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RESEARCH ARTICLE

APPL1 gates long-term potentiation through its plekstrin homology domain

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ABSTRACT

Hippocampal synaptic plasticity involves both membrane trafficking events and intracellular signaling, but how these are coordinated is far from clear. The endosomal transport of glutamate receptors in and out of the postsynaptic membrane responds to multiple signaling cascades triggered by synaptic activity. In this work, we have identified adaptor protein containing a plekstrin homology domain, phosphotyrosine-binding domain and leucine zipper motif 1 (APPL1) as a crucial element linking trafficking and signaling during synaptic plasticity. We show that APPL1 knockdown specifically impairs PI3K-dependent forms of synaptic plasticity, such as long-term potentiation (LTP) and metabotropic-glutamate-receptor-dependent long-term depression (mGluR-LTD). Indeed, we demonstrate that APPL1 is required for the activation of the phosphatidylinositol triphosphate (PIP_3) pathway in response to LTP induction. This requirement can be bypassed by membrane localization of PI3K and is related to phosphoinositide binding. Interestingly, inhibitors of PDK1 (also known as PDPK1) and Akt have no effect on LTP expression. Therefore, we conclude that APPL1 gates PI3K activation at the plasma membrane upon LTP induction, which is then relayed by downstream PIP_3 effectors that are different from PDK1 and Akt.

KEY WORDS: APPL1, Synaptic plasticity, PI3K, Trafficking

INTRODUCTION

Synaptic plasticity is widely thought to underlie learning and memory (Bliss and Collingridge, 1993; Martin et al., 2000). Accordingly, alterations in synaptic plasticity have been implicated in the pathology of several cognitive disorders, including Alzheimer disease (Selkoe, 2002) and several forms of mental retardation (Newey et al., 2005). Therefore, it is expected that unravelling the mechanisms for synaptic plasticity will help in understanding the pathophysiology of these human disorders.

An important mechanism involved in synaptic plasticity in the hippocampus is the regulated movement of neurotransmitter receptors in and out of the synaptic membrane during long-term potentiation (LTP) and long-term depression (LTD). This trafficking is controlled by the coordinated regulation of intracellular signaling (activation of specific Ca^{2+}-dependent kinases and phosphatases), together with the recruitment of multiple membrane trafficking molecules and synaptic scaffold proteins (Shepherd and Huganir, 2007; Huganir and Nicoll, 2013). Nevertheless, the mechanistic connection between activity-dependent signaling pathways and the membrane trafficking machinery is far from clear. One potential link might be provided by phosphorylated derivatives of phosphatidylinositol or phosphoinositides. Different phosphoinositides are specifically distributed at the plasma membrane and distinct intracellular compartments, where they can function both as second messengers and as key spatial cues for the recruitment of additional molecules (Di Paolo and De Camilli, 2006). Alterations in phosphoinositide metabolism are involved in several neurological disorders such as Charcot–Marie Tooth syndrome type 4J (Chow et al., 2007), amyotrophic lateral sclerosis (ALS) and primary lateral sclerosis (Chow et al., 2009), Down syndrome (Voronov et al., 2008), Lowe syndrome (Olivos-Glander et al., 1995) and Alzheimer disease (Berman et al., 2008). Phosphoinositide dynamics are increasingly being appreciated as regulators of neurotransmitter receptor trafficking (Man et al., 2003; Opazo et al., 2003; Arendt et al., 2010; Jurado et al., 2010; Kim et al., 2011; McCartney et al., 2014). Particularly, in hippocampal neurons, we have recently shown that the synthesis of PI(3,4,5)-triphosphate (PIP_3) by PI3K at the postsynaptic terminal is triggered upon NMDA receptor (NMDAR) activation during LTP induction (Arendt et al., 2014) and is necessary for AMPA receptor (AMPAR) synaptic accumulation (Arendt et al., 2010). Nevertheless, the link between PI3K activation and endosomal trafficking during synaptic plasticity remains to be elucidated.

One interesting candidate for such a link is adaptor protein containing a plekstrin homology (PH) domain, phosphotyrosine-binding domain (PTB) domain and leucine zipper motif 1 (APPL1). APPL1 is a phosphoinositide-interacting protein, whose structure reveals a function in both intracellular trafficking and signaling. Indeed, APPL1 contains a BAR domain, which induces curvature in plasma membrane, a PH domain, which mediates phosphoinositide binding and association to membrane compartments, and a PTB domain (Habermann, 2004; Zhu et al., 2007; Chial et al., 2008). This molecular structure suggests that APPL1 links endocytic trafficking with phosphotyrosine and/or phosphoinositide signaling. Indeed, APPL1 is a Rab5 effector, and acts upstream in the early endocytic process (Zoncu et al., 2009). APPL1 has also been associated with the PI3K–Akt pathway in response to growth factor signaling (Lin et al., 2006; Varsano et al., 2006; Schenck et al., 2008). Specifically, in neurons, APPL1 has been found to modulate the PI3K–Akt pathway during synaptogenesis (Majumdar et al., 2011) and NMDA-receptor-dependent prosurvival signaling (Wang et al., 2012). However, a potential role of APPL1 during synaptic plasticity has never been tested.

In this study, we show for the first time that APPL1 is required for specific forms of synaptic plasticity, particularly those in which PI3K signaling is involved. Thus, knockdown of APPL1 in hippocampal CA1 neurons impairs both NMDA-receptor-dependent LTP and mGluR-dependent LTD (both requiring PI3K signaling; Hou and Klann, 2004), whereas NMDA-receptor-

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dependent LTD was surprisingly unaffected. In addition, we demonstrate that activation of the PI3K–Akt pathway upon induction of synaptic plasticity depends on the presence of APPL1. Intriguingly, we found that inhibition of the canonical PIP_3 downstream effectors [PDK1 (also known as PDPK1)] and Akt family proteins had no effect on LTP, suggesting that APPL1-dependent activation of the PI3K pathway might be relayed by alternative molecules for the induction of synaptic potentiation.

RESULTS

APPL1 partially localizes with synaptic proteins and is not required for basal synaptic transmission

APPL1 is highly expressed in brain tissue (Tan et al., 2010) and is localized in dendrites of neurons (Majumdar et al., 2011; Wang et al., 2012). Using immunocytochemical techniques, we have found that APPL1 is ubiquitously expressed in neurites and spines of hippocampal neurons in primary cultures, where it partially colocalizes with excitatory synaptic markers, such as PSD95 (also known as DLG4), AMPARs (GluA1 subunit; also known as Gria1) and NMDARs (GluN1 subunit; also known as Grin1) (Fig. 1A). In addition, APPL1 also localizes intracellularly with its binding partner Rab5, mostly enriched in dendrites (Fig. 1A, yellow arrows), although some spines contain both Rab5 and APPL1 (Fig. 1A, white arrow). As shown in Fig. 1B, individual spines show enrichment of APPL1 in the postsynaptic compartment, partially colocalizing with AMPARs and NMDARs, and with the synaptic scaffold protein PSD95, as previously shown (Wang et al., 2012). This synaptic distribution, together with its molecular structure (Fig. S1A), supports a potential role of APPL1 in synaptic function. Thus, we decided to develop a range of molecular tools to investigate whether APPL1 is involved in the regulation of function and plasticity of synapses (Fig. S1).

We used knockdown and protein replacement approaches to address this issue. As shown in Fig. 2A and in Fig. S1B, we developed a very effective small hairpin (sh)RNA to knockdown APPL1 expression (shAPPL1). Cultured neurons that had been infected with a lentivirus expressing shAPPL1 showed a decrease in total APPL1 of around 90% as compared to controls ('empty' lentivirus), analyzed by western blotting (Fig. S1B). In addition, we prepared similar lentiviral vectors with a hairpin resistant (HR) version of APPL1 fused to the red fluorescent protein mRFP (Fig. S1C).

To assess the role of APPL1 in synaptic function, we infected organotypic slices of hippocampus with these lentiviruses at 1 day *in vitro*. They were maintained for at least 10 days in order to perform immunostaining and electrophysiology as stated. Fig. 2A shows the CA1 region of the hippocampus that had been injected with the lentivirus that drives the expression of shAPPL1 together with mRFP (top panels) or with the mRFP-tagged hairpin-resistant recombinant APPL1 (mRFP–HR-APPL1, bottom panels). Red fluorescence is shown in the leftmost panels (note nuclear exclusion of the mRFP–HR-APPL1 fusion protein). Effective knockdown of endogenous APPL1 and rescue expression in infected neurons was confirmed by immunostaining with an antibody against APPL1 (middle panels and green channel in merged panels). We then performed electrophysiology recordings in these infected neurons to test whether basal transmission mediated by AMPARs or NMDARs was affected by the APPL1 depletion. As shown in Fig. 2B,C, neither AMPAR nor NMDAR synaptic responses, nor the AMPA:NMDA ratio, significantly changed upon APPL1 knockdown. These results indicate that APPL1 is not required for the maintenance of glutamatergic transmission in CA1 hippocampal neurons.

