Loss of aPKC\textsubscript{\lambda} in Differentiated Neurons Disrupts the Polarity Complex but Does Not Induce Obvious Neuronal Loss or Disorientation in Mouse Brains

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Abstract

Cell polarity plays a critical role in neuronal differentiation during development of the central nervous system (CNS). Recent studies have established the significance of atypical protein kinase C (aPKC) and its interacting partners, which include PAR-3, PAR-6 and Lgl, in regulating cell polarization during neuronal differentiation. However, their roles in neuronal maintenance after CNS development remain unclear. Here we performed conditional deletion of aPKC\textsubscript{\lambda}, a major aPKC isoform in the brain, in differentiated neurons of mice by camk2a-cre or synapsinI-cre mediated gene targeting. We found significant reduction of aPKC\textsubscript{\lambda} and total aPKCs in the adult mouse brains. The aPKC\textsubscript{\lambda} deletion also reduced PAR-6\textsubscript{\beta}, possibly by its destabilization, whereas expression of other related proteins such as PAR-3 and Lgl-1 was unaffected. Biochemical analyses suggested that a significant fraction of aPKC\textsubscript{\lambda} formed a protein complex with PAR-6\textsubscript{\beta} and Lgl-1 in the brain lysates, which was disrupted by the aPKC\textsubscript{\lambda} deletion. Notably, the aPKC\textsubscript{\lambda} deletion mice did not show apparent cell loss/regeneration in the brain. In addition, neuronal orientation/distribution seemed to be unaffected. Thus, despite the polarity complex disruption, neuronal deletion of aPKC\textsubscript{\lambda} does not induce obvious cell loss or disorientation in mouse brains after cell differentiation.

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Introduction

In mammals, neuronal cells are polarized in multiple steps of cell differentiation. These include apical-basal polarity of neuronal progenitor epithelial cells, asymmetric division of the progenitors, directed cell migration, axon-dendrite specification and dendritic spine formation. These cell polarizations are fundamental to proper development of the central nervous system (CNS).

Atypical protein kinase C (aPKC) is a Ser/Thr kinase that is structurally different from other typical PKC subfamily kinases; that is, it lacks binding regions for calcium and phosphol ester in its regulatory domain, but contains a protein binding PBl domain at its N-terminus [1]. aPKC forms an evolutionarily conserved protein complex with the PDZ-containing proteins PAR-3 and PAR-6, and it localizes asymmetrically within a cell to regulate polarization. This has been observed in various types of cells, such as C. elegans one-cell embryos, Drosophila epidermis and mammalian epithelial cells [2–4]. aPKC also forms a complex with Lgl, a protein that contains WD repeats. This complex forms independently of PAR-3 and regulates aPKC/PAR-3/PAR-6-mediated polarization of epithelial cells [5–8]. Recent studies of gene knockout or knockdown in mice have established the in vivo significance of aPKC\textsubscript{\lambda} and PAR-3 for epithelial tissue morphogenesis and its maintenance in mammals [9–14].

Genetic studies using Drosophila have further identified critical roles of aPKC/PAR-3/PAR-6 and Lgl in CNS development through the regulation of asymmetric division of neuronal progenitors (neuroblasts) [15–17]. Previously, we found that conditional knockout of an aPKC isoform—aPKC\textsubscript{\lambda}—in mice using a nestin-cre transgene induces disruption of apical-basal polarity of neuronal progenitor cells (neuroepithelial cells) in mouse brain cortex [18]. Although the role of aPKC\textsubscript{\lambda} in neuronal progenitor differentiation was not clarified by this study, possibly because gene knockout was done at a relatively late stage (E15), knockout of PAR-3 at earlier stages (E12–13) enhances neuronal progenitor differentiation whereas ectopic expression of PAR-3 or PAR-6 suppresses it in mouse brains [19,20]. In contrast, knockout of the Lgl isoform Lgl-1 suppresses progenitor differentiation and induces its continuous proliferation, leading to neoplasia formation [21], suggesting that neuronal progenitor
differentiation is differentially regulated by PAR-3 and Lgl-1 in mammals. The importance of aPKC for neural progenitor proliferation/differentiation is shown during neurogenesis in Xenopus [22,23] and zebrafish [24] embryos. As for neuronal migration, overexpression of the PAR-6 isoform PAR-6β has been shown to suppress migration of cerebellar granule neurons by disturbing cytoskeletal organization [25,26]. Thus, aPKC and/or its interactors are involved in multiple steps of CNS development from progenitor maintenance/differentiation to cell migration by regulating cell polarization.

