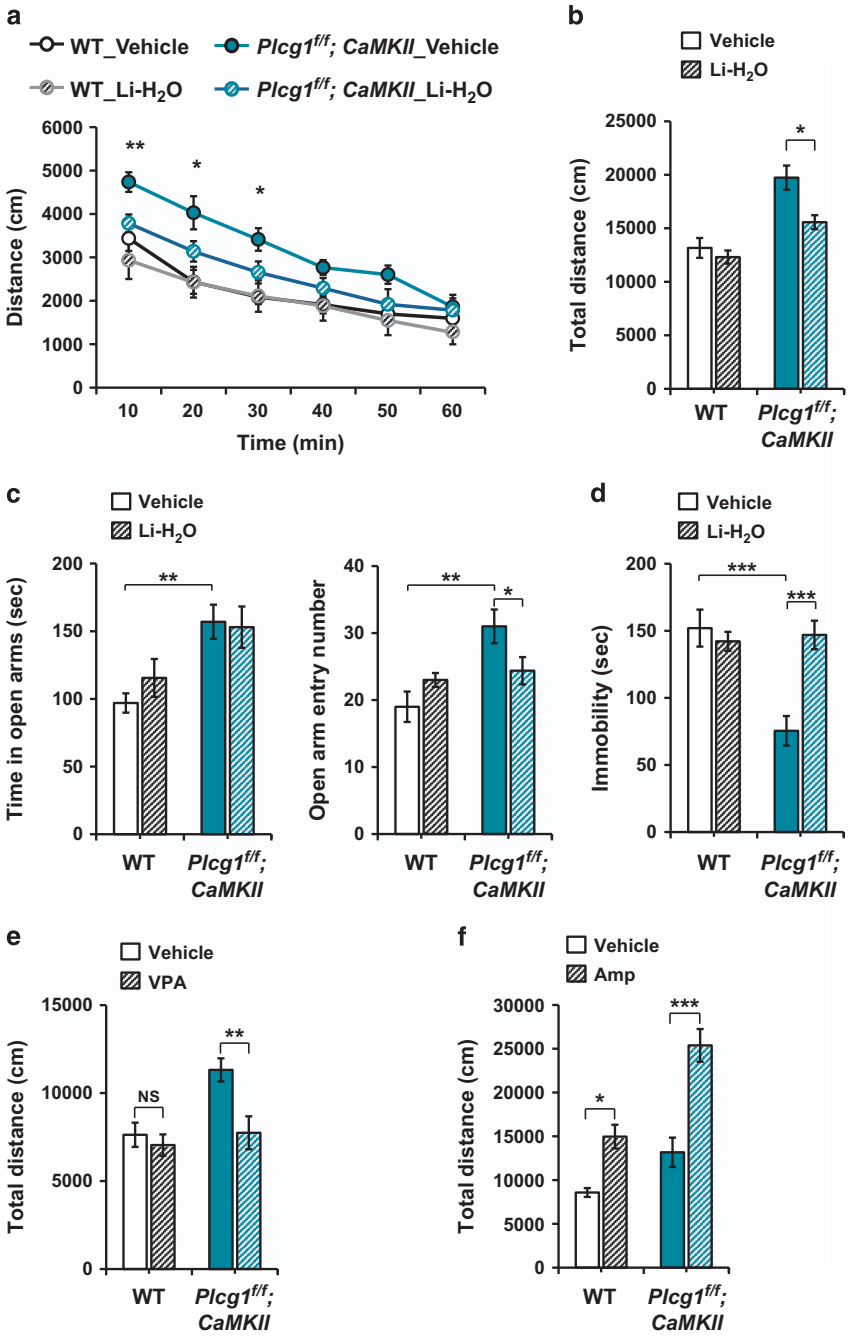

Figure 5. Behavior of *Plcg1*$^{f/f}$*; CaMKII* mice is normalized by lithium chloride (LiCl) or valproic acid (VPA), but not by amphetamine (Amp). (**a**–**d**) Locomotor activity of wild-type (WT) and *Plcg1*$^{f/f}$*; CaMKII* mice ($n = 7$–11 per group) in the open field (**a** and **b**), elevated plus maze (**c**) and forced swimming test (**d**) after LiCl treatment (300 mg l^{-1} for 14 days). (**e**) Locomotor activity in the open field after injection of saline or VPA ($n = 9$–10 per group). (**f**) Locomotor activity in the open field after injection of saline or Amp ($n = 7$–9 per group). Error bars represent ± s.e.m. ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$. NS, nonsignificant.