APPL1 is necessary for PI3K-dependent forms of synaptic plasticity

We then decided to test whether APPL1 is involved in synaptic plasticity. APPL1 interacts with Rab5 and participates in endocytic signaling (Miaczynska et al., 2004; Zoncu et al., 2009). We have

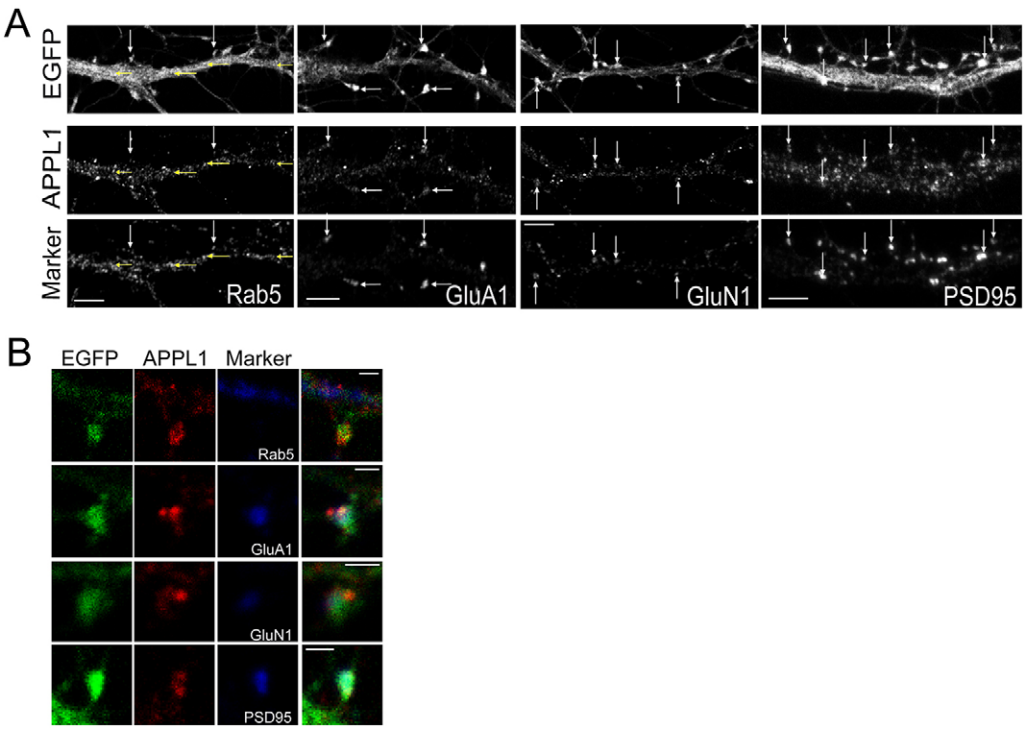

Fig. 1. APPL1 is ubiquitously expressed in hippocampal neurons in dendrites and spines. (A) Neuronal cultures of rat hippocampus were infected with Sindbis virus driving the expression of EGFP in order to distinguish the dendrites and spines of the infected neuron. After 24 h of infection, neurons were fixed and immunostained with antibodies against the indicated cell markers together with an antibody against APPL1. Panels show dendrites of infected neurons and markers used in the immunostaining. White arrows, colocalization of APPL1 with intracellular and synaptic markers in spines; yellow arrows, dendritic colocalization of APPL1 with Rab5. Scale bars: 5 µm. (B) Detail of spines from the immunostaining in A. Merged images are shown on the right. Scale bars: 1 µm.

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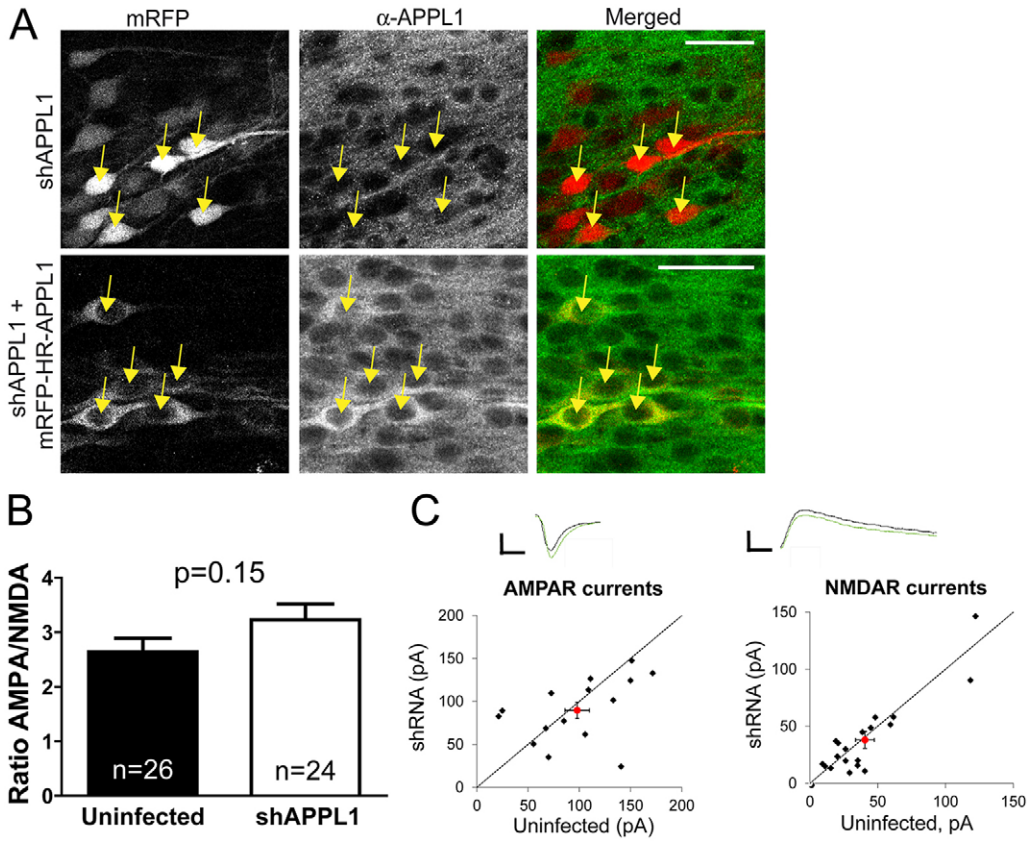

Fig. 2. APPL1 depletion does not affect basal synaptic transmission of hippocampal neurons. (A) Rat hippocampal slices in organotypic cultures were injected in the CA1 region with lentivirus driving the expression of shRNA against APPL1 mRNA and mRFP (shAPPL1, upper panels; yellow arrows show infected neurons) or shAPPL1 plus hairpin-resistant mRFP-tagged APPL1 (mRFP–HR-APPL1, lower panels). Slices were kept in culture for 10 days, fixed and immunostained with an antibody against APPL1 to confirm knockdown of the protein and the expression levels of the recombinant APPL1. Yellow arrows mark some infected neurons. Scale bars: 50 µm. (B) Electrophysiological recordings of CA1 neurons from hippocampal slices that had been infected with the lentivirus driving the expression of shRNA against APPL1. AMPA:NMDA ratios were calculated by using whole-cell voltage clamp recordings of CA3-to-CA1 synaptic responses from infected and non-infected neurons. Data are mean±s.e.m.; *n* values represent the number of cells; *P*-values were calculated with a two-tailed Mann–Whitney test. (C) Simultaneous recordings of infected neurons and nearby non-infected cells were made, and with the same stimulus in order to compare isolated AMPA receptor currents (cells recorded at −60 mV; left panel) or NMDAR currents (cells recorded at +40 mV; right panel). The red data point represents mean±s.e.m. value. Scale bars: 10 ms (horizontal in upper panel of C); 50 pA (vertical in upper panel of C).

previously shown that Rab5-driven endocytosis mediates the removal of AMPARs from synapses during NMDAR-dependent LTD (Brown et al., 2005). Therefore, we first tested whether APPL1 is required for this form of LTD. Surprisingly, both uninfected and shAPPL1-infected neurons displayed strong NMDAR-dependent LTD (Fig. 3A,B). Therefore, we conclude that the endocytic functions of APPL1 are not required for AMPAR synaptic removal during NMDAR-dependent LTD.

APPL1 also binds to signaling proteins such as PI3K and growth factor receptors (Lin et al., 2006; Nechamen et al., 2007; Tan et al., 2010; Wang et al., 2012). Interestingly, another form of LTD, induced by metabotropic glutamate receptors (mGluR-LTD), does involve activation of PI3K signaling, in addition to AMPAR internalization (Snyder et al., 2001; Hou and Klann, 2004). We decided to test whether APPL1 is involved in this type of plasticity. In organotypic slices, mGluR-LTD is induced by a short application of the mGluR agonist (R,S)-3,5-dihydroxyphenylglycine (DHPG) (Huber et al., 2001). As shown in Fig. 3C,D, DHPG application produced a consistent and long-lasting depression of synaptic transmission in control neurons. In contrast, depletion of APPL1 (shAPPL1) virtually abolished mGluR-LTD immediately after induction with DHPG. Interestingly, the role of APPL1 in mGluR-LTD appears to occur upstream from AMPAR endocytosis, because blocking Rab5-dependent endocytosis with a Rab5 dominant-negative mutant (Brown et al., 2005) only impaired mGluR-LTD maintenance, not its initial onset (Fig. S2A,B). Finally, a control lentivirus to replace endogenous APPL1 with a hairpin-resistant version of APPL1 tagged to mRFP (mRFP–HR-APPL1) rescued mGluR-LTD, confirming the specificity of the APPL1 shRNA effect (Fig. 3C,D).