Studies using in vitro cultured rat hippocampal neurons further suggest the involvement of aPKC/PAR-3/PAR-6 in later stages of differentiation [27,28]. One of them is axon specification, during which these proteins localize to the tip of the growing axon and regulate axonal growth of rat cortical neurons during mouse brain development [33,34]. Lgl-1, which these proteins localize to the tip of the growing axon and regulate axonal growth by interacting with several molecules such as KIF3A, APC and Tiam1 [29–32]. In addition, TGF-β signaling and Smurfl E3 ligase regulate PAR-6 by its phosphorylation and degradation, respectively, and play a role in axonal growth of cortical neurons during mouse brain development [33,34]. Lgl-1 has also been shown to regulate axonal growth of rat cortical neurons in vivo [35]. PAR-3, aPKC and PAR-6 are required for dendritic spine morphogenesis in in vitro cultured hippocampal neurons [36,37], and the potential in vivo significance of this is suggested by evidence that BAII interacts with PAR-3 to recruit it to dendritic spines in mice [30]. In addition, analysis of mutant zebrafish has revealed that aPKCα is required for dendritic specification of Purkinje cells during development [39]. Thus, although these observations contradict those observed in Drosophila [40], at least in mammals (and possibly also in zebrafish), aPKC and its interactors are involved in axon/dendrite specification and morphogenesis in later stages of neuronal differentiation.

In contrast to the significance of aPKC and its interactors for neuronal differentiation during CNS development, their roles in neuronal maintenance after CNS development remain unknown. To clarify this, we established mice in which aPKCα is deleted specifically in differentiated neurons. We found a significant reduction of aPKCθ and the polarity complex in the brains of these mice. However, the mice were healthy and did not show clear brain weight loss or cell degeneration. In addition, staining of several markers suggested that neuronal orientation/distribution was totally unaffected in these mice. Thus, despite the disruption of the polarity complex, our analysis did not detect obvious cell loss or disorientation by neuronal deletion of aPKCθ after cell differentiation.

Results
Promoter- and age-dependent DNA recombination in brain neurons by cre transgenes
To examine the role of aPKCθ in differentiated mouse neurons, we used a cre-loxP system to establish mouse lines with conditional deletion of aPKCθ in differentiated neurons [41]. For cre expression, we used two transgenic mouse lines, synapsin1-cre (S1-cre) and camk2a-cre (C2-cre), which express cre specifically in differentiated, postmitotic neurons of the brain [41,42]. We first checked cre expression by these transgenes using RNZ reporter mice that express LacZ in nuclei by cre-mediated DNA recombination [43]. LacZ staining using X-gal as substrate revealed that S1-cre induced LacZ expression in whole brain regions, especially in layers IV/V of cortex, CA3 and dentate gyrus of the hippocampus, thalamus and brain stem (Figure S1A, B). In contrast, C2-cre induced LacZ expression specifically in the forebrain, especially in layers II-IV of cortex and CA1/3 and dentate gyrus of the hippocampus (Figure S1C, D).

We also checked cre-mediated LacZ expression by staining with anti-LacZ antibody. In S1-cre; RNZ mice, LacZ-positive cells were strongly detected in the hippocampus and cortex but very few were seen in the cerebellum at 16 weeks (Figure 1A), consistent with the above LacZ staining data. The specificity of these signals was confirmed using RNZ mice without the cre transgene in which distinct anti-LacZ signal was not detected (Figure 1A). Notably, the LacZ expression became wider at a later stage (23 weeks); anti-LacZ positive cells were more broadly detected in cortex and hippocampus (Figure 1A, B). Especially in cerebellum, significant anti-LacZ signals were detected in Purkinje and granular cells at 23 weeks this stage (Figure 1A, B). Similarly, cells with high LacZ expression were more broadly detected in cortex and hippocampus of C2-cre; RNZ mice at 24 weeks of age compared with those at 8 weeks of age, although the expression was restricted to the forebrain region in these mice (Figure 1C, D). Thus, cre expression in S1-cre or C2-cre is promoter dependent and become wider with age in mouse brain.

Generation of mutant mice with conditional aPKCθ deletion in differentiated neurons
The cre transgenic mice were crossed with aPKCθfloxed mice in which exon 5 of aPKCθ genes is flanked by loxP sequences [18]. To generate aPKCθ conditional deletion mice under the C2-cre transgene (aPKCθC2-cko), we crossed aPKCθfloxed mice with aPKCθfloxed mice. Resultant pups were aPKCθC2-cko (floxed/floxed; C2-cre) mice at the expected Mendelian ratio in addition to mice with other genotypes (Table S1). The aPKCθ conditional deletion mice under the S1-cre transgene (aPKCθS1-cko) were generated by a similar strategy. In this case, however, we occasionally obtained mice with a deleted aPKCθ allele, possibly due to its recombinination in the germine during generation [44]. As a consequence, two types of aPKCθS1-cko mice were obtained; floxed/floxed; S1-cre and floxed+/−; S1-cre (Table S2), although the ratio for these cko mice was a little higher than expected, for an unknown reason. Thus, we obtained two lines of differentiated neuron-specific aPKCθ conditional deletion mice, aPKCθS1-cko and aPKCθC2-cko mice.

Neuronal deletion of aPKCθ results in reduction of total aPKCs and PAR-6β in mouse brain
To check the expression of aPKCθ and its related proteins in aPKCθS1-cko and aPKCθC2-cko mice, we first performed Western blot analysis. Because of age-dependent cre expression in the mouse brain as described above, we sampled brains at later stages (7-month-old aPKCθS1-cko mouse and 13-month-old aPKCθC2-cko mouse) to delete aPKCθ in broad types of cells in the brain. The brains were then separated into 5 regions: striatum (Str), hippocampus (Hpc), cortex (Cor), other remaining cerebrum regions (Other) and cerebellum (Cbl), and analyzed by Western blotting.