Figure 13). Although PLCγ1 is an essential mediator of BDNF-TrkB signaling for synaptic features of inhibitory transmission,[51,52] our finding that PLCγ1 has a role in the translocation of CaMKIIα to inhibitory synapses provides a mechanistic basis for how BDNF-TrkB signaling promotes the postsynaptic expression of functional $GABA_A$ receptors.[53] Emerging evidence indicates that the imbalance between excitatory and inhibitory inputs (E/I) is a major cause of various neuropsychiatric disorders[54,55] and the GABAergic dysfunction observed in the hippocampi of BD patients.[56] Therefore, PLCγ1 deletion is highly likely to alter the E/I balance in forebrain circuits such as the hippocampus and striatum, leading to behavioral abnormalities. Lithium or VPA increases inhibitory input into hippocampal neurons.[57,58] Thus, it is reasonable that lithium or VPA enhances basal inhibitory activity in *Plcg1*$^{f/f}$*; CaMKII* mice and thereby normalizes the E/I balance, which can ameliorate the hyperactive behavior.

Interestingly, we observed that loss of PLCγ1 affects BDNF-mediated ERK, CaMKII, CaMKIV and cAMP-response element-binding protein activation, but not the phosphoinositide 3-kinase/AKT signaling pathway (Figure 4b). It is highly likely that deleting

PLCγ1 disrupts BDNF-mediated TRPC channel activation, which in turn interferes with the activation of ERK. Our data are consistent with those of a previous study showing that activation of ERK, CaMKIV and cAMP-response element-binding protein is mediated by Ca^{2+} influx via PLCγ1-triggered TRPC3/6 channels and IP_3 generation in cerebellar granule neurons.[17] Moreover, we cannot exclude the possibility that receptor tyrosine kinase-mediated cascades[11,59] other than the BDNF-TrkB pathway control synaptic functions and lead to behavioral changes in *Plcg1$^{f/f}$; CaMKII* mice. Indeed, neurotrophins stimulate mitogen-activated protein kinase, phosphoinositide 3-kinase and PLCγ1 through respective Trk receptors, and neuregulins (NRG1–6) can activate diverse signaling pathways, including the phosphoinositide 3-kinase, mitogen-activated protein kinase and PLCγ1 pathways.[59] Interestingly, *NRG1* and *NRG3* SNPs are also associated with BD.[60,61] Given the conceivable alterations of TrkB- or other receptor tyrosine kinases-mediated signaling pathways in *Plcg1$^{f/f}$; CaMKII* mice, the underlying molecular mechanisms and functional consequences merit subsequent investigation.

Trkb$^{PLC/PLC}$-knock-in mice lacking PLCγ1 docking sites substantiated the requirement of PLCγ1 activity for normal synaptic plasticity and associative memory.[20,21] However, in contrast to *Plcg1$^{f/f}$; CaMKII* mice, *Trkb$^{PLC/PLC}$*-knock-in mice showed normal locomotor activity and anxiety-like behavior. Although it should be clearly clarified how the two types of mice display disparate behavioral phenotypes, it may simply be due to their different genetic backgrounds or perhaps distinct signaling pathways in which either *Trkb$^{PLC/PLC}$*-knock-in or *Plcg1$^{f/f}$; CaMKII* mice are defective. For example, it is possible that PLCγ1 could be activated through its catalytic domains and by multidomains responsible for protein–protein and protein–lipid interactions[62] in *Trkb$^{PLC/PLC}$*-knock-in mice, but not directly by TrkB. The residual activity of PLCγ1 would possibly support the normal locomotion and anxiety observed in *Trkb$^{PLC/PLC}$*-knock-in mice.

Here we provide substantial evidence that the BDNF-mediated PLCγ1 signaling is required for the formation and function of inhibitory synapses, and that dysfunction of PLCγ1 in the forebrain contributes to hyperactive behavior. As *Plcg1$^{f/f}$; CaMKII* mice exhibited a constellation of manic-like behaviors, but neither sensory-gating deficits nor depression-like behavior, we argue that mice deficient in PLCγ1 would be a reliable model for the manic phase of BD. Considering the necessity and importance of genetic animal models for neuropsychiatric diseases, *Plcg1$^{f/f}$; CaMKII* mice may be a reliable and representative model of manic episodes of BD and may have potential use in future drug development studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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