Protein kinase C regulates AMPA receptor auxiliary protein Shisa9/CKAMP44 through interactions with neuronal scaffold PICK1

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Synaptic α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors are essential mediators of neurotransmission in the central nervous system. Shisa9/cysteine-knot AMPAR modulating protein 44 (CKAMP44) is a transmembrane protein recently found to be present in AMPA receptor-associated protein complexes. Here, we show that the cytosolic tail of Shisa9/CKAMP44 interacts with multiple scaffold proteins that are important for regulating synaptic plasticity in central neurons. We focussed on the interaction with the scaffold protein PICK1, which facilitates the formation of a tripartite complex with the protein kinase C (PKC) and thereby regulates phosphorylation of Shisa9/CKAMP44 C-terminal residues. This work has implications for our understanding of how PICK1 modulates AMPAR-mediated transmission and plasticity and also highlights a novel function of PKC.

The ionotropic glutamate receptors are of particular importance for fast synaptic transmission in the central nervous system. Among these ligand-gated receptors, the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors are especially important for regulating changes in synaptic strength; elucidating the mechanisms by which these receptors are regulated is therefore critical for our molecular understanding of information storage in the brain. Recent studies indicate that they are found in complex with additional transmembrane proteins that modulate receptor function, including the Shisa/cysteine-knot AMPAR modulating protein (CKAMP) family of proteins, among others.

Shisa/CKAMP proteins were originally described for their role in head formation [1] and were named Shisa after a form of sculpture with a large head (similar to the Egyptian Sphinx) that is found in southern Japan. The first study on Shisa family transcript expression in the mouse brain focussed on the transcript for Shisa2; since then, high-throughput in situ analyses have confirmed that several of the Shisa transcripts, including Shisa5, 6, 7 and 9, exhibit high expression in the brain, and each one has restricted expression in a subset of CNS neurons (see Allen Mouse Brain Atlas: http://mouse.brain-map.org) [2], suggesting that these proteins may be involved in specific neuronal functions. Indeed, more recent studies indicated that Shisa9 is present in AMPA receptor complexes and that its presence there regulates synaptic transmission by modulating AMPA receptor channel properties [3]. In addition, it has now been shown that not only Shisa9 but also Shisa6, 7 and 8 are

Abbreviations
AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; CaMKII, Ca2+/calmodulin-dependent protein kinase II; CKAMP44, cysteine-knot AMPAR modulating protein 44; MAG11, membrane-associated guanylate kinase inverted 1; MAGUK, membrane-associated guanylate kinase; nPIST, neuronal protein interacting specifically with Tc10; PDZ, PSD-95 Dlg1 and ZO-1; PICK1, protein interacting with C kinase 1; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; PSD-95, postsynaptic density protein 95; SAP102, synapse-associated protein 102; TARP, transmembrane AMPAR regulatory protein; Y2H, yeast two-hybrid.
capable of influencing receptor function [4,5]. In the light of these new studies, this subgroup of Shisa proteins is now also referred to as the CKAMP family, based on their common ability to modulate AMPAR synaptic transmission. Another property that these CKAMP proteins share is the presence of a conserved C-terminal sequence typical for proteins that have been shown to bind a postsynaptic density protein 95 (PSD-95) Dlg1 and ZO-1 (PDZ) domain, the 80–90 AA structurally conserved motif originally identified for its presence in PSD-95, Dlg1 and ZO-1 proteins.

Numerous cytosolic proteins at synapses harbour PDZ domains and are capable of interacting directly with the cytoplasmic tails of transmembrane receptors, and it has been shown that such PDZ–ligand interactions are important for the trafficking of AMPA receptors to and from the cell surface [6]. Notably, it has become clear in recent years that direct interactions between PDZ domain proteins and AMPA receptor subunits themselves are not exclusively responsible for this regulation of AMPAR surface expression. Cumulative data from several groups suggest that transmembrane AMPAR-interacting proteins are essential for this function (for review, see [7]). As several of these proteins also influence the gating properties of isolated AMPA receptors, they are often referred to collectively as AMPA receptor auxiliary proteins (for recent reviews, see [7,8]). Among these transmembrane AMPAR auxiliary proteins, the transmembrane AMPAR regulatory protein (TARP) family has been studied in most depth: it is well established that TARPs are involved in the modulation of AMPAR desensitisation and also play a critical role in AMPAR trafficking to the surface. Importantly, the latter function of TARPs clearly relies on the C-terminal cytosolic region that binds to PDZ domains of specific synaptic scaffold molecules [9–11], illustrating that the cytosolic C-terminal tail of AMPAR auxiliary proteins can play a decisive role in AMPA receptor function via this mechanism.

It has further been established that phosphorylation of the C-terminal regions of ionotropic glutamate receptor subunits themselves, and also the TARPs family of auxiliary proteins, can dramatically influence receptor function, presumably by influencing interactions between AMPAR complexes and cytosolic proteins or specific cellular components. It has been shown, for example, that serine residues within the C-terminal cytosolic region of AMPAR subunits GluA1 and GluA2 are phosphorylated [by, e.g., protein kinase A (PKA), PKC and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII)] and that the phosphorylation status influences channel conductance and opening probability (for recent review, see [12]). Also relevant is the fact that modulating the phosphorylation status of the TARPs C terminus can dramatically influence its localisation and function [13–15].