Staining with aPKCθ-specific antibody revealed that aPKCθ expression, which was widely detected in the brain, was reduced in aPKCθS1-cko mouse brain (Figure 2A). Similar reduction was also observed when we used an antibody recognising both aPKCθ and another aPKC isoform, aPKCβ [18] (Figure 2A), suggesting that expression of all aPKC isoforms was reduced in these brain regions of aPKCθS1-cko mice. In contrast, we found that expression of PAR-6β, a PAR-6 isoform that binds to the Pβ1 domain of aPKCθ to form a functional protein complex for cell polarization [3,4,45], was also reduced in aPKCθS1-cko mouse brains (Figure 2A). In contrast, p62, another Pβ1-interacting protein of aPKCθ [46,47], did not show altered expression after
aPKC deletion [Figure 2A]. Similar patterns of altered aPKCs, total aPKCs and PAR-6β expression, but not p62, were observed in aPKC S1-cko mice, although the alterations were specific to forebrain regions including striatum, hippocampus and cortex (Figure 2B), consistent with the C2-cre expression described above (Figure 1, S1). These data support the region-dependent deletion of aPKC by the S1-cre or C2-cre transgene, which accompanies reductions of total aPKCs and PAR-6β in the brain.

The reduction of aPKCα, total aPKCs, and PAR-6β was also observed when we used cerebri of aPKC S1-cko (Figure 2C, D) or C2-cko (Figure 2E, F) mice. In contrast, expression of other aPKCα-interacting polarity proteins, such as PAR-3 and Lgl-1, as well as p62, was not altered (Figure 2C–F). In addition, PKMε, an alternative isoform of aPKCζ lacking its N-terminal regulatory domain [48], did not show altered expression in aPKC deletion cerebra (Figure 2C–F). Thus, aPKCα deletion by S1-cre or C2-cre induces specific reduction of aPKCα, total aPKCs and PAR-6β without affecting expressions of PAR-3, Lgl-1, p62 and PKMε in the cerebrum. Taken together, these data support the notion of aPKCα gene knockout by cre transgenes, which results in ~50% reduction of total aPKCs in the brain. The remaining aPKCs after aPKCα conditional deletion might be expressed in non-neuronal cells such as glia and/or neurons without cre expression.

**Figure 1. Detection of DNA recombination by synapsinI-cre or camk2a-cre transgene in mouse brain.** Transgenic mice for synapsinI-cre (S1-cre) or camk2a-cre (C2-cre) were crossed with RNZ mice. RNZ male mice harboring S1-cre or C2-cre at indicated weeks of age were subjected to anti-LacZ staining to detect cre-mediated DNA recombination. RNZ mice without a cre transgene were used as controls. (A) In S1-cre; RNZ mice at 16 weeks of age, LacZ-positive cells were strongly detected in dentate gyrus and CA3 in hippocampus and some cortical cells, but very few were seen in cerebellum. At 23 weeks, LacZ expression became wider in the cortex and hippocampus, and was clearly detected in cerebellum. No distinct LacZ expression was detected in the control RNZ mice. (B) High magnification of boxed region in (A). LacZ expression was broadly detected in multiple layers of cortex and Purkinje and granular cells of cerebellum in 23 week-old S1-cre; RNZ mice. (C) LacZ-positive cells were broadly detected in brains of 8-week-old C2-cre; RNZ mice, especially in layer II/III of cortex and CA1 of hippocampus. It became wider at 24 weeks of age. Again, no distinct LacZ expression was detected in the control RNZ mice. (D) High magnification of boxed region in (C), indicating detection of LacZ-positive cells in multiple layers of cortex and Purkinje and granular layers of cerebellum. Bars are 1 mm (A, C) and 0.4 mm (B, D).

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aPKCζ is a major aPKC isoform in mouse brain and its deletion did not affect transcription of its related gene

We next examined mRNA levels of aPKCζ and other related genes in the cerebrum of aPKCζ deletion mice by quantitative RT-PCR. First, we made a primer set for aPKCζ, targeting its regulatory domain (RD) and two primer sets for aPKCζ targeting its RD and kinase domain (KD) [see Materials and Methods]. To check the specificity of these primer sets, we used plasmid DNA containing mouse aPKCζ or aPKCζ cDNA for quantitative PCR. As shown in Figure 3A and B, the aPKCζ primer set efficiently amplified only aPKCζ cDNA, whereas aPKCζ primer sets (RD and KD) efficiently amplified only aPKCζ cDNA. Quantification confirmed specific detection of the target genes by these primer sets (Figure 3C). Thus, the primers we used are available for aPKC isoform-specific detection by quantitative PCR. RT-PCR using the aPKCζ primer set indicates around 50% reduction of aPKCζ in cerebrum of aPKCζ S1-cko or C2-cko mice (Figure 3D, E), which is compatible with the Western blot data (Figure 2D, F). In contrast, the aPKCζ deletion did not affect mRNA expressions of PAR-6β, PAR-3, Lgl-1 and PAR-6ζ, another PAR-6 isoform (Figure 3D, E). No reduction of PAR-6β mRNA in contrast to its protein reduction by aPKCζ deletion suggests that PAR-6β protein reduction is not caused by its reduced transcription, but rather by other unknown mechanisms such as destabilization.