Altogether, this set of experiments strongly suggests that the function of APPL1 in LTD is mainly related to the transduction of intracellular cascades, rather than the regulated endocytosis of AMPARs (given that this a common element for both mGluR- and NMDAR-dependent LTD). If this is the case, it was then relevant to investigate whether APPL1 is also involved in NMDAR-dependent LTP, where the PI3K pathway is activated, in this case resulting in the delivery of AMPARs to synapses (Raymond et al., 2002; Sanna et al., 2002; Opazo et al., 2003; Arendt et al., 2010). NMDAR-dependent LTP was induced in organotypic hippocampal neurons by pairing postsynaptic depolarization of 0 mV with a presynaptic stimulation of 3 Hz. As shown in Fig. 4A,B, this protocol produced substantial potentiation in control neurons. In contrast APPL1-depleted neurons displayed a reduced form of potentiation, as compared to uninfected neurons. Importantly, this effect was due to the specific knockdown of APPL1 and not to viral infection because

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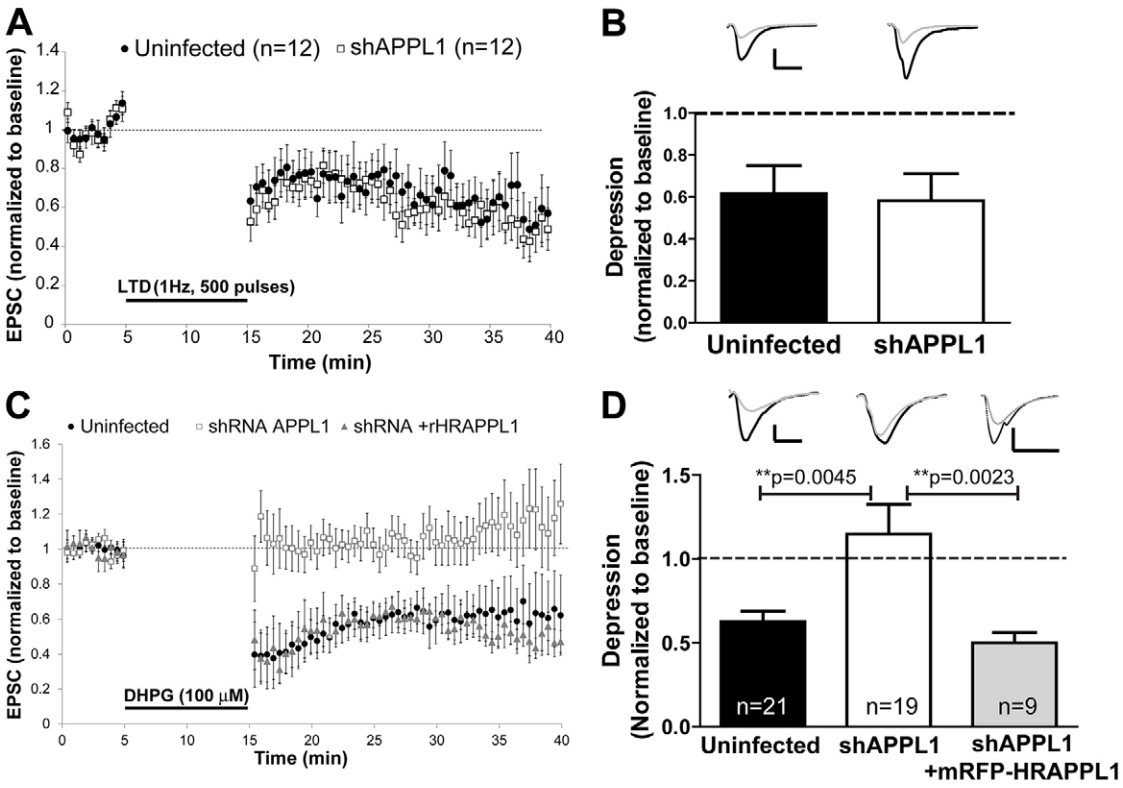

Fig. 3. APPL1 depletion impairs mGluR-LTD, but not NMDAR-dependent LTD. Hippocampal slices were infected with shAPPL1 in the CA1 region, and CA3-to-CA1 synaptic responses were recorded from infected and non-infected CA1 neurons by performing whole-cell voltage clamping. (A) NMDAR-dependent LTD was induced by pairing postsynaptic depolarization to −40 mV and stimulation of Schaffer collateral fibers for 8.3 min at 1 Hz. (B) Quantification of excitatory postsynaptic potential current depression from the final 5 min of recordings (35–40 min in panel A) normalized to basal levels. Representative recording traces: black line baseline, gray line LTD 35–40 min. Scale bars: 50 pA (upper panels, vertical); 20 ms (upper panels, horizontal). (C) mGluR-LTD is induced by 10 min incubation of slices with type-I mGluR agonist DHPG while switching recordings to current clamp. Hippocampal slices were also infected with lentivirus driving the expression of shAPPL1 together with the hairpin-resistant version of APPL1 tagged with mRFP (mRFP–HR-APPL1) as a control for infection. (D) Quantification of excitatory postsynaptic potential current depression from the final 5 min of recordings (35–40 min). Representative recording traces: black line, baseline; gray line, LTD 35–40 min. Data are mean±s.e.m., *P*-values were calculated with a two-tailed Mann–Whitney test. *n* values, number of recorded pathways. Scale bars: 50 pA (upper panels vertical); 20 ms (upper panels horizontal).

coexpression of HR-APPL1 together with shAPPL1 rescued LTP expression.

These combined results suggest that APPL1 is involved in PI3K-dependent forms of synaptic plasticity, irrespective of their synaptic outcome (potentiation or depression). We then proceeded to further investigate the mechanism of action of APPL1.

APPL1 is required for activation of the PI3K pathway upon LTP induction

The activation of the PI3K pathway during LTP has been well established biochemically and pharmacologically (Raymond et al., 2002; Sanna et al., 2002; Man et al., 2003; Kim et al., 2011). Thus, LTP induction triggers an increase in PIP_3 synthesis (Arendt et al., 2014) and Akt phosphorylation (Sanna et al., 2002), and LTP is abolished with the PI3K inhibitors LY294002 and wortmannin (Passafaro et al., 2001; Sanna et al., 2002), as well as by quenching PIP_3 with a specific PH domain (Arendt et al., 2010).

We then tested whether APPL1 participates in the activation of the PI3K–Akt pathway during LTP. To this aim, we employed a pharmacological approach for LTP induction (chemLTP, Otmakhov et al., 2004). This protocol drives strong NMDAR-dependent synaptic potentiation, simultaneously maximizing the number of synapses undergoing plasticity to facilitate biochemical and imaging analyses (Otmakhov et al., 2004; Kopec et al., 2006; Arendt et al., 2014). We then monitored Akt activation by immunostaining using an antibody against the activated form of Akt phosphorylated at residue Ser473 (in the Akt1 isoform, equivalent to Ser474 for Akt2 and Ser472 for Akt3) in hippocampal slices that had been infected with the lentivirus driving the expression of shAPPL1 with or without HR-APPL1. Using this approach, we were able to discriminate neurons that had been depleted of APPL1 from normal cells in the same slice that had been stimulated to undergo LTP and to compare them by measuring the average fluorescence intensity of phospho-Akt staining. As shown in Fig. 5A,D (low-magnification representative pictures of at least three experiments, and quantification in C and F), chemLTP triggered an overall increase in phospho-Akt staining in the CA1 region of the hippocampus, as expected. However, when measuring infected areas of neurons that expressed shAPPL1 (infected area delimited by yellow outline in A), phospho-Akt levels remained similar to control (not induced) conditions (yellow arrows in B). This result indicates that APPL1-depleted neurons failed to activate Akt in response to the LTP stimulus. Interestingly, shAPPL1 did not affect basal phospho-Akt levels in the absence of LTP induction (Fig. 5B, compare uninfected and infected areas under control conditions). Importantly, when the endogenous protein was replaced by HR-APPL1, LTP-induced Akt activation was recovered in the infected neurons (Fig. 5D, low-magnification representative picture, and E yellow arrows), strongly supporting that this effect was specific for APPL1. In addition, staining of

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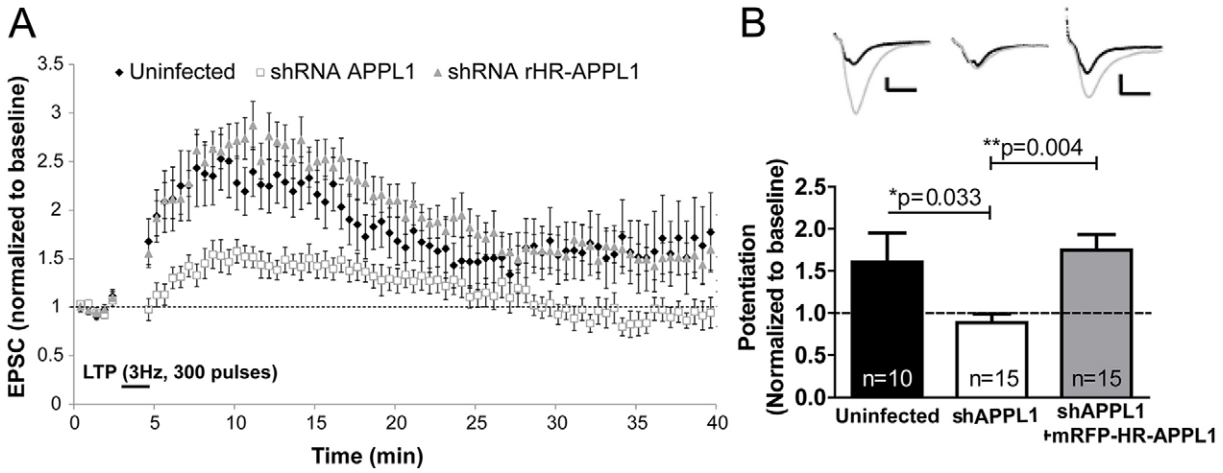