In this study, we focus on Shisa9/cysteine-knot AMPAR modulating protein 44 (CKAMP44), a small protein with only one transmembrane domain (TM). It was initially proposed that its effects on AMPAR-mediated transmission are mediated by a set of six N-terminal extracellular cysteines, as this region resembles that of the snail conotoxin (Cys-ikot-ikot) that disrupts AMPAR desensitisation [3]. It has since been shown that the C-terminal intracellular domain is also functionally important. Like the TARPs, Shisa9/CKAMP44 harbours a long C-terminal cytosolic tail that is presumably a target for both interactions with cytosolic scaffolds and regulation by associated kinases. Given that Shisa9/CKAMP44 binds to AMPARs and modulates specific functions at the membrane, we considered it highly relevant to explore how this protein is regulated by other protein–protein interactions. In this study, we have screened for and identified cytosolic interaction partners of Shisa9/CKAMP44, including the synaptic scaffold molecule interacting with C kinase 1 (PICK1). More importantly, we demonstrate that this interaction facilitates regulation of Shisa9/CKAMP44 by PKC, which highlights a novel function of PKC in neurons that will significantly influence future investigations into PICK1 and PKC-mediated effects on AMPAR composition and function.

**Results**

**Shisa9/CKAMP44 interacts with several synapse-associated PDZ-containing scaffold proteins**

In order to determine putative cytosolic binding partners of Shisa9/CKAMP44, we used this region of the protein as bait in a yeast two-hybrid (Y2H) assay and identified several putative interaction partners (summarised in Fig. 1A). Within this list were several scaffold proteins harbouring PDZ domains [e.g. PICK1, DLG1/SAP97, DLG2/PSD-93, DLG3/synapse-associated protein 102 (SAP102), DLG4/PSD-95, MPP5, membrane-associated guanylate kinase inverted 1 (MAGI1), MAGI3, LNX1, LNX2 and HTRA1], suggesting that the C-terminal region of Shisa9/CKAMP44 is indeed a PDZ ligand, as predicted by sequence analysis [3] and likewise observed in more recent work [5,16,17]. We validated an interaction of the synapse-associated PDZ domain proteins PICK1, MAGI1, PSD-95 and SAP102 with the full-length Shisa9/
CKAMP44 by coimmunoprecipitation (Fig. 1B–E), and confirmed that Shisa9/CKAMP44 localises to synaptic sites in primary neurons (Fig. 1F–H). Further experiments indicated that the interaction with membrane-associated guanylate kinase (MAGUK) scaffolds relies exclusively on the C-terminal PDZ-binding motif of Shisa9/CKAMP44 and the two most N-terminal MAGUK PDZ domains (see Fig. S1), highlighting a clear binding specificity for PDZ domains 1 and 2.

The Shisa9/CKAMP44 interaction with PICK1 facilitates Shisa9/CKAMP44 phosphorylation by PKC

In the following experiments, we focussed on the interaction of Shisa9/CKAMP44 with the protein PICK1, which has been shown previously to bind directly with AMPA receptor subunit C-terminal tails in a ligand–PDZ domain interaction [18,19]. Unexpectedly, we observed that the Shisa9/CKAMP44-PICK1 interaction is not exclusively dependent on the PDZ-binding motif in Shisa9/CKAMP44, which differs from the interaction with PSD-95 and SAP102 (Fig. S1) as demonstrated in coimmunoprecipitation experiments (Fig. 2B). To further narrow down the interaction region, we deleted a more proximal region of the cytosolic tail and observed an even further reduced interaction of PICK1 with Shisa9/CKAMP44 (Fig. 2B; for schematic diagram of deletion constructs used, see Fig. 2A). This proximal region of the cytoplasmic tail of Shisa9/CKAMP44 contains an amino acid (AA) stretch that is highly conserved between species and...
also across Shisa/CKAMP family proteins [4] and has been shown to bind GluA1 in co-immunoprecipitation (IP) experiments [17]. In this context, it is interesting that this region also has an influence on PICK1-Shisa9/CKAMP44 binding.

These data led us to hypothesise that the binding between PICK1 and Shisa9/CKAMP44 is an important, high-affinity interaction that could be subject to temporal or signal-dependent regulation. PICK1 was named for its role as an interaction partner for the protein kinase PKC [20,21]. We therefore asked whether Shisa9/CKAMP44 might be a target of PKC-mediated phosphorylation. Using the Phos-tag system, we demonstrated that wild-type Shisa9/CKAMP44 is indeed modified following expression in heterologous cells and that the observed mobility shift was inhibited following phosphatase treatment (Fig. 2C), confirming that we indeed observed a phosphorylation-dependent shift. In addition, phosphorylation could be modulated using phorbol-12-myristate-13-acetate (PMA), which
activates PKC signal cascades. Moreover, while PMA treatment amplified the relative phospho-Shisa9/CKAMP44 signals, PKC inhibitors, including GF109203X, reduced total phosphorylation of Shisa9/CKAMP44 (Fig. 2C,D) in this system, supporting the idea that Shisa9/CKAMP44 can be regulated by PKC. Importantly, we also observe these PKC-dependent changes in the phosphorylation of Shisa9/CKAMP44 in a neuronal environment (see Fig. 3), and we demonstrated that Shisa9/CKAMP44 is indeed a direct PKC target using purified PKCα with the GST-tagged Shisa9/CKAMP44 cytosolic tail as a substrate in an in vitro kinase assay (Fig. 2E).

**Shisa9/CKAMP44 forms a complex with PICK1 and PKC that is regulated by activation of PKC signalling cascades in neurons**

We next confirmed that Shisa9/CKAMP44, PICK1 and PKC indeed form a tripartite complex in coimmunoprecipitation experiments (Fig. S2). Interestingly, activated PKC binds more effectively than inactive PKC to the PICK1-Shisa9/CKAMP44 complex (Fig. 3A; co-IP with or without PMA stimulation), which is in line with the dramatic phosphorylation of Shisa9/CKAMP44 observed following PMA stimulation in the presence of both PICK1 and wild-type PKCα (Fig. 3B; lane 3 of Phos-tag panel). A similar phospho-dependent mobility shift of Shisa9/CKAMP44 in response to PKC activation was observed in primary hippocampal neurons (Fig. 3C; compare lanes 3 and 4 of the Phos-tag panel), confirming the importance of endogenous PKC in the regulation of this AMPAR auxiliary protein at synapses. In line with these data, we observe a strong increase in colocalisation of neuronal PICK1 with synaptic Shisa9/CKAMP44 following activation of endogenous PKC with PMA (see representative images in Fig. 3D). Digital analysis of overlapping signals (synaptic FLAG-CKAMP44-FL with endogenous PICK1 puncta) indicated approximately a 1.4-fold increase in colocalisation in response to treatment with PMA (1.388 ± 0.086; see data points and quantification of observations in the Fig. 3D bar diagram).