Using the plasmid DNA of aPKCζ, we estimated the relative amount of aPKCζ (full-length) with that of PKMε in mouse brain.
The RD and KD primer sets for aPKCζ were used to detect aPKCζ and both aPKCζ/PKMζ, respectively. As shown in Figure 3F, PCR amplification by the aPKCζ RD primer set was hardly observed compared with that by the aPKCζ KD primer set. Thus, PKMζ is the abundant isoform in adult mouse brain, which is consistent with previous observations [18,48,49]. We also observed that expressions of aPKCζ and PKMζ were not altered in aPKCζ S1-cko brains (Figure 3G), suggesting no compensatory
induction of aPKC\(_l\) by aPKC\(_l\) deletion. Taken together with the Western blot data described above (Figure 2), these data support the notion that aPKC\(_l\) is a major full-length aPKC isoform expressed in adult mouse brain and that aPKC\(_l\) deletion mostly reflects loss of total aPKCs in neurons.

Neuronal deletion of aPKC\(_l\) disrupts polarity protein complex in mouse brain cortex

For regulation of cell polarity, aPKC\(_l\) works as a protein complex with other polarity proteins including PAR-6, Lgl-1 and PAR-3 [3,4,7]. To examine the effect of aPKC\(_l\) deletion on the protein complex formation, we lysed the cortex of aPKC\(_l\) S1-cko mice and subjected it to gel filtration. As shown in Figure 4A, most of the aPKC\(_l\) was solubilized in this condition. Gel filtration revealed that aPKC\(_l\) and Lgl-1 were mainly found in fractions 13–24 (referred to as Fr. II in Figure 4A). Reduction of aPKC\(_l\) in Fr. II was observed in aPKC\(_l\) S1-cko cortical lysates. In contrast, p62 was found in earlier fractions 5–12 (referred to as Fr. I in Figure 4A), suggesting that it incorporates into a large protein complex. Detailed analysis of fractions 1–12 suggests that p62 was mainly contained in Fr. I where aPKC\(_l\) was hardly detected (Figure 4B). Thus, aPKC\(_l\) and Lgl-1 were contained in Fr. II and...
Neuronal aPKC deletion after Cell Differentiation

Neuronal deletion of aPKCΔ did not induce apparent neuronal loss/degeneration in mouse brain

Although above data clearly suggest that conditional deletion of aPKCΔ reduces total aPKCs and disrupts the polarity protein complex in mouse brain, neither aPKCΔ S1-cko nor C2-cko mice showed any alteration in their appearance, body size or behavior (data not shown). Survival may not have been affected either (mean life spans of aPKCΔ S1-cko and C2-cko female mice are 94±19 weeks (n=6) and 98±14 weeks (n=4), respectively). Notably, hematoyxlin staining of cerebral coronal sections suggested no clear alteration in overall cell population in aPKCΔ deletion mice (Figure 6A, B). In addition, total brain weights were not changed (Figure 6C, D). We also stained the sections with anti-NeuN, a neuronal marker, and found that NeuN-positive neurons seemed to be preserved in aPKCΔ S1-cko and C2-cko mice (Figure 7A, B, Table S3). Furthermore, anti-GFAP staining revealed no induction of astrocytosis, an indicator of neurodegeneration (Figure 7C, D). The absence of GFAP induction was also confirmed by quantitative RT-PCR (Figure 5D, E). These data suggest that aPKCΔ conditional deletion in differentiated neurons did not lead to obvious neuronal loss/degeneration in mouse brain.

Neuronal deletion of aPKCΔ may not affect neuronal orientation/distribution in mouse brain

We next examined distribution of neural structures of the aPKCΔ deletion mouse brain by staining with antibodies for MAP2, phospho-neurofilaments (pNF) and synaptophysin (SYP) — markers for dendrites, axons and synapses, respectively. As shown in Figure 8A, staining patterns of these proteins were not clearly altered in brains of aPKCΔ S1-cko and C2-cko mice. Detailed analysis of the cortex of aPKCΔ S1-cko mice suggests that distribution of dendrites and axons in layers II/III region may not be affected (Figure 8B, S2E). No distinct alteration in staining patterns of dendrites, axons and synapses was observed in aPKCΔ S1-cko or C2-cko mice in later stages (Figure S2A, C, E). These data suggest that neuronal deletion of aPKCΔ does not affect distribution of these neural structures in mouse brain cortex.

We next checked cell orientation by staining with anti-GM130, a Golgi marker, and noticed that Golgi locations in layer V cortical neurons seemed to be preserved in aPKCΔ S1- and C2-cko mice (Figure 8C, S2B, D, E). Detailed analysis suggested that Golgi was concentrated to the superior part of the cell body in a majority of these neurons, both in control and aPKCΔ S1- or C2-cko mice (Figure 9A, B). In addition, the Nav1.6 voltage-gated sodium channel, an axon initial segment marker [30,31], was observed at the inferior region of the neurons in both control and aPKCΔ S1- or C2-cko mice (Figure 9A). Taken together, these data suggest that the orientation of layer V cortical neurons was unaffected in the aPKCΔ deletion mice. We further analyzed Purkinje cells in aPKCΔ S1-cko cerebellum. Calbindin and pNF staining suggest that dendritic and axonal distribution around Purkinje cells may not be affected (Figure 10A, B). In addition, the concentration of Golgi to the molecular layer side of these cells seemed to be preserved in aPKCΔ S1-cko mouse (Figure 10A, C). Taken together, these data suggest that neuronal deletion of aPKCΔ does not affect neuronal orientation/distribution in cortex and cerebellum.