Fig. 4. APPL1 depletion impairs LTP in hippocampal neurons. (A) Hippocampal slices were infected with lentivirus driving the expression of shAPPL1 or shAPPL1 together with the mRFP–HR-APPL1 in the CA1 region, and CA3-to-CA1 synaptic responses were recorded from CA1 neurons by whole-cell voltage clamping. NMDAR-dependent LTP was induced by pairing postsynaptic depolarization to 0 mV and stimulation of Schaffer collaterals for 1.7 min at 3 Hz. (B) Quantification of excitatory postsynaptic potential current potentiation from the final 5 min of recordings (35–40 min in panel A) normalized to basal levels. Representative recording traces: black line, baseline; gray line, LTP 35–40 min. Data are mean±s.e.m. *P*-values were calculated with a two-tailed Mann–Whitney test. *n* values, number of recorded pathways. Scale bars: 50 pA (vertical in upper panels); 20 ms (horizontal in upper panels).

phospho-Akt truly reflected PI3K activation because slices that had been preincubated with the PI3K inhibitor LY294002 showed a substantial reduction in phospho-Akt increase upon LTP induction (gray column in Fig. S3A). It is also noteworthy that we observed that the requirement of APPL1 for basal PI3K activation was different in primary cultures of dissociated neurons. Indeed, dissociated neurons displayed higher levels of phospho-Akt than organotypic slice cultures, under basal conditions (Fig. S3B,C). Accordingly, APPL1 depletion did reduce phospho-Akt levels in non-induced dissociated neurons (Fig. S3D,E). This lower PI3K activation was also associated with a decrease in total GluA1 AMPAR expression in APPL1-depleted neurons, as monitored by western blotting (Fig. S3D,E) and immunocytochemistry analyses (Fig. S3F,G). The reason for this differing response to APPL1 depletion between dissociated neurons and organotypic slices is unclear, thus we decided to continue our characterization of APPL1-dependent signaling in hippocampal slices as this is a more established system for synaptic plasticity.

LTP induction is also known to trigger the activation of the Erk1 and Erk2 pathway (also known as MAPK3 and MAPK1; Erk1/2) (English and Sweatt, 1996; Opazo et al., 2003). Indeed, both the Erk and PI3K pathways could be activated separately, downstream of NMDAR activation (Qin et al., 2005). Therefore, we tested whether APPL1 depletion also affects the Erk1/2 signaling pathway upon LTP induction, or whether the effects are specific to the PI3K pathway. As shown in Fig. 5G,H, LTP induction produced a large (about tenfold) and widespread activation of Erk1/2, as monitored by immunostaining for phosphorylated Erk1/2, which showed no changes upon APPL1 depletion. Altogether, these results demonstrate that APPL1 is specifically involved in the activation of the PI3K–Akt pathway upon LTP induction.

APPL1 is required for LTP independently of PDK1–Akt activation

As mentioned previously, it is well established that NMDAR activation upon LTP induction leads to Akt activation. Because APPL1 knockdown impairs LTP (Fig. 4) and prevents Akt activation (Fig. 5), it is reasonable to propose that APPL1 is required for LTP because it is needed for Akt activation and further downstream signaling. Therefore, we needed to test whether Akt activation is required for LTP, because to our knowledge, this issue has not been explored before. To this end, we tested the effect of two different Akt inhibitors (Yang et al., 2004; Barnett et al., 2005) on LTP induction electrophysiologically in hippocampal slices (similar to Fig. 4). These slices were first preincubated with the inhibitor for at least 1 h, and the inhibitors were present in the perfusion solution during recordings. As shown by western blot analysis (Fig. S4A,B), both inhibitors blocked Akt activation (phospho-Akt) after LTP induction without altering AMPAR phosphorylation by CaMKII and PKC (GluA1 Ser831).

Surprisingly, LTP induction or maintenance was not impaired by the Akt inhibitors in hippocampal slices (Fig. 6A,B). This result suggests that, although the activation of Akt accompanies LTP induction, it might not be required for synaptic potentiation. Therefore, the role of APPL1 in PI3K during LTP appears to occur upstream or separately from Akt activation. Akt is phosphorylated and activated by PDK1, which is directly activated by PIP_3 binding. Therefore, we reasoned that alternative substrates of PDK1 (different from Akt) were responsible for the APPL1 requirement during LTP. We then proceeded to test whether PDK1 itself was required for LTP. We preincubated hippocampal slices with a PDK1 inhibitor (Najafov et al., 2011) and performed LTP experiments in the presence of the inhibitor. Incubation of organotypic slices for 1 h with 10 µM of the PDK1 inhibitor GSK2334470 completely blocked Akt activation (Fig. S4C,D). Nevertheless, and similar to the results with Akt inhibition, the PDK1 inhibitor did not impair LTP (Fig. 6C,D). This result is consistent with a recent publication that revealed PDK1-knockout mice display normal LTP (Sperow et al., 2012).

Altogether, this set of experiments suggests that PI3K activation upon chemLTP induces PDK1–Akt cascades, but these might not be directly involved in synaptic potentiation after LTP induction. Nevertheless, the activation of these cascades was reduced by APPL1 depletion, and APPL1 was required for LTP, which suggests that (1) APPL1 acts in PI3K signaling upstream of PDK1–Akt activation and that (2) APPL1 is needed for LTP independently from these downstream effectors.

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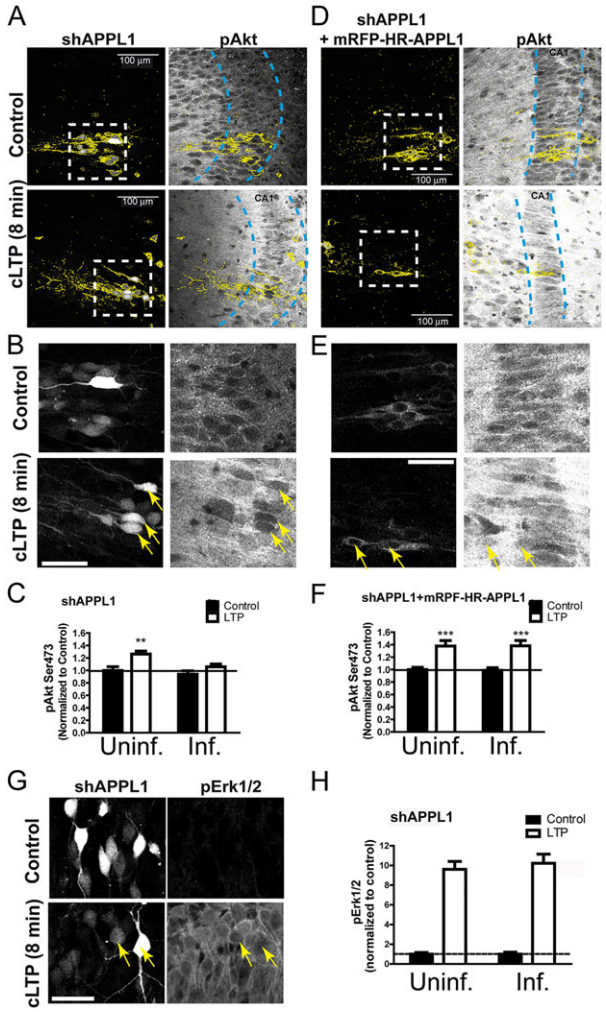

Fig. 5. APPL1 depletion prevents Akt activation that is induced by LTP. (A) Low-magnification representative pictures of hippocampal slices infected (Inf.) with lentivirus driving the expression of shAPPL1 and that had been subjected to chemLTP (cLTP) for 8 min (blue outline shows CA1 pyramidal region). Slices were fixed and immunostained with an antibody against phospho-Akt (Ser473). A ROI of infected area (yellow outline) was selected from a binary mask created by thresholding (see Materials and Methods). This ROI was then transferred onto the phospho-Akt staining channel and measured. (B) High-magnification picture of selected area in A. Yellow arrows point to shAPPL1-expressing neurons. Scale bar: 50 µm. (C) Quantification of the fluorescence intensity of phospho-Akt staining normalized to that in the non-infected (Uninf.) region under control conditions. n=9 slices for control and n=14 slices for LTP conditions from three independent experiments. (D–F) Similar to A–C, hippocampal slices that had been infected with lentivirus driving the expression of shAPPL1 together with the mRFP–HR-APPL1. Quantification from n=12 slices for both control and LTP conditions, from three independent experiments. (G) Hippocampal slices were infected with lentivirus driving the expression of shAPPL1 and subjected to chemLTP for 8 min. Slices were fixed and immunostained with an antibody against phospho-Erk1/2 (pErk1/2). Slices were processed as described in A, and phospho-Erk1/2 staining was measured and analyzed with ImageJ. Scale bar: 50 µm. (H) Quantification of the fluorescence intensity of phospho-Erk1/2 staining normalized to that under control conditions. n=7 slices for control and n=9 slices for LTP conditions from three independent experiments. Data are mean±s.e.m. $^{**}P$<0.005, $^{***}P$<0.001 (two-way ANOVA and Bonferroni post-hoc test).