These data support a model proposed earlier [19], in which activation of PKC stabilises the interaction between PKCα and PICK1. We further demonstrate here that PKC activation promotes localisation of PICK1 towards Shisa9/CKAMP44 at synaptic sites, which enables an inducible and dynamic PKC-mediated regulation of the Shisa9/CKAMP44 C terminus. These novel ideas are summarised in Fig. 4.

**Discussion**

In this study, we have explored the molecular interactions mediated by the cytosolic C-terminal region of the CNS-expressed Shisa9/CKAMP44 protein. We have identified several Shisa9/CKAMP44 binding proteins, including several scaffold molecules that play an established role in the regulation of cell–cell communication, for example PICK1 and the MAGUK family members PSD-95, SAP102 and MAGI1. In addition to the synaptic MAGUK proteins (PDZ-dependent interaction), the scaffold molecule PICK1 exhibited strong binding to the cytosolic region of the AMPAR auxiliary protein Shisa9/CKAMP44.

Protein interacting with C kinase 1 and protein kinase C (PKC) – as their names indicate – have an established biochemical connection, and several studies have confirmed that PICK1 cooperates with PKC to execute various regulatory functions in neurons [22–25]. Moreover, a role for both PICK1 and PKC in the regulation of AMPAR-mediated plasticity has been explored [19,26–28]. In the context of these studies, our data highlighting a strong interaction between PICK1 and the AMPAR receptor auxiliary protein Shisa9/CKAMP44 and the subsequent discovery that this protein is a novel PKC target, are of interest. Our observation that the overlapping subcellular localisation of PICK1 and Shisa9/CKAMP44 in neurons is dynamically regulated by activation of PKC signalling cascades supports the idea that the interaction between these two proteins may be a target of finely tuned temporal or activity-dependent regulation by intracellular signals. Further supporting this idea, we found that the formation of a Shisa9/CKAMP44-PICK1-PKC complex in response to PKC activation leads to changes in the phosphorylation status of Shisa9/CKAMP44 in primary neurons.

To date, there have been relatively few investigations into the function of Shisa9/CKAMP44, but it has been demonstrated that the N-terminal extracellular domain of Shisa9/CKAMP44 is important for regulating AMPAR desensitisation kinetics [3,17]. In the context of our data, investigations into Shisa9/CKAMP44-mediated modulation of AMPAR trafficking to the surface, which have so far yielded diverse results, are of particular interest. In the hippocampal CA1 region, Shisa9/CKAMP44-deficient neurons did not exhibit a reduction in synaptic AMPARs [3]; however, in dentate gyrus granule cells, where endogenous Shisa9/CKAMP44 is expressed at higher levels, studies on KO animal tissue suggest that the trafficking of AMPARs to the synaptic membrane involves both TARP and CKAMP auxiliary proteins [17]. It has
Fig. 3. Shisa9/CKAMP44-PICK1 complex formation and regulation by active PKC in neurons. (A) PKC activation status influences CKAMP44/PICK1/PKCα complex formation: communoprecipitation experiments following expression of Shisa9/CKAMP44 (FLAG-CKAMP44-FL), PICK1 (MYC-PICK1) and PKCα (EGFP-PKCα-WT) in COS-7 cells indicate that the three proteins form a complex. After pull-down FLAG-CKAMP44-FL, coprecipitated proteins are detected by WB, as indicated. mIgG serve as a negative pull-down control. Comparison (with or without PMA activation, 1 μM, 30 min) indicates that EGFP-PKCα association with FLAG-CKAMP44-FL/MYC-PICK1 protein complex is modulated by PKC activation status. (B) Phosphorylation of overexpressed Shisa9/CKAMP44 (FLAG-CKAMP44-FL) in COS-7 cells in the presence of either wild-type or kinase-dead PKCα (EGFP-PKCα-WT and EGFP-PKCα-DN; compare lanes 3 and 4) with or without PICK1 (MYC-PICK1; compare lanes 3 and 7) is shown (top panel; Phos-tag: phosphorylated proteins observed by mobility shift). PKC was activated by PMA (1 μM, 30 min); controls for relevant protein levels (actin: loading control) are shown below (Laemmli SDS/PAGE and WB). Antibodies used are indicated on the right. (C) In primary hippocampal neurons, activation of PKC by PMA (0.2 μM, 15 min) likewise affects Shisa9/CKAMP44 phosphorylation status (compare lanes 3 and 4 in the Phos-tag blot, left panel). Shisa9/CKAMP44 proteins were observed by WB following viral transduction of FLAG-CKAMP44-FL, with or without activation by PMA. Neurons were harvested at DIV22; on the right, control levels of related proteins are observed via SDS/PAGE. (D) Overlapping localisation of Shisa9/CKAMP44 and PICK1 in primary hippocampal neurons after activation of PKC: neurons were infected (DIV 10–15) with virus expressing Shisa9/CKAMP44 (FLAG-CKAMP44-FL) and fixed at DIV 20–24 without a treatment (upper panel) or after activation of PKC by PMA (0.2 μM, 15 min; lower panel). Immunofluorescence with a FLAG antibody (cyan), an antibody to endogenous PICK1 (magenta) and an antibody to endogenous MAP2 (blue) is shown. Shisa9/CKAMP44–PICK1 overlapping puncta are indicated. Merged images are shown on the right. Scale bar 5 μm. The colocalisation of FLAG-CKAMP44-FL and endogenous PICK1 in rat hippocampal neurons was quantified with the Puncta Analyzer (IMAGEJ plugin) in dendritic segments of FLAG-CKAMP44-FL-infected neurons (untreated: n = 49 neurons from N = 4 cultures; PMA treated: n = 48 neurons from N = 4 cultures) and normalised to the mean of untreated condition. The colocalisation increased significantly after PMA treatment (0.2 μM PMA for 15 min) by the factor of 1.388 ± 0.086 (** P = 0.0008, two-tailed Mann–Whitney *U*-test, *U* = 716.5) compared to untreated neurons (1.0 ± 0.074). Data are represented as mean ± SEM.
PKC phosphorylates CKAMP44 via PICK1