Discussion

In this study, we first developed mice with conditional deletion aPKCΔ in brain differentiated neurons by camk2a-cre or synapsin1-cre mediated gene recombination. We found that aPKCΔ is the aPKC isoform that is almost exclusively expressed in mouse brain, not aPKCΔ, as previously suggested [18,49], and that neuronal deletion of aPKCΔ induced reduction of the total fraction of aPKCs without inducing expression of aPKCβ and PKMΔ. Biochemical analyses suggested that the aPKCΔ deletion accompanied destabilization of PAR-6β and decrease in the protein complex containing aPKCΔ, PAR-6β and Lgl-1. Despite the significant reductions of total aPKCs and the polarity complex, aPKCΔ deletion did not induce apparent neuronal loss or degeneration in the brain, even in aged mice. In addition, staining of several markers suggested that overall neuronal orientation/distribution may be unaffected in these mice. Thus, although aPKCΔ deletion in differentiated neurons disrupts the polarity complex in mouse brain, it does not induce obvious cell degeneration or neuronal disorientation, implying that aPKCΔ and the polarity complex are not indispensable for neuronal survival and organized cell distribution in adult mouse brain.

Our observations stand in contrast to the critical roles of aPKC in several steps of neuronal differentiation during CNS development. One possibility is that aPKC is required only for cell polarization processes during neuronal differentiation but not for maintenance. Indeed, in vitro studies using cultured epithelial cells have shown that aPKC suppression affects cell polarity only after re-polarization [43,52]. In addition, so far there has been no report of axonal/dendritic degeneration by suppressing aPKC or its related proteins after axonal specification in cultured hippocampal neurons [36,37]. Alternatively, alterations are structurally too small to be detected by our tissue-based microscopic analysis. These could include dendritic spines, small protrusions forming postsynaptic structures, as aPKC and its related proteins are critical for maintenance of spine structure as well as morphogenesis in cultured hippocampal neurons [36,37]. The essential role of aPKCΔ in tissue structural maintenance is also evidenced by podocytes-specific knockout mice in which the maintenance of slit
diaphragms, only detectable under an electron microscope in renal glomeruli, are disorganized, resulting in renal dysfunction [10]. Further detailed analysis is necessary for final conclusion.

One possible way to identify a clear significance of aPKC\(\lambda\) in differentiated neurons is examination of its role in neurological disease conditions. In epithelial cells, suppression of aPKC\(\lambda\), PAR-5 or PAR-6 modulates tumorigenesis of several tissues in vivo [53–59]. Morphological abnormalities of dendritic spines are reported in a variety of neurological diseases such as Fragile-X mental retardation syndrome and Alzheimer’s neurodegenerative disease.

Figure 4. Gel filtration of cortical lysates of aPKC\(\lambda\) conditional deletion mice. Cortex of 7-month-old female mice harboring aPKC\(\lambda\) flox/−; S1-cre (Cho) or flox/+ (Cont) was homogenized with lysis buffer. After centrifugation and removal of pellets (Ppt), supernatants (Sup) were subjected to gel filtration, and a total of 40 fractions were collected. Molecular weight markers were detected in Fr. 13–14 (669 kDa; thyroglobulin), Fr. 17–18 (440 kDa; ferritin), Fr. 23 (67 kDa; bovine serum albumin) and Fr. 29 (25 kDa; RNase). (A) Western blot analysis of Ppt, Sup, and mixture of four sequential fractions using antibodies for aPKC\(\lambda\), Lgl-1 and p62. aPKC\(\lambda\) and Lgl-1 were detected mainly in fractions 13–24 (referred to as Fr. II) in the control cortex, whereas p62 was detected exclusively in fractions 5–12 (Fr. I). (B) Western blot analysis of Sup and fractions 1–12 using antibodies for aPKC\(\lambda\) and p62. p62 but not aPKC\(\lambda\) was highly detected in the Fr. I in control cortex. (C) Western blot analysis of Sup and fractions 13–24 using antibodies for aPKC\(\lambda\), PAR-6\(\beta\), Lgl-1, PKM\(\gamma\) (sc-216) and PAR-3. aPKC\(\lambda\) was broadly detected in fractions 15–24 in the control cortex, which could be separated into two fractions; Fr. II containing PAR-6\(\beta\) and Lgl-1, and Fr. IIb without containing aPKC\(\lambda\)-interacting proteins examined here. (D) Schematic model of potential protein compositions in the cortical lysates. In the control mouse, aPKC\(\lambda\) was incorporated into two major fractions: the Fr. Ia containing aPKC\(\lambda\) in a protein complex with PAR-6\(\beta\) and Lgl-1, and the Fr. IIb containing complex-free aPKC\(\lambda\) monomer. In contrast, aPKC\(\lambda\) was not clearly detected in the Fr. I containing large protein complex composed of p62 oligomer and some of its interacting proteins (indicated by an X). aPKC\(\lambda\) deletion induces reductions of aPKC\(\lambda\) in complex (IIa) as well as free aPKC\(\lambda\) (IIb), resulting in PAR-6\(\beta\) reduction and Lgl-1 dissociation from the complex.