Membrane targeting of PI3K bypasses the requirement of APPL1 for pathway activation

It is known that PI3K targeting to the plasma membrane is enough to activate PIP_3 signaling (Klippel et al., 1996), and this form of activation leads to synaptic potentiation (Arendt et al., 2010). Therefore, we tested whether PI3K membrane targeting was sufficient to rescue loss of APPL1 in PIP_3 signaling. To this end, we depleted APPL1 and simultaneously targeted PI3K to the plasma membrane by co-infecting individual neurons with a lentivirus driving shAPPL1 expression and a Sindbis virus expressing the catalytic subunit of PI3K p110α (also known as PIK3CA) tagged with a myristoylation signal at the N-terminus and with EGFP at the C-terminus (myr–P110–EGFP). Doubly infected neurons were identified from the green fluorescence of the myr–P110–EGFP construct and the red fluorescence of mRFP accompanying expression of shAPPL1. Then, the extent of activation of the PIP_3 pathway at individual neurons was monitored using phospho-Akt immunostaining, as described above. Therefore, under this configuration, we could compare pathway activation in neurons with membrane-targeted PI3K in the presence of APPL1 (green-only neurons), with membrane-targeted PI3K and lack of APPL1 (green and red neurons), and under basal conditions (non-fluorescent neurons). As shown in Fig. 7, myr–P110-EGFP expression produced a consistent increase in phospho-Akt signal, as compared to uninfected neurons (blue and green channels in merged panel in Fig. 7A; green column in panel Fig. 7B), but this increase was independent from APPL1 (yellow arrows pointing to green and red neurons in Fig. 7A; brown column in Fig. 7B). In addition, knockdown of APPL1 did not affect basal phospho-Akt signal (red column in Fig. 7B), similar to our previous results (Fig. 5B, control). Therefore, this result supports the notion that the role of APPL1 in PIP_3 signaling involves PI3K membrane localization and can be bypassed by anchoring of PI3K to the membrane.

APPL1 function in LTP is dependent on the PH domain

APPL1 is not typically associated with the plasma membrane, but it does contain multiple interaction domains that might affect its localization and function. The BAR-PH domain is crucial for the association of APPL1 with pre-endosomal compartments close to the plasma membrane, before the recruitment of early endosomal factors and fusion with sorting endosomes (Zoncu et al., 2009; Gan et al., 2013). Therefore, we tested whether this element was important for APPL1 function during LTP. Thus, we generated a lentivirus to knockdown endogenous APPL1 and replace it with a mutant lacking the PH domain. Fig. S1C shows the effectiveness of the molecular replacement strategy with these lentiviruses. As shown in Fig. 8A,B, replacement of endogenous APPL1 with a full-length shRNA-resistant APPL1 rescued LTP (similar to results in Fig. 4). In contrast, replacement with truncated APPL1 that lacked the PH domains failed to support stable potentiation. Therefore, we conclude that the PH domain of APPL1 (and possibly its membrane association) is required for APPL1 function during LTP.

DISCUSSION

In this work, we provide new insight into the coupling of endosomal proteins and intracellular signaling during synaptic plasticity. In the last decade, many efforts have been made to decipher the interaction between trafficking and signaling in the context of synaptic function. We now propose that APPL1 is one of such coupling mechanisms. This is based on four main lines of evidence: (1) APPL1 is specifically required for synaptic plasticity forms that rely on PI3K signaling, such as mGluR-LTD and NMDAR-dependent LTP; (2) in agreement with this observation, APPL1 is necessary for activation of the PIP_3 pathway upon induction of synaptic plasticity; (3) the requirement of APPL1 for PIP_3 pathway activation can be bypassed by membrane localization of PI3K; (4) the association of

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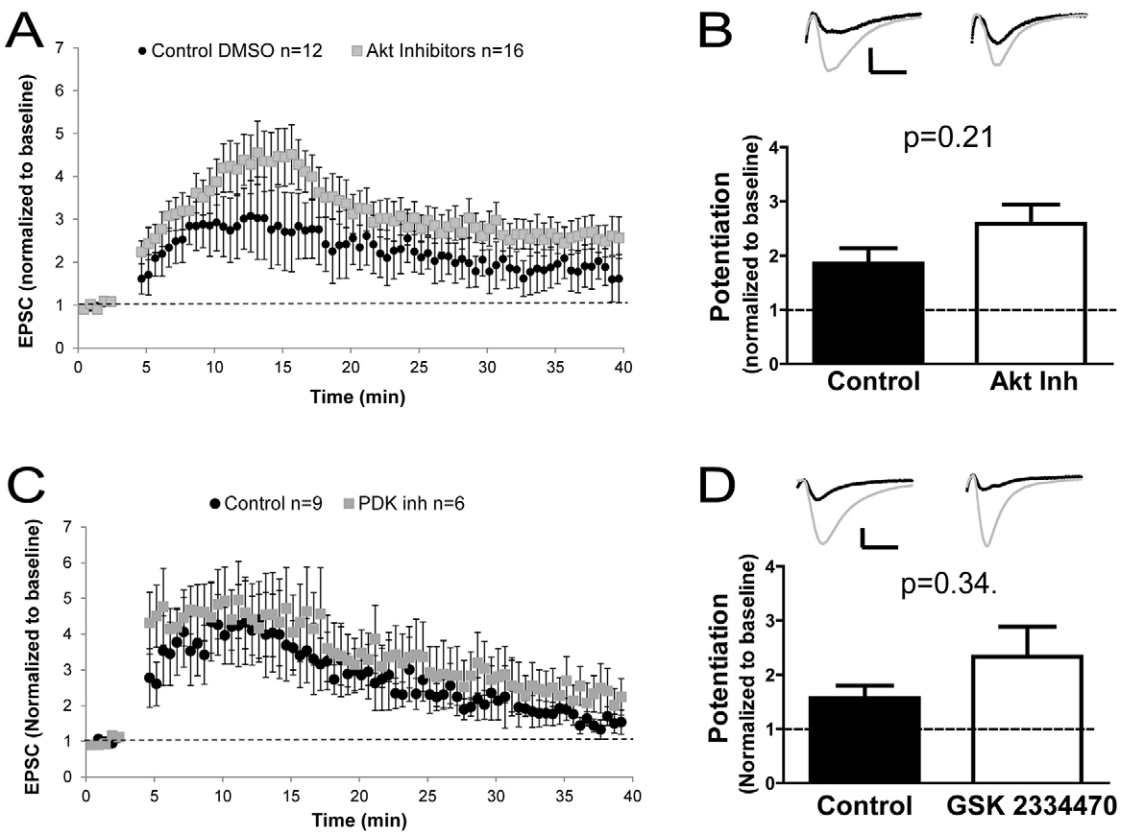

Fig. 6. Inhibition of the PI3K downstream effectors PDK1 and Akt has no effect on LTP expression. (A) CA3-to-CA1 synaptic responses were recorded under whole-cell configuration from hippocampal slices that had been preincubated with Akt inhibitors (0.7 µM AktV or 5 µM AktVIII) and compared to vehicle (DMSO) pretreated slices. LTP was induced as described in Fig. 4. Akt inhibitors (Akt Inh) were also present during the recordings. Similar results were obtained with the inhibitors AktV and AktVIII, and the data have been pooled for simplicity. (B) Quantification of excitatory postsynaptic potential current potentiation of the final 5 min of recordings (35–40 min in panel A) normalized to basal levels. Representative recording traces: black line, baseline; gray line, LTP 35–40 min. Scale bar: 50 pA (vertical in upper panels); 20 ms (horizontal in upper panels). (C,D) Similar to A,B, with 10 µM of the PDK1 inhibitor GSK2334470. Data are mean±s.e.m. *P*-values were calculated with a two-tailed Mann–Whitney test.

APPL1 with membrane compartments, mediated by its PH domain, is required for its function during LTP.

The observation that the common factor for the requirement of APPL1 in synaptic plasticity is PI3K signaling rather than endocytic trafficking was initially surprising. This is because APPL1 acts as a Rab5 effector during endocytosis (Miaczynska et al., 2004), and it associates with clathrin-derived endocytic vesicles (Zoncu et al., 2009). In addition, the Rab5-mediated acceleration of endocytosis that is found in fibroblasts from individuals with Alzheimer disease or Down syndrome depends on abnormal recruitment of APPL1 by an APP metabolite (Kim et al., 2015). However, NMDAR-dependent LTD, which clearly relies on clathrin-dependent endocytosis (Carroll et al., 1999; Man et al., 2000; Lee et al., 2002) and Rab5-driven endosomal trafficking (Brown et al., 2005), did not require APPL1 function. We have also observed that Rab5 participates in a late phase of mGluR-LTD, whereas APPL1 is required immediately after induction. Therefore, APPL1 is an interesting regulator of synaptic function that mediates certain forms of synaptic plasticity depending on the intracellular signaling that is engaged, rather than on the outcome of the synaptic change (potentiation versus depression).