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Fig. 4. A model illustrating the Shisa9/CKAMP44–PICK1-PKCα protein complex. PICK1 can dimerise and is likely able to bind Shisa9/CKAMP44 and PKCα at the same time, thereby enabling PKCα-mediated phosphorylation of Shisa9/CKAMP44 upon kinase activation.

been shown clearly that this function of TARP family auxiliary proteins relies on the cytosolic tail [9–11]; it is therefore plausible that CKAMP family members likewise participate in this process via intracellular molecular mechanisms.

Our observations that the Shisa9/CKAMP44 intracellular domain is a target of phospho-regulation and interacts differentially with certain scaffold proteins suggest that there may be parallels between how TARPs and CKAMP family proteins influence receptor trafficking. It has been shown that phosphorylation-dependent interactions of TARPs can regulate synaptic content of both TARPs and AMPAR subunits: the phosphorylation status of the TARPγ2 C terminus influences its association with the membrane lipid bilayer [15] and also affects its ability to interact with PSD-95 [13,14]; these changes have implications for AMPAR-mediated synaptic transmission. It has also been shown that — in addition to the most C-terminal region responsible for interacting with synaptic MAGUKs — other cytosolic regions of TARPs can influence receptor trafficking [29]. In addition, the interaction between TARPγ2 and the intracellular scaffold neuronal protein interacting specifically with Tc10 (nPIST; also known as GOPC/CAL), a Golgi-enriched protein [30,31], can modulate the synaptic targeting of AMPAR complexes [32]. In this case, it is not the C-terminal PDZ-binding region of TARPγ2 that is responsible for the function but a more proximal cytosolic region close to the TM. These authors propose a mechanism that involves — early on in the secretory process — a PDZ-independent interaction between TARPγ2 and nPIST, which is followed by a PDZ domain-mediated interaction with PSD-95 that ensures synaptic localisation. Both of these interactions are important for the function of TARPs as mediators of AMPAR trafficking, and it is plausible that a similar model applies to Shisa9/CKAMP44, which is able to bind PICK1 and also PSD-95-family synaptic MAGUKs.

Although Shisa9/CKAMP44 clearly binds to PICK1 via its PDZ-binding motif, we demonstrate that at least one additional region, namely the N-terminal region of its intracellular domain, which is also important for its effects on AMPAR modulation [17], is also involved in binding to this scaffold protein. This leads to the possibility that PICK1 and GluA1 might compete for this binding region of Shisa9/CKAMP44. An additional possibility is that these interactions are temporally and/or spatially separated; for example, a brief interaction between Shisa9/CKAMP44 and PICK1 during early trafficking could be followed by PSD-95-guided synaptic localisation and subsequent functional interaction with AMPAR subunits.

Importantly, our observation that there are dramatic effects on the phospho-regulation of Shisa9/CKAMP44 specifically when it is in a complex with PICK1 and PKC suggests that formation of this tripartite complex is important. The fact that the complex can be dynamically regulated suggests that it may have a transitory role, perhaps comparable to that proposed for the TARPγ2/nPIST interaction, which is relevant at specific time points in the secretion and trafficking of receptor complexes en route to the membrane. Indeed, numerous investigations illustrate a role for PICK1 in both basal (e.g. see [27,33]) and activity-dependent receptor trafficking (e.g. see [34–37]; for review, see [38]). It is possible that the Shisa9/CKAMP44-PICK1 interaction and subsequent PKC-mediated Shisa9/CKAMP44 phosphorylation that we observe might influence early phases of AMPAR trafficking in a subset of excitatory neurons. Interestingly, a role for PICK1 in the export of AMPA receptors from the endoplasmic reticulum (ER) has recently been reported [39], and PKC-mediated phosphorylation of other ionotropic glutamate receptor subunits participates in the regulation of their export from the ER [40–42]. In addition, it has been shown that the interaction between PICK1 and active PKCα is transient, and primarily important for initial targeting of PICK1 to the membrane [43], where the transition of assembled AMPAR complexes to other membrane-associated PDZ domain scaffolds could take place. Moreover, there is evidence that trans-Golgi network-derived organelles in the dendrites of hippocampal
neurons undergo regulated exocytosis in response to calcium signals [44], further supporting a role for the calcium sensing PICK1/PKC complex in this process.

In summary, this study highlights a novel molecular mechanism that may contribute to PKC association with AMPARs: via interaction with the PICK1-Shisa9/CKAMP44 complex. It thereby lays the groundwork for further investigations into the functional modulation of Shisa9/CKAMP44 and associated AMPAR protein complexes via PKC signalling cascades that involve PICK1.

Materials and methods

Y2H screen

The Y2H screen (Hybrigenics ULTImate Y2H) was performed by HYBRIGENICS (Paris, France), using the cytoplasmic tail of mouse Shisa9/CKAMP44 (171-424 AA), cloned into pB27 (N-LexA-bait-C, Cter-free) with an adult mouse brain cDNA library.