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[58,61], and notably functional interaction of FMR1, a causative gene of Fragile-X syndrome, with Lgl is reported in Drosophila [62]. Tau-mediated neuropathology is also possible because PAR-1, a downstream target of aPKC [63,64], is shown to be involved in hyperphosphorylation of tau which links to Alzheimer’s disease [65]. Axon regeneration after CNS injury may also be interesting for analysis because roles of aPKC in axonal elongation and guidance have been reported [66–68]. Very recently, Ren et al. have shown that knockdown of aPKC in hippocampal neurons suppresses expression of long-term potentiation (LTP), and in this case aPKC cooperates with p62 for phosphorylation of AMPA receptors to mediate its synaptic incorporation [69]. Because p62 is another protein that binds to the PB1 domain of aPKC in addition to PAR-6 [46,47], it seems that this aPKC function is different from that in the cell polarity complex with PAR-6. An aPKC inhibitory peptide, aPKC pseudosubstrate (PS) peptide, suppresses PKM and induces LTP suppression and memory perturbation [48]. However, two groups have recently reported that knockout of aPKC or PKM does not affect LTP and learning/memory in mice whereas aPKC-PS peptide is still effective in these mice [49,70]. In addition, aPKC-PS peptide is also shown to suppress aPKC at physiological concentrations [49,69]. Thus, it is likely that aPKC is also the physiological target of aPKC-PS peptide and involved in memory function by regulating LTP in mouse brain. Our mutant mice may be useful in examining polarity-independent functions of aPKC, which would identify novel mechanisms underlying maintenance of long-term memory in vivo.

Materials and Methods

Mice

The mouse experiments were approved by the animal experiment committee at RIKEN Brain Science Institute. Mice were maintained and bred in accordance with RIKEN guidelines. The generation of aPKC flox mice maintained on a C57BL6 (B6) background was described previously [18]. The transgenic mice for camk2a-cre (C2-cre) harboring a cre transgene under the camk2a promoter (B6.Cg-Tg (Syn1-cre) 671Jxm/J) [41] and mice for synapsinI-cre (S1-cre) harboring a cre transgene under the synapsinI promoter (B6.Cg-Tg (Camk2a-cre) T29-qStl/J) [42] were obtained from the Jackson Laboratory (Bar Harbor, ME). RNZ (ROSA26-LoxP-STOP-loxP-nlsLacZ) knock-in (KI) mice that express LacZ under cre-mediated recombination [43] were generously provided by Dr. Itohara (RIKEN BSI). All mice were maintained on a B6 background. For generation of C2-cre-mediated aPKC conditional deletion (aPKC C2-cko) mice, we crossed aPKC flox/flox mice with aPKC flox/+; C2-cre mice.

Figure 5. Immunoprecipitation assay using aPKC conditional deletion mouse brains. (A) Cerebra of 11-month-old male mice harboring aPKC flox/flox (Cont; n = 3) or aPKC flox/flox; S1-cre (aPKC S1-cko; n = 3) were lysed (Input) and subjected to immunoprecipitation (IP) with anti-Lgl-1 antisera. IP without antisera (-) was used as a negative control. The input and IP samples were analyzed by Western blotting using antibodies for Lgl-1 and aPKC. (B) Bands of IP samples in (A) were quantified and plotted. (C) Cerebra of 20-month-old male mice harboring aPKC flox/flox (Cont; n = 3) or aPKC flox/flox; C2-cre (aPKC; n = 3) were subjected to IP and analyzed as in (A). (D) Bands of IP samples in (C) were quantified and plotted. Note the significant reduction of aPKC co-immunoprecipitated with Lgl-1 in these aPKC deletion mouse cerebra. Values are means ±SD (*P<0.05, **P<0.001).

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For generation of S1-cre-mediated aPKCα-mediated conditional deletion (aPKCα S1-cko) mice, we crossed aPKCα flox/flox mice with aPKCα flox(-)/+; C2-cre. The (-) indicates a deleted allele of aPKCα detected in some mice when crossed with S1-cre during generation, possibly due to germline recombination [44]. As a consequence, mice with a deleted aPKCα allele (-) instead of the flox allele were occasionally obtained in the generation of aPKCα S1-cko mice. The sequences of primers used for genotyping are listed in Table S4.