After having established that the common factor for APPL1 involvement in synaptic plasticity is PI3K-dependent signaling, we turned to investigate at what level APPL1 participates. Using the phosphorylation of Akt as a reporter, we established that APPL1 is required for the activation of the PIP_3 pathway upon LTP induction. APPL1 has been recently shown to interact with PSD95 and PI3K, and in this manner to couple the PIP_3 pathway to NMDAR signaling during neuroprotection (Wang et al., 2012). A similar mechanism might operate for PI3K activation during NMDAR-dependent LTP. Nevertheless, the mechanism might need to be more general because our data suggest that APPL1 is also engaged for mGluR signaling (mGluR-LTD). In addition, APPL1 has been shown to couple with a variety of receptors that are linked to PI3K signaling, such as TrkA (Lin et al., 2006; Varsano et al., 2006) and the receptors for adiponectin (Mao et al., 2006) and follicle-stimulating hormone (Nechamen et al., 2004).

In the case of LTP, it has been shown that activation of NMDARs leads to the activation of Ras (Kim et al., 2003; Harvey et al., 2008). Ras, in turn, can activate PI3K through direct interaction with the p110α catalytic subunit of class-I PI3Ks, leading to the activation of its lipid kinase activity and synthesis of PIP_3 (Suire et al., 2002). Whether PI3K is recruited to Ras or NMDAR signaling complexes during LTP remains to be established. Nevertheless, it is interesting that APPL1 is no longer required for activation of PIP_3 signaling when PI3K is 'passively' targeted to the membrane with a myristoylation tag. The notion of a role for APPL1 based on its membrane localization is also supported by the result that APPL1 cannot mediate LTP when its membrane localization domain is missing (ΔPH). Admittedly, APPL1 is not thought to be targeted to the plasma membrane but is, instead, thought to sense curved endosomal membranes through its combined BAR-PH domain

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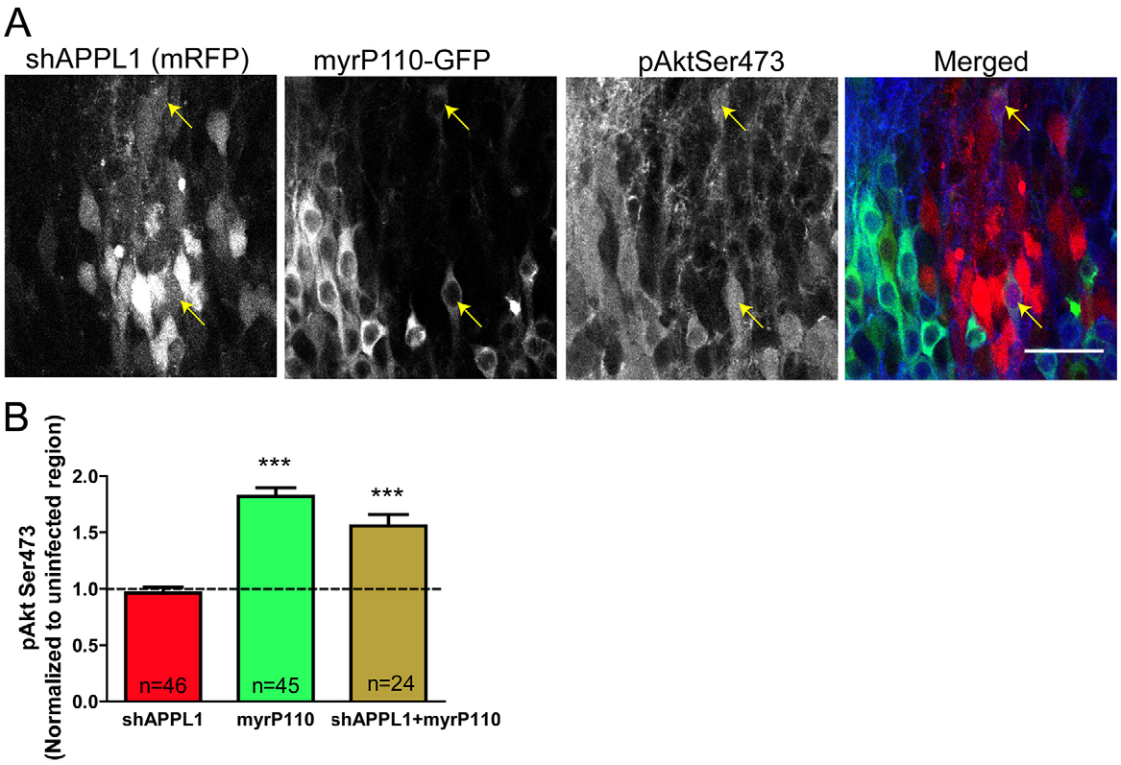

Fig. 7. APPL1 depletion does not affect Akt activation induced by a membrane-targeted version of PI3K. (A) Hippocampal slices were infected with lentivirus driving the expression of shAPPL1, left in the incubator for 9 days and then co-infected with Sindbis virus driving the expression of a membrane-attached (constitutively active) form of PI3K, myr–P110–EGFP, for 24 h. Slices were fixed and immunostained with an antibody against phospho-Akt (Ser473; pAktSer473). Confocal stack images were analyzed by using ImageJ. Three regions from the same stack were selected: shAPPL1-infected region (shAPPL1, red-only neurons), myr–P110–EGFP region (myrP110, green-only neurons) and shAPPL1–myr–P110–EGFP co-infected region (shAPPL1+myrP110, red and green neurons, arrows). The fluorescence intensity of phospho-Akt was measured in the different regions and compared to that in a non-infected region. Scale bar: 50 µm. (B) Quantification of the fluorescence intensity of the different regions normalized to non-infected regions. *n* values, number of cells from two independent experiments; ***P<0.0001 (two-tailed Mann–Whitney test).

(Zhu et al., 2007). Nevertheless, APPL1 is considered to operate very close to the plasma membrane, in pre-endosomal compartments before early endosomal sorting and fusion (Zoncu et al., 2009; Gan et al., 2013). Therefore, the localization of APPL1 at the interphase between the plasma membrane and early endosomes might facilitate its participation in synaptic plasticity events that engage both exocytic (NMDAR-dependent LTP) and endocytic (mGluR-LTD) trafficking (see proposed model in Fig. S4C). In agreement with this interpretation, APPL1 has been recently described as an important factor for bidirectional sorting of endosomal cargo that is destined for plasma membrane delivery or for endocytic processing (Kalaidzidis et al., 2015).

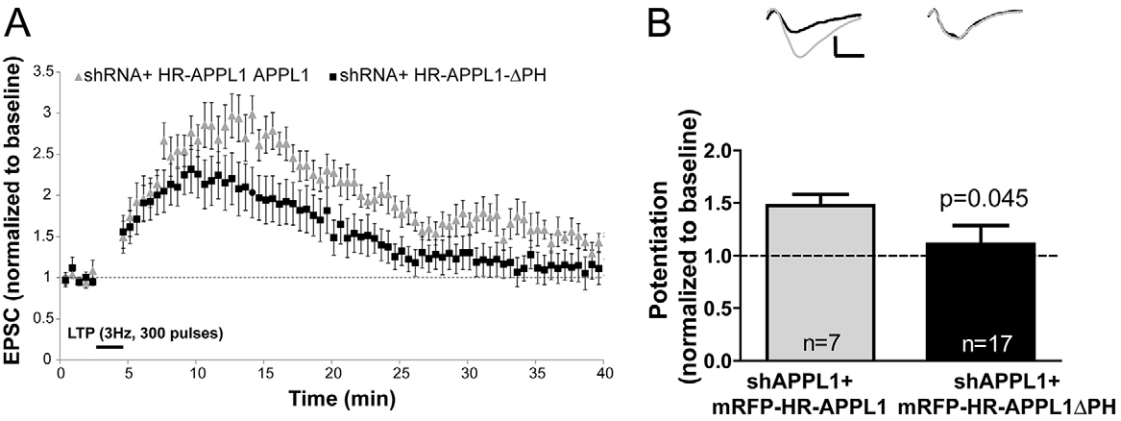

Fig. 8. The PH domain of APPL1 is necessary for LTP expression. (A) Hippocampal slices were infected with lentivirus driving the expression of shAPPL1 together with recombinant mRFP–HR-APPL1 or with the mutated version lacking the PH domain (mRFP–HR-APPL1-ΔPH) in the CA1 region, and whole-cell voltage clamp measurements of neurons were recorded. LTP was induced as described in Fig. 4. (B) Quantification of excitatory postsynaptic potential current potentiation from the final 5 min of recordings (35–40 min in panel A) normalized to basal levels. Representative recording traces: black line, baseline; gray line, LTP 35–40 min. Data are mean±s.e.m.; *n* values, number of recorded pathways; *P*-values were calculated with a two-tailed Mann–Whitney test. Scale bars: 50 pA (vertical in upper panels); 20 ms (horizontal in upper panels).