Constructs

The CKAMP44 constructs used in this study are based on mouse SHISA9/CKAMP44 (Uniprot Q9CZN4; 424AA). All constructs were generated using standard cloning techniques. For full-length FLAG- or MYC-tagged CKAMP44 DNA constructs in the pCMV vector, we cloned the coding sequence for the FLAG tag (AAs DYKDDDDK) or the MYC tag (AAs EQKLISEEDL) behind the coding sequence for the first N-terminal 29 AAs of CKAMP44 (including the signal peptide). The constructs containing the cytoplasmic tail of CKAMP44 express the following AA sequences as either EGFP or GST fusion proteins: pEGFP-C1-CKAMP44 (171-424AA), pEGFP-C1-CKAMP44-ΔC (171-415AA), pEGFP-C1-CKAMP44-ΔNΔC (199-415AA), pGEX-6P1-CKAMP44 (186-424AA), pGEX-6P1-CKAMP44-ΔC (186-415AA).

The constructs pCMV2A-FLAG-PSD-95 and pCMV2A-FLAG-SAP102 were described previously [45], and the constructs pCMV2A-FLAG-PSD-95-PDZ3-SH3-GK and pCMV2A-FLAG-PSD-95-PDZ123mut have also been described [46]; the constructs pCMV2A-FLAG-PSD-95-PDZ12mut and pCMV2A-FLAG-PSD-95-PDZ3mut were cloned in similar fashion using standard recombinant DNA technology. We cloned pCMV3A-MYC-PICK1 (Uniprot Q9NRD5, human, 415 AA) from human cDNA. A construct expressing FLAG-MAGI1 (mouse, 1432 pcDNA3 flag MAGI1c) was obtained from Addgene (Cambridge, MA, USA; clone #10714). For the pEGFP-C1-PKCα-WT construct in our study, we used pHACE-PKCalphaWT (Addgene clone #21232, human, Uniprot P17252, 672 AA) as a cloning template, and for the kinase-deficient PKCα mutant DN (K368R), we used pHACE-PKCalphaDN (Addgene clone #21235). For generation of virus particles, FLAG-CKAMP44 was cloned into a lentiviral shuttle vector under the control of a human synapsin-1 promoter.

Cells/treatment/transfection

COS-7 cells (purchased from ATCC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Lonza, Verviers, Belgium) supplemented with 10% FBS (Sigma-Aldrich, Taufkirchen, Germany), 2 mM l-glutamine and penicillin/streptomycin at 37 °C with 5% CO2. Transient transfections were performed using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s recommendations. COS-7 cells were treated with 1 μM PMA for 30 min. For the inhibitor experiment (Fig. 2D), cells were treated for 120 min with 3.5 μM or 7 μM PKC inhibitor GF109203X followed by 60-min treatment with 0.2 μM PMA. Primary neuronal cultures were generated essentially as described previously [45], with minor modifications: for primary rat hippocampal neuronal cultures, embryonic E18 Wistar Unilever rats were used. Following decapitation, hippocampi from 5 to 10 pups were isolated and collected in ice-cold DMEM (Lonza). Tissue was partially digested in trypsin/EDTA (Lonza) at 37 °C for 5 min. After stopping the reaction with 10% FBS (Biochrom, Berlin, Germany) in DMEM and subsequent washing in DMEM to remove traces of trypsin, the hippocampal tissue was suspended in neuron culture medium (Neurobasal A supplemented with B27 and 0.5 mM glutamine) and dissociated mechanically; neurons were then plated onto glass coverslips coated with a mixture of poly-d-lysine (Sigma) and laminin (Sigma) at a density of approximately 10^5 cells per cm². Cell debris was removed after healthy neurons adhered to the cover slips, and neurons were maintained for 2–4 weeks with minimal perturbation at 37 °C with 5% CO2 in neuron culture medium. Lentiviral particles were prepared by the NeuroCure Virus Core Facility essentially as described by Lois et al. [47]. Primary neurons were transduced at DIV10–15 and analysed after fixation at DIV20–24. PMA treatment of neurons was with 0.2 μM PMA for 15 min. All animals used in this study were treated according to German regulations on the use of animals for research purposes and reported under the permit T0280/10.

Coimmunoprecipitation

Shisa9/CKAMP44 proteins were overexpressed in COS-7 cells together with their candidate interaction partners. Twenty-four hours post-transfection, cells were harvested in IP buffer [50 mM Tris pH 7.5, 100 mM NaCl, 0.1 – 1% Triton-X and Mini Complete Inhibitors (Roche, Basel, Switzerland)] and lysed with 5–10 strokes using a 30-G needle. Lysates were cleared by two centrifugations for 10 min at...
20 000 g. For IP, lysates were incubated for 3 h at 4 °C with 2 μg of the respective antibody and transfected proteins were pulled down with protein-G agarose (Roche) for 1 h. Beads were washed three times for 5 min at 4 °C. Samples were analysed by SDS/PAGE and western blot (WB).

**GST pull-down assays and GST protein purification**

GST-tagged proteins were expressed and purified according to the manufacturer’s recommendations (GE Healthcare, Freiburg, Germany). Briefly, BL21 *Escherichia coli* transformed with the regarding GST protein constructs were cultured O/N. Expression of GST proteins was induced with 0.1 mM IPTG, and cells were lysed in TBS containing 1% Triton-X. After lysate clearing at 20 000 g, GST-tagged proteins were purified using glutathione agarose (Thermo Fisher Scientific). For GST pull-down assays, the GST-tagged proteins (bound to the glutathione agarose beads) were incubated with lysate from adult mouse brains or from transfected COS-7 cells. For brain lysate pull-down experiments, adult C57Bl6 mice were sacrificed by cervical dislocation; whole brains were removed and placed directly in ice-cold TN buffer [50 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton-X. After lysate clearing at 20 000 g, GST-tagged proteins were washed three times and centrifugation (20 000 g). After 3 h of incubation at 4 °C, the glutathione agarose beads were washed three times and the bound proteins were analysed by SDS/PAGE and WB. For the *in vitro* kinase assay, GST fusion proteins were purified using glutathione agarose and eluted with 50 mM glutathione in 200 mM Tris (pH 8). They were desalted using a Zeba Spin Desalting Column (40 K MWCO; Thermo Scientific).