Antibodies
Rabbit polyclonal antibodies for PAR-6β (BC31AP) and Lgl-1 (C-2AP) were described previously [6]. Rabbit polyclonal antibody for Nav1.6 was generously provided by Dr. Ogiwara and Dr. Yamakawa (RIKEN BSI) [50,51]. Antibodies for aPKCα (610175) and GM130 (610822) were from BD (Transduction); and synaptophysin (SYP, MAB3258), Calbindin D-28K (AB1778) and NeuN (MAB377) were from MILLIPORE (Chemicon). The following antibodies were also used: β-actin (A5441, Sigma-Aldrich), aPKCα/ζ (C-20) (sc-216, Santa Cruz), GFAP (Z0334, DAKO); LacZ (200-4136, Rockland), MAP2 (M4403, Sigma-Aldrich), p62 (PM045, MBL), PAR-3 (07-330, Upstate) and phospho-neurofilament (pNF) (SMI 31, Covance (Sternberger Monoclonals Inc)).

Histological analysis
Mice were perfused with 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS), cryoprotected with 20% sucrose/PBS and processed for cryosectioning (10 μm or 20 μm). Hematoxylin staining was performed using Mayer’s Hematoxylin. Immunohistochemistry and immunofluorescence microscopy were performed as described previously [71,72], and images were obtained by a CCD camera-equipped Olympus microscope (AX80) or Keyence microscope (BZ-9000). Quantitative analyses (counting of anti-NeuN-positive cells and measurement of anti-GM130 fluorescence intensities) were performed using ImageJ software [73]. For LacZ staining, fixed whole brains by perfusion were cut into 2-mm sections using brain matrix, and further fixed in 4% PFA/PBS for 2 hr at 4°C. After rinsing with 100 mM NH₄Cl/PBS and detergent solution (2 mM MgCl₂, 0.01% deoxycholate, 0.02%...
NP-40 in PBS), sections were incubated in X-gal solution (1 mg/ml X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide in detergent solution) overnight at 37°C. Images were obtained using a digital camera-equipped Leica stereo microscope (MZFLIII).

Quantitative reverse transcription (RT)-PCR
Preparations of total RNA, reverse transcription and cDNA synthesis from mouse tissue were performed as described previously [71]. Primers for quantitative real-time PCR were designed based on Primer Express software (Applied Biosystems). Real-time PCR was performed by Roche FastStart Universal SYBR Green Master (ROX) using LightCycler 480 (Roche) according to the manufacturer’s protocol. All values obtained were normalized with respect to levels of GAPDH mRNA. Primers used for RT-PCR are listed in Table S5. Plasmid DNA for mouse aPKC\(l\) or mouse aPKC\(f\) in SRD vector was used to check specificities of primers for detection of aPKC\(l\) and aPKC\(f\) and to compare the amount of full-length aPKC\(f\) with that of PKM\(f\) in mouse brain.

Gel filtration, immunoprecipitation and Western blotting
For gel filtration, isolated brain cortices were homogenized in phosphate-buffered saline (PBS) containing 0.1% triton X-100 and complete protease inhibitor on ice. After centrifugation at 14 krpm for 30 min and filtration 250 µg of protein were separated by gel filtration (superose 6) using a SMART system (GE Pharmacia) at a speed of 40 µl/min. Total 40 fractions (40 µl/tube) were collected from 18 min after the sample loading. For immunoprecipitation, brain cortebra were homogenized in lysis buffer containing 20 mM Hepes at pH 7.2, 150 mM NaCl, 0.5% triton X-100, 10% glycerol and complete protease inhibitor. After centrifugation at 14 krpm for 30 min, the lysates containing 2 mg of protein were co-incubated with anti-Lgl-1 antisera (C-2) conjugated with protein A sepharose. After washing with the lysis buffer three times, the immunoprecipitates were eluted with SDS sample buffer. SDS-PAGE and Western blotting were performed as described previously [71]. Chemiluminescent signals were obtained and quantified using ImageQuant LAS-4000 (GE).

Statistical analysis
For comparison between two sample groups, data were first analyzed by F-test. For \(P<0.05\), the data were analyzed by unpaired Student's t-test (two-tailed); otherwise data were analyzed by Welch's t-test (two-tailed). We considered the difference between comparisons to be significant when \(P<0.05\) for all statistical analyses.
Supporting Information

Figure S1 LacZ staining of RNZ mice harboring synapsinI-cre or camk2a-cre. RNZ mice harboring synapsinI-cre (S1-cre) or camk2a-cre (C2-cre) were subjected to LacZ staining using X-gal as a substrate to detect cre-mediated DNA recombination. (A) Wide distribution of LacZ-positive cells in brain of 18 week-old S1-cre; RNZ female mouse. (B) Magnified images of cortex, hippocampus and cerebellum shown in (A). (C) Forebrain-specific distribution of LacZ-positive cells in brain of 8 week-old C2-cre; RNZ female mouse. Age-matched RNZ female mouse (without cre transgene) was used as a negative control. (D) Magnified images of cortex, striatum and hippocampus shown in (C). Cor (cortex), Str (striatum), Hpc (hippocampus), Th (thalamus), Cbl (cerebellum), BS (brain stem), and DG (dentate gyrus). Bars are 5 mm (A, C) and 1 mm (B, D).