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Finally, as a corollary to the study of APPL1 and PI3K signaling for LTP, we have found that inhibitors of the immediate downstream effectors of this pathway, PDK1 and Akt, do not affect synaptic potentiation. This is a surprising result because they have been often assumed as integral components of the signaling events leading to potentiation (Raymond et al., 2002; Sanna et al., 2002; Opazo et al., 2003; Arendt et al., 2010). Nevertheless, our results are consistent with the absence of synaptic plasticity deficits in the PDK1-knockout mouse (Sperow et al., 2012). It should also be noted that our experimental conditions refer to early phases of LTP, which mostly rely on membrane trafficking and post-translational modifications. There is abundant evidence for the role of the Akt–mTOR pathway during the late phases of LTP, which stimulate new protein synthesis (Tang et al., 2002; Kelleher et al., 2004; Gobert et al., 2008; Stoica et al., 2011). Therefore, our results point to a signaling bifurcation immediately after PIP_3 synthesis, with the PDK1–Akt–mTOR branch being responsible for protein-synthesis-dependent late LTP, and alternative effectors mediating more acute changes in the structure and composition of the synaptic membrane. What are these alternative PIP_3 effectors? There are actually multiple possibilities and several of them are likely to act in parallel. For example, the small GTPase Cdc42 is activated through PIP_3-sensitive guanine nucleotide exchange factors (GEFs), such as Vav2 and Vav3 (Aoki et al., 2005). This is relevant for LTP because Cdc42 is activated upon LTP induction (Murakoshi et al., 2011), and is required for synaptic potentiation and spine structural plasticity (Kim et al., 2014). Additionally, PIP_3 might act directly at the synaptic scaffold because PIP_3 depletion reduces the accumulation of PSD95 at spines (Arendt et al., 2010) and PI3K activation (in this case upon stimulation with BDNF) triggers the mobilization of PSD95 in dendrites (Yoshii and Constantine-Paton, 2007). But even among synaptic scaffolds, it is difficult to predict the key PIP_3 sensor (or sensors) because multiple PDZ domains have phosphoinositide-binding capabilities (Zimmermann, 2006).

In conclusion, these new data have revealed a new molecular link between endosomal trafficking and intracellular signaling during synaptic plasticity, and shed light on the complexities of PIP_3 signaling in synaptic compartments.

MATERIALS AND METHODS

Materials

Chemicals were purchased from Sigma-Aldrich when not specified. Antibodies against APPL1, phosphorylated Akt at Ser473, phosphorylated Akt at Thr308, Akt (pan), phosphorylated Erk1/2 and Erk1/2 were obtained from Cell Signaling Technology (1:1000; mAb#3858, mAb#4060, mAb#4056, #mAb2920, mAb#9106 and mAb#9102, respectively). The antibody against the GluA1 receptor was from Abcam (1:1000; AB31232), and the antibody against phosphorylated GluA1 at Ser831 was from Merck Millipore (1:1000; AB5847). Akt inhibitors V and VIII were purchased from Merck Millipore. DHPG and GSK2334470 were from Tocris Biosciences.

Cloning of lentiviral vectors and lentivirus production

The APPL1 mRNA target sequence was selected from Mao et al. (2006) (5′-AAGAGTGGATCTGTACAATAA-3′). Briefly, two complementary oligonucleotides with the sequences of the target, hairpin loops and restriction sites (5′-AAGAGTGGATCTGTACAATAATTCAAGAGATT-ATTGTACAGATCCACTCTTTTTTTTGT-3′) were annealed and cloned into the KH1-LV vector (*Sma*I–*Xba*I sites). This vector contains also a fluorescence tag controlled by a ubiquitin promoter, allowing us to monitor the viral infection. Alternatively, and for control and rescue experiments, cDNA of a hairpin-resistant version of APPL1 was cloned from a pcDNA vector right after the fluorescent tag, thus replacing the endogenous APPL1 with an exogenous fluorescent protein. The primers used in PCR for deletion of the PH domain (amino acids 281–378) from the mRFP–APPL1–EGFP sequence were 5′-CCCGGCGCCTACAAGACCG-3′ and 5′-GGC-GCGGCTTAAGTACTTTCGGGTTAAATTTCGATTAACAGG-3′. These vectors were transfected into HEK-293FT cells together with helper pMD2.8 and pCMV vectors containing viral genes. At 2 days after transfection, media containing viral particles were collected and ultracentrifuged to concentrate the virus.

Primary cultures and infection

Dissociated hippocampal neurons were prepared from embryonic day 18 Wistar rats, as previously described (Fernandez-Monreal et al., 2009). Organotypic cultures were prepared from Wistar rats (postnatal day 5 to 6, both sexes) and placed on porous membranes. Slices were infected in the CA1 area through pulse injection of the lentivirus with the help of a picospritzer at 1 day *in vitro* and maintained for 10 days in an incubator at 35°C under 5% CO_2. Protein extracts from neuronal cultures and organotypic slices were obtained with a lysis buffer containing 10 mM HEPES, 0.5 M NaCl, 10 mM EDTA, 1% Triton X-100 and protease and phosphatase inhibitors (tablets with protease inhibitor cocktail and PhosSTOP™ from Roche).

Electrophysiology

The recording chamber was perfused with artificial cerebrospinal fluid (ACSF; 119 mM NaCl, 2.5 mM KCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM glucose at pH 7.4) with 0.1 mM picrotoxin, and 4 µM 2-chloroadenosine, and continuously gassed with 5% CO_2 and 95% O_2. Patch recording pipettes (4–6 MΩ) for voltage clamps were filled with 115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM $MgCl_2$, 4 mM Na_2ATP, 0.4 mM Na_3GTP, 10 mM sodium phosphocreatine and 0.6 mM EGTA at pH 7.25. For current clamp experiments with DHPG incubation, pipettes were filled with a solution containing 115 mM potassium gluconate, 20 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, 4 mM Na_2ATP and 0.3 mM Na_3GTP at pH 7.25. Synaptic responses were evoked with bipolar electrodes using single-voltage pulses (200 µs, up to 20 V). The stimulating electrodes were placed over Schaffer collateral fibers between 300 and 500 µm from the recorded cells. Synaptic AMPAR-mediated responses were measured at −60 mV and NMDAR-mediated responses at +40 mV, at 100 ms, when AMPAR responses had fully decayed. NMDAR-dependent LTD was induced using a pairing protocol by stimulating Schaffer collateral fibers at 1 Hz (300 pulses) while depolarizing the postsynaptic cell to −40 mV. mGluR-LTD was induced through incubation with the group-I mGluR agonist DHPG (100 µM) while switching to current clamp recordings during the incubation. NMDAR-dependent LTP was induced by pairing stimulation of Schaffer collateral fibers at 3 Hz (300 pulses) with depolarization of the postsynaptic cell to 0 mV.

Chemical LTP

Slices were preincubated in gassed ACSF for 10 min at 35°C. They were then transferred to a gassed Mg^{2+}-free ACSF chamber with a mix of 100 µM picrotoxin, 50 µM forskolin and 0.1 µM rolipram, and incubated for 8 min. Control conditions were performed in ACSF containing normal levels of Mg^{2+} with DMSO as vehicle (Otmakhov et al., 2004).

Immunofluorescence in organotypic slices

Slices were subjected to chemLTP and fixed overnight with a solution of 4% paraformaldehyde, 4% of sucrose in PBS containing phosphatase inhibitors (PhosphoSTOP, Roche) at 4°C. Slices were then incubated with blocking solution (0.2% fish gelatin and 0.3% Triton X-100 in PBS), also containing phosphatase inhibitors, for 2 h. Antibodies were applied in blocking solution and incubated overnight at 4°C. Slices were incubated with fluorescently tagged secondary antibodies (Life Technologies, Alexa-Fluor®) for 1–3 h at room temperature and mounted with Prolong Gold antifade medium (Life Technologies). 3-µm *z*-stack fluorescence images were obtained with a LSM710 Zeiss confocal microscope and analyzed with ImageJ free software. Infected areas were selected by thresholding the fluorescent channel to create a binary mask and a single region of interest (ROI) (Edition>Create selection, yellow outline in Fig. 5A,D). This ROI

was saved using the ROI manager function and transferred to the channel of interest (phospho-Akt and phospho-Erk staining) to measure average fluorescence intensity. The uninfected area was defined by the corresponding inverted ROI (Edition>Make inverse), whose average intensity was also measured. The results were normalized to the uninfected area of the control condition. Control and LTP conditions were compared in the same stack of the slices in each experiment (a depth of around 15 µm).

Statistical analyses

All graphs represent mean±s.e.m. Statistical differences were calculated according to non-parametric tests. When significant differences were observed, *P*-values for pairwise comparisons were calculated according to two-tailed Mann–Whitney tests (for unpaired data) or Wilcoxon's tests (for paired data). Data from Fig. 5 were analyzed using two-way ANOVA and Bonferroni post test.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.F.-M. and J.A.E. designed the research approach and wrote the manuscript. M.F.-M. performed the biochemical, imaging and electrophysiology experiments. C.S.-C. performed some of the biochemistry experiments.

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Supplementary information

Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.183475.supplemental

References

Aoki, K., Nakamura, T., Fujikawa, K. and Matsuda, M. (2005). Local phosphatidylinositol 3,4,5-trisphosphate accumulation recruits Vav2 and Vav3 to activate Rac1/Cdc42 and initiate neurite outgrowth in nerve growth factor-stimulated PC12 cells. *Mol. Biol. Cell* **16**, 2207-2217.