**In vitro PKCα kinase assay**

Purified and desalted GST-CKAMP44 protein was used for an *in vitro* PKCα kinase assay (SignalChem, Richmond, Canada; #P61–18G) according to the manufacturer’s instructions. Samples were analysed with Phos-tag SDS/PAGE followed by WB.

**Phos-tag SDS/PAGE**

*Shisa9/CKAMP44 phosphorylation was assessed using the Phos-tag system (Wako, Neuss, Germany), which facilitates phosphorylation-dependent retarded protein migration through a polyacrylamide gel, and subsequent WB. Phos-tag gel electrophoresis (6–8% polyacrylamide supplemented with 50 μM Phos-tag) was established using an optimised protocol with Zn²⁺ in a bis/tris-buffered neutral pH gel system either as described previously [48] or according to the manufacturer’s recommendations. Phos-tag gels were generally blotted using a wet transfer blotting set-up. As a control for phosphorylated proteins, the proteins were dephosphorylated using a thermosensitive alkaline phosphatase (FastAP; Thermo Fisher Scientific). The transfected cells were lysed in 1× FastAP buffer (Thermo Fisher Scientific) containing 1% Triton-X and Mini Complete protease inhibitors without EDTA (Roche). The lysates were cleared by centrifugation and proteins were subsequently dephosphorylated for 2 h at 37 °C using FastAP.**

**Immunofluorescence/colocalisation analysis**

After 2–4 weeks in culture, differentiated neurons were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 min and immunofluorescence was performed as described previously [49] according to standard IF protocols. Briefly, cells were permeabilised in 0.2% Triton-X in PBS for 5 min, washed in PBS and blocked with 4% bovine serum albumin (BSA) in PBS for 1 h at room temperature. They were then incubated overnight with the primary antibodies in the same solution at 4 °C, washed with PBS and subsequently incubated with secondary antibodies in blocking solution. After final washes in PBS, coverslips were mounted with Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Images were acquired with a Leica (Wetzlar, Germany) laser-scanning confocal microscope (Leica TCS-SP5 II) using a 63× objective. Dendritic segments of rat hippocampal neurons were analysed for colocalisation of FLAG-CKAMP44 (FL, cyan, Alexa488) with endogenous PICK1 (magenta, Alexa568). Regions of interest (ROIs) of merged images were selected blind to the conditions of the experiments and analysed with the IMAGEJ (NIH, Bethesda, MD, USA) plugin Puncta Analyzer as described previously [50]. Number of cells (*n*) and number of cultures (*N*) are stated in the figure legend. FLAG-CKAMP44-FL puncta (cyan) were analysed for colocalised PICK1 (magenta). Data are normalised to the mean of the untreated condition and presented as mean ± SEM (standard error of the mean). Statistics were calculated using GRAPHPAD Prism 7 (GraphPad Software, La Jolla, CA, USA) (two-tailed Mann–Whitney *U*-test for unpaired data).

**Antibodies**

Antibodies used in this study include GFP (mouse, Roche 11814460010; goat, Abcam, Cambridge, UK, AB6673, WB 1 : 5000), FLAG (mouse, rabbit, Sigma F1804 and F7425, respectively, WB 1 : 5000), FLAG-HRP (Sigma A8592, WB 1 : 5000), MYC (mouse, Clontech, Mountain View,
Canada, 631206; rabbit, Cell Signaling, Danvers, MA, USA, 2272S, WB 1 : 5000), MYC-HRP (Abcam AB6298, WB 1 : 5000), GST (goat, GE Healthcare, 27457701V, WB 1 : 5000), CKAMP44 (rabbit, custom made by David’s Biotechnology, Regensburg, Germany, WB 1 : 1000), actin (rabbit, Sigma A2066, WB 1 : 3000), GAPDH (rabbit, Abcam AB9485, WB 1 : 2000), GluA1 (rabbit, Millipore, Billerica, MA, USA, AB1504, WB 1 : 2000), MAP2 (rabbit, Millipore AB5622; guinea pig, Synaptic Systems, 188004), PICK1 (rabbit, Abcam AB3420, WB 1 : 2000), PKCζ (mouse, Millipore 05–154, WB 1 : 2000), PSD-95 (mouse, UC Davis/NIH NeuroMab Facility, Davis, CA, USA, 75–028, WB 1 : 2000), SAP102 (mouse, UC Davis/NIH NeuroMab Facility 75–058, WB 1 : 2000) and vGlut1 (mouse, UC Davis/NIH NeuroMab Facility 75–066). For communoprecipitation experiments, 2 μg of the respective antibodies was used. Unspecific mouse IgGs (mIgG), as required (SantaCruz, Biotechnology, Dallas, TX, USA, SC-2025, 2 μg), were used for negative controls in communoprecipitation studies. All primary and secondary antibodies were diluted in 5% milk/PBST for WB or in 4% BSA/PBS for immunofluorescence experiments (1 : 1000). For immunofluorescence experiments, we used anti-guinea-pig-Alexa405 (Abcam, ab175678), anti-mouse-Alexa488 and anti-rabbit-Alexa568 or anti-mouse-Alexa568 and anti-rabbit-Alexa488 (Thermo Fisher Scientific A-21200 and A-11036 or A-11031 and A-21441, respectively).

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Author contributions

SAK and SAS designed the study. SAK, NR and HZ performed experiments and collected the data. SAK, NR and SAS analysed data. SAK and SAS wrote the manuscript. All authors reviewed the manuscript.