Figure 8. Neural marker staining of aPKC\(L\) deletion mouse cerebrum. Immunohistochemical analysis of 7-month-old aPKC\(L\) flox/\(-\); S1-cre (S1-cko) or flox/+ (Cont) female mice (left two panels), or 15-month-old aPKC\(L\) flox/flox; C2-cre (C2-cko) or flox/+; C2-cre (Cont) male mice (right two panels). (A) Staining of coronal sections with antibodies for microtubule-associated protein-2 (MAP2), phospho-neurofilament (pNF) and synaptophysin (SYP), markers for dendrites, axons and synapses (pre-synapses), respectively. (B) Enlarged images for cortical layer II/III region of 7-month-old female mice stained with anti-MAP2 or anti-pNF antibody shown in (A). (C) Staining of coronal sections of 7-month-old female mice with antibody for GM130, a Golgi marker. Images for cortical layer V region are shown, and insets are enlarged images for layer V neurons. Note no distinct alteration in neuronal marker staining and Golgi location in aPKC\(L\) deletion mice. Cor (cortex) and Hpc (hippocampus). Bars are 1 mm (A), 100 \(\mu\)m (B, C) and 40 \(\mu\)m (insets in C).

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Figure S2 Neural marker staining of cerebrum of aged aPKC\(L\) deletion mice. Immunohistochemical analysis of 18-month-old aPKC\(L\) flox/\(-\); S1-cre (S1-cko) or flox/+ (Cont) male mice (A, B), or 26-month-old aPKC\(L\) flox/flox; C2-cre (C2-cko) or
flox/+ (Cont) female mice (C, D). (A, C) Staining of coronal sections with antibodies for microtubule-associated protein-2 (MAP2), phospho-neurofilament (pNF) and synaptophysin (SYP), markers for dendrites, axons and synapses (pre-synapses), respectively. Images for cortical layer II/III region are shown. (B, D) Staining of coronal sections with antibody for GM130, a Golgi marker. Images for cortical layer V region are shown (insets are enlarged images of layer V neurons). Note no distinct alteration in neuronal marker staining and Golgi localization in aPKC^l deletion mouse. (E) Cortical areas shown in (A, C) containing layers II/III and in (B, D) containing layer V, Cor (cortex) and Hpc (hippocampus). Bars are 100 μm (A–D) and 40 μm (insets in B, D).

Figure 9. Cell orientation of cortical layer V neurons in aPKC^l deletion mice. (A) Coronal sections of 7-month-old aPKC^l flox/−; S1-cre (S1-cko) or flox/+ (Cont) female mice (left two panels), or 15-month-old aPKC^l flox/flox; C2-cre (C2-cko) or flox/+; C2-cre (Cont) male mice (right two panels) were stained with a Golgi marker GM130 (red) and an axon initial segment (AIS) marker Nav1.6 (green). Nuclei were stained with TOTO-3. Cortical layer V neurons are shown. Note that Golgi was abundant at superior part of the neurons (arrows) whereas AIS was detected in inferior region (arrowheads) in both control and aPKC^l deletion mice. (B) Immunofluorescence data in (A) were used for quantification of relative GM130 fluorescence intensities in superior region to those in whole cell region (n means number of analyzed cells). Values are means ± SD. A majority of the layer V neurons showed superior accumulation of GM130, which was not significantly affected in aPKC^l deletion mice. Bar is 20 μm (A). doi:10.1371/journal.pone.0084036.g009

Table S1 Born ratio of aPKC^l C2-cko mice. (PDF)

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<th>aPKC^l, C2-cko</th>
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<td>n.s. (P = 0.104)</td>
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<td>Inferior</td>
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<td>n.s. (P = 0.104)</td>
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Table S2 Born ratio of aPKC^l S1-cko mice. *The (−) means deleted allele of aPKC^l detected in some mice when crossed with S1-cre possibly due to its recombination in germline. +Mice with aPKC^l deleted allele (−) instead of flox allele were occasionally obtained during generation. (PDF)

Table S3 Quantification of anti-NeuN stained cells in brain cortex. *Coronal sections of indicated control or aPKC^l deletion mice were stained with anti-NeuN. The NeuN-positive cells in all layers of cortex (60 μm in width) in left and right hemisphere were quantified. Mean cell number and ratio to control for each pair were also indicated. (PDF)

Table S4 List of primers used for genotyping. (PDF)

Table S5 List of primers used for quantitative RT-PCR. (PDF)
Acknowledgments
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Author Contributions
Conceived and designed the experiments: TY NN. Performed the experiments: TY AT MK. Analyzed the data: TY AT. Contributed reagents/materials/analysis tools: KA TH SO NH. Wrote the paper: TY NN.

References

Figure 10. Neural marker staining of aPKC\lambda deletion mouse cerebellum. (A) Coronal sections of cerebellum of 7-month-old aPKC\lambda flox/−; S1-cre (S1-cko) or flox/+ (Cont) female mice were stained with antibodies for calbindin, phospho-neurofilament (pNF) and GM130, markers for Purkinje cells, axons and Golgi apparatus, respectively. Insets are enlarged images of boxed regions. (B, C) The sections were stained with calbindin (green) together with pNF (red; B) or GM130 (red; C). Nuclei were stained with TOTO-3. GM130 was relatively concentrated to molecular layer side in Purkinje cells, whereas pNF was highly detected in granular layer. PC (Purkinje cell), ML (molecular layer) and GL (granular layer). Bars are 200 \mu m (A) and 50 \mu m (insets of A, B, C).

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