References

1 Filipe M, Goncalves L, Bento M, Silva AC and Belo JA (2006) Comparative expression of mouse and chicken Shisa homologues during early development. Dev Dyn 235, 2567–2573.
2 Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bansinger A, Bernard A, Boe AF, Boguski MS, Brockway KS, Byrnes EJ et al. (2007) Genome-wide atlas of gene expression in the adult mouse brain. Nature 445, 168–176.
3 von Engelhardt J, Mack V, Sprengel R, Ravenstok N, Li KW, Stern-Bach Y, Smit AB, Seeburg PH and Monyer H (2010) CKAMP44: a brain-specific protein attenuating short-term synaptic plasticity in the dentate gyrus. Science 327, 1518–1522.
4 Farrow P, Khodosevich K, Sapir Y, Schulmann A, Aslam M, Stern-Bach Y, Monyer H and von Engelhardt J (2015) Auxiliary subunits of the CKAMP family differentially modulate AMPA receptor properties. Elife 4, e09693.
5 Klaassen RV, Stroeder J, Courten F, Hafner AS, Petersen JD, Renancio C, Schnitzl JJ, Normand E, Lodder JC, Rotaru DC et al. (2016) Shisa6 traps AMPA receptors at postsynaptic sites and prevents their desensitization during synaptic activity. Nat Commun 7, 10682.
6 Anggono V and Huganir RL (2012) Regulation of AMPA receptor trafficking and synaptic plasticity. Curr Opin Neurobiol 22, 461–469.
7 Sumioka A (2013) Auxiliary subunits provide new insights into regulation of AMPA receptor trafficking. J Biochem 153, 331–337.
8 Haering SC, Tapken D, Pahl S and Hollmann M (2014) Auxiliary subunits: shepherding AMPA receptors to the plasma membrane. Membranes (Basel) 4, 469–490.
9 Bats C, Groc L and Choquet D (2007) The interaction between Stargazin and PSD-95 regulates AMPA receptor surface trafficking. Neuron 53, 719–734.
10 Chen L, Chetkovich DM, Petralia RS, Sweeney NT, Kawasaki Y, Wenthold RJ, Breit DS and Nicoll RA (2000) Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. Nature 408, 936–943.
11 Schnell E, Sizemore M, Karimzadegan S, Chen L, Breit DS and Nicoll RA (2002) Direct interactions between PSD-95 and stargazin control synaptic AMPA receptor number. Proc Natl Acad Sci USA 99, 13902–13907.
12 Lussier MP, Sanz-Clemente A and Roche KW (2015) Dynamic regulation of N-Methyl-d-aspartate (NMDA) and alpha-Amino-3-hydroxy-5-methyl-4-isoxazolpropionic Acid (AMPA) receptors by posttranslational modifications. J Biol Chem 290, 28596–28603.
13 Chetkovich DM, Chen L, Stocker TJ, Nicoll RA and Breit DS (2002) Phosphorylation of the postsynaptic density-95 (PSD-95)/discs large/zona occludens-1 binding site of stargazin regulates binding to PSD-95.
and synaptic targeting of AMPA receptors. J Neurosci 22, 5791–5796.

14 Stein EL and Chetkovich DM (2010) Regulation of stargazin synaptic trafficking by C-terminal PDZ ligand phosphorylation in bidirectional synaptic plasticity. J Neurochem 113, 42–53.

15 Sumioka A, Yan D and Tomita S (2010) TARP phosphorylation regulates synaptic AMPA receptors through lipid bilayers. Neuron 66, 755–767.

16 Karataeva AR, Klassen RV, Stroder J, Ruiperez-Alonso M, Hjorth JJ, van Nierop P, Spijker S, Mansvelder HD and Smit AB (2014) C-terminal interactors of the AMPA receptor auxiliary subunit Shisa9. PLoS ONE 9, e87360.

17 Khodosevich K, Jacobi E, Farrow P, Schulmann A, Rusu A, Zhang L, Sprengel R, Monyer H and von Engelhardt J (2014) Coexpressed auxiliary subunits exhibit distinct modulatory profiles on AMPA receptor function. Neuron 83, 601–615.

18 Xia J, Zhang X, Staudinger J and Huganir RL (1999) Clustering of AMPA receptors by the synaptic PDZ domain-containing protein PICK1. Neuron 22, 179–187.

19 Perez JL, Khatri L, Chang C, Srivastava S, Osten P and Ziff EB (2014) PICK1 targets activated protein kinase C-alpha to AMPA receptor clusters in spines of hippocampal neurons and reduces surface levels of the AMPA-type glutamate receptor subunit. J Neurosci 21, 5417–5428.

20 Staudinger J, Zhou J, Burgess R, Elledge SJ and Olson EN (1995) PICK1: a perinuclear binding protein and substrate for protein kinase C isolated by the yeast two-hybrid system. J Cell Biol 128, 263–271.

21 Staudinger J, Lu J and Olson EN (1997) Specific interaction of the PDZ domain protein PICK1 with the COOH terminus of protein kinase C-alpha. J Biol Chem 272, 32019–32024.

22 Deval E, Salinas M, Baron A, Linguedola E and Lazdunski M (2004) ASIC2b-dependent regulation of ASIC3, an essential acid-sensing ion channel subunit in sensory neurons via the partner protein PICK1. J Biol Chem 279, 19531–19539.

23 Hu ZL, Huang C, Fu H, Jin Y, Wu WN, Xiong QJ, Xie N, Long LH, Chen JG and Wang F (2010) Disruption of PICK1 attenuates the function of ASICs and PKC regulation of ASICs. Am J Physiol Cell Physiol 299, C1355–C1362.

24 Wang Z, Wang YN, Sun CL, Yang D, Su LD, Xie YJ, Zhou L, Wang Y and Shen Y (2013) C-terminal domain of ICA69 interacts with PICK1 and acts on trafficking of PICK1-PKCalpha complex and cerebellar plasticity. PLoS ONE 8, e83862.

25 Suh YH, Pelkey KA, Lavezzi G, Roche PA, Huganir RL, McBain CJ and Roche KW (2008) Corequirement of PICK1 binding and PKC phosphorylation for stable surface expression of the metabotropic glutamate receptor mGluR7. Neuron 58, 736–748.

26 Daw MI, Chittajallu R, Bortolotto ZA, Dev KK, Duprat F, Henley JM, Collingridge GL and Isaac JT (2000) PDZ proteins interacting with C-terminal GluR2/3 are involved in a PKC-dependent regulation of AMPA receptors at hippocampal synapses. Neuron 28, 873–886.

27 Lu W, Khatri L and Ziff EB (2014) Trafficking of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptor subunit GluA2 from the endoplasmic reticulum is stimulated by a complex containing Ca2+/calmodulin-activated kinase II (CaMKII) and PICK1 protein and by release of Ca2+ from internal stores. J Biol Chem 289, 19218–19230.

28 Roche KW, O’Brien RJ, Mammen AL, Bernhardt J and Huganir RL (1996) Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. Neuron 16, 1179–1188.

29 Milstein AD and Nicoll RA (2009) TARP modulation of synaptic AMPA receptor trafficking and gating depends on multiple intracellular domains. Proc Natl Acad Sci USA 106, 11348–11351.

30 He J, Bellini M, Xu J, Castleberry AM and Hall RA (2004) Interaction with cystic fibrosis transmembrane conductance regulator-associated ligand (CAL) inhibits beta1-adrenergic receptor surface expression. J Biol Chem 279, 50190–50196.

31 Xu Z, Oshima K and Heller S (2010) PIST regulates the intracellular trafficking and plasma membrane expression of cadherin 23. BMC Cell Biol 11, 80.

32 Cuadra AE, Kuo SH, Kawasaky Y, Bredt DS and Chetkovich DM (2004) AMPA receptor synaptic targeting regulated by stargazin interactions with the Golgi-resident PDZ domain protein nPST. J Neurosci 24, 7491–7502.

33 Volk L, Kim CH, Takamiya K, Yu Y and Huganir RL (2010) Developmental regulation of protein interacting with C kinase 1 (PICK1) function in hippocampal synaptic plasticity and learning. Proc Natl Acad Sci USA 107, 21784–21789.

34 Citri A, Bhattacharyya S, Ma C, Morishita W, Fang S, Rizo J and Malenka RC (2010) Calcium binding to PICK1 is essential for the intracellular retention of AMPA receptors underlying long-term depression. J Neurosci 30, 16437–16452.

35 Jaafari N, Henley JM and Hanley JG (2012) PICK1 mediates transient synaptic expression of GluA2-lacking AMPA receptors during glycine-induced AMPA receptor trafficking. J Neurosci 32, 11618–11630.

36 Sossa KG, Court BL and Carroll RC (2006) NMDA receptors mediate calcium-dependent, bidirectional changes in dendritic PICK1 clustering. Mol Cell Neurosci 31, 574–585.
37 Terashima A, Pelkey KA, Rah JC, Suh YH, Roche KW, Collingridge GL, McBain CJ and Isaac JT (2008) An essential role for PICK1 in NMDA receptor-dependent bidirectional synaptic plasticity. Neuron 57, 872–882.

38 Hanley JG (2006) Molecular mechanisms for regulation of AMPAR trafficking by PICK1. Biochem Soc Trans 34, 931–935.

39 Mignogna ML, Giannandrea M, Gurgone A, Fanelli F, Raimondi F, Mapelli L, Bassani S, Fang H, Van Anken E, Alessio M et al. (2015) The intellectual disability protein RAB39B selectively regulates GluA2 trafficking to determine synaptic AMPAR composition. Nat Commun 6, 6504.

40 Scott DB, Blanpied TA, Swanson GT, Zhang C and Ehlers MD (2001) An NMDA receptor ER retention signal regulated by phosphorylation and alternative splicing. J Neurosci 21, 3063–3072.

41 Scott DB, Blanpied TA and Ehlers MD (2003) Coordinated PKA and PKC phosphorylation suppresses RXR-mediated ER retention and regulates the surface delivery of NMDA receptors. Neuropharmacology 45, 755–767.

42 Cui-Wang T, Hanus C, Cui T, Helton T, Bourne J, Watson D, Harris KM and Ehlers MD (2012) Local zones of endoplasmic reticulum complexity confine cargo in neuronal dendrites. Cell 148, 309–321.

43 Masukawa K, Sakai N, Ohmori S, Shirai Y and Saito N (2006) Spatiotemporal analysis of the molecular interaction between PICK1 and PKC. Acta Histochem Cytochem 39, 173–181.

44 Maletic-Savatic M and Malinow R (1998) Calcium-evoked dendritic exocytosis in cultured hippocampal neurons. Part I: trans-Golgi network-derived organelles undergo regulated exocytosis. J Neurosci 18, 6803–6813.

45 Kunde SA, Rademacher N, Tzschach A, Wiedersberg E, Ullmann R, Kalscheuer VM and Shoichet SA (2013) Characterisation of de novo MAPK10/JNK3 truncation mutations associated with cognitive disorders in two unrelated patients. Hum Genet 132, 461–471.

46 Rademacher N, Kunde SA, Kalscheuer VM and Shoichet SA (2013) Synaptic MAGUK multimer formation is mediated by PDZ domains and promoted by ligand binding. Chem Biol 20, 1044–1054.

47 Lois C, Hong EJ, Pease S, Brown EJ and Baltimore D (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science 295, 868–872.

48 Kinoshita E and Kinoshita-Kikuta E (2011) Improved Phos-tag SDS-PAGE under neutral pH conditions for advanced protein phosphorylation profiling. Proteomics 11, 319–323.

49 Kunde SA, Musante L, Grimme A, Fischer U, Muller E, Wanker EE and Kalscheuer VM (2011) The X-chromosome-linked intellectual disability protein PQBP1 is a component of neuronal RNA granules and regulates the appearance of stress granules. Hum Mol Genet 20, 4916–4931.

50 Ippolito DM and Eroglu C (2010) Quantifying synapses: an immunocytochemistry-based assay to quantify synapse number. J Vis Exp 45, pii:2270. https://doi.org/10.3791/2270.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. PDZ-dependent Shisa9/CKAMP44 protein–protein interactions.

Fig. S2. Shisa9/CKAMP44, PICK1 and PKC form a protein complex.