Arendt, K. L., Royo, M., Fernandez-Monreal, M., Knafo, S., Petrok, C. N., Martens, J. R. and Esteban, J. A. (2010). PIP3 controls synaptic function by maintaining AMPA receptor clustering at the postsynaptic membrane. *Nat. Neurosci.* **13**, 36-44.

Arendt, K. L., Benoist, M., Lario, A., Draffin, J. E., Munoz, M. and Esteban, J. A. (2014). PTEN counteracts PIP3 upregulation in spines during NMDA-receptor-dependent long-term depression. *J. Cell Sci.* **127**, 5253-5260.

Barnett, S. F., Defeo-Jones, D., Fu, S., Hancock, P. J., Haskell, K. M., Jones, R. E., Kahana, J. A., Kral, A. M., Leander, K., Lee, L. L. et al. (2005). Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. *Biochem. J.* **385**, 399-408.

Berman, D. E., Dall'Armi, C., Voronov, S. V., McIntire, L. B. J., Zhang, H., Moore, A. Z., Staniszewski, A., Arancio, O., Kim, T.-W. and Di Paolo, G. (2008). Oligomeric amyloid-beta peptide disrupts phosphatidylinositol-4,5-bisphosphate metabolism. *Nat. Neurosci.* **11**, 547-554.

Bliss, T. V. P. and Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**, 31-39.

Brown, T. C., Tran, I. C., Backos, D. S. and Esteban, J. A. (2005). NMDA receptor-dependent activation of the small GTPase Rab5 drives the removal of synaptic AMPA receptors during hippocampal LTD. *Neuron* **45**, 81-94.

Carroll, R. C., Beattie, E. C., Xia, H., Luscher, C., Altschuler, Y., Nicoll, R. A., Malenka, R. C. and von Zastrow, M. (1999). Dynamin-dependent endocytosis of ionotropic glutamate receptors. *Proc. Natl. Acad. Sci. USA* **96**, 14112-14117.

Chial, H. J., Wu, R., Ustach, C. V., McPhail, L. C., Mobley, W. C. and Chen, Y. Q. (2008). Membrane targeting by APPL1 and APPL2: dynamic scaffolds that oligomerize and bind phosphoinositides. *Traffic* **9**, 215-229.

Chow, C. Y., Zhang, Y., Dowling, J. J., Jin, N., Adamska, M., Shiga, K., Szigeti, K., Shy, M. E., Li, J., Zhang, X. et al. (2007). Mutation of FIG4 causes neurodegeneration in the pale tremor mouse and patients with CMT4J. *Nature* **448**, 68-72.

Chow, C. Y., Landers, J. E., Bergren, S. K., Sapp, P. C., Grant, A. E., Jones, J. M., Everett, L., Lenk, G. M., McKenna-Yasek, D. M., Weisman, L. S. et al. (2009). Deleterious variants of FIG4, a phosphoinositide phosphatase, in patients with ALS. *Am. J. Hum. Genet.* **84**, 85-88.

Di Paolo, G. and De Camilli, P. (2006). Phosphoinositides in cell regulation and membrane dynamics. *Nature* **443**, 651-657.

English, J. D. and Sweatt, J. D. (1996). Activation of p42 mitogen-activated protein kinase in hippocampal long term potentiation. *J. Biol. Chem.* **271**, 24329-24332.

Fernandez-Monreal, M., Oung, T., Hanson, H. H., O'Leary, R., Janssen, W. G., Dolios, G., Wang, R. and Phillips, G. R. (2009). Gamma-protocadherins are enriched and transported in specialized vesicles associated with the secretory pathway in neurons. *Eur. J. Neurosci.* **32**, 921-931.

Gan, Z., Ram, S., Ober, R. J. and Ward, E. S. (2013). Using multifocal plane microscopy to reveal novel trafficking processes in the recycling pathway. *J. Cell Sci.* **126**, 1176-1188.

Gobert, D., Topolnik, L., Azzi, M., Huang, L., Badeaux, F., Desgroseillers, L., Sossin, W. S. and Lacaille, J.-C. (2008). Forskolin induction of late-LTP and up-regulation of 5′ TOP mRNAs translation via mTOR, ERK, and PI3K in hippocampal pyramidal cells. *J. Neurochem.* **106**, 1160-1174.

Habermann, B. (2004). The BAR-domain family of proteins: a case of bending and binding? *EMBO Rep.* **5**, 250-255.

Harvey, C. D., Yasuda, R., Zhong, H. and Svoboda, K. (2008). The spread of Ras activity triggered by activation of a single dendritic spine. *Science* **321**, 136-140.

Hou, L. and Klann, E. (2004). Activation of the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling pathway is required for metabotropic glutamate receptor-dependent long-term depression. *J. Neurosci.* **24**, 6352-6361.

Huber, K. M., Roder, J. C. and Bear, M. F. (2001). Chemical induction of mGluR5- and protein synthesis–dependent long-term depression in hippocampal area CA1. *J. Neurophysiol.* **86**, 321-325.

Huganir, R. L. and Nicoll, R. A. (2013). AMPARs and synaptic plasticity: the last 25 years. *Neuron* **80**, 704-717.

Jurado, S., Benoist, M., Lario, A., Knafo, S., Petrok, C. N. and Esteban, J. A. (2010). PTEN is recruited to the postsynaptic terminal for NMDA receptor-dependent long-term depression. *EMBO J.* **29**, 2827-2840.

Kalaidzidis, I., Miaczynska, M., Brewińska-Olchowik, M., Hupalowska, A., Ferguson, C., Parton, RG, Kalaidzidis, Y. and Zerial, M. (2015). APPL endosomes are not obligatory endocytic intermediates but act as stable cargo-sorting compartments. *J. Cell Biol.* **211**, 123-144.

Kelleher, R. J., Govindarajan, A. and Tonegawa, S. (2004). Translational regulatory mechanisms in persistent forms of synaptic plasticity. *Neuron* **44**, 59-73.

Kim, J. H., Lee, H. K., Takamiya, K. and Huganir, R. L. (2003). The role of synaptic GTPase-activating protein in neuronal development and synaptic plasticity. *J. Neurosci.* **23**, 1119-1124.

Kim, J.-I., Lee, H.-R., Sim, S.-E., Baek, J., Yu, N.-K., Choi, J.-H., Ko, H.-G., Lee, Y.-S., Park, S.-W., Kwak, C. et al. (2011). PI3Kγ is required for NMDA receptor-dependent long-term depression and behavioral flexibility. *Nat. Neurosci.* **14**, 1447-1454.

Kim, I. H., Wang, H., Soderling, S. H. and Yasuda, R. (2014). Loss of Cdc42 leads to defects in synaptic plasticity and remote memory recall. *Elife* **3**, e02839.

Kim, S., Sato, Y., Mohan, P. S., Peterhoff, C., Pensal, A., Rigoglioso, A., Jiang, Y. and Nixon, R. A. (2015). Evidence that the rab5 effector APPL1 mediates APP-β CTF-induced dysfunction of endosomes in Down syndrome and Alzheimer's disease. *Mol. Psychiatry* **21**, 707-716.

Klippel, A., Reinhard, C., Kavanaugh, W. M., Apell, G., Escobedo, M. A and Williams, L. T. (1996). Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways. *Mol. Cell. Biol.* **16**, 4117-4127.

Kopec, C. D., Li, B., Wei, W., Boehm, J. and Malinow, R. (2006). Glutamate receptor exocytosis and spine enlargement during chemically induced long-term potentiation. *J. Neurosci.* **26**, 2000-2009.

Lee, S. H., Liu, L., Wang, Y. T. and Sheng, M. (2002). Clathrin adaptor AP2 and NSF interact with overlapping sites of GluR2 and play distinct roles in AMPA receptor trafficking and hippocampal LTD. *Neuron* **36**, 661-674.

Lin, D. C., Quevedo, C., Brewer, N. E., Bell, A., Testa, J. R., Grimes, M. L., Miller, F. D. and Kaplan, D. R. (2006). APPL1 associates with TrkA and GIPC1 and is required for nerve growth factor-mediated signal transduction. *Mol. Cell. Biol.* **26**, 8928-8941.

Majumdar, D., Nebhan, C. A., Hu, L., Anderson, B. and Webb, D. J. (2011). An APPL1/Akt signaling complex regulates dendritic spine and synapse formation in hippocampal neurons. *Mol. Cell. Neurosci.* **46**, 633-644.

Man, H.-Y., Ju, W., Ahmadian, G. and Wang, Y. T. (2000). Intracellular trafficking of AMPA receptors in synaptic plasticity. *Cell. Mol. Life Sci.* **57**, 1526-1534.

Man, H.-Y., Wang, Q., Lu, W.-Y., Ju, W., Ahmadian, G., Liu, L., D'Souza, S., Wong, T. P., Taghibiglou, C., Lu, J. et al. (2003). Activation of PI3-kinase is required for AMPA receptor insertion during LTP of mEPSCs in cultured hippocampal neurons. *Neuron* **38**, 611-624.

Mao, X., Kikani, C. K., Riojas, R. A., Langlais, P., Wang, L., Ramos, F. J., Fang, Q., Christ-Roberts, C. Y., Hong, J. Y., Kim, R.-Y. et al. (2006). APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. *Nat. Cell Biol.* **8**, 516-523.

Martin, S. J., Grimwood, P. D. and Morris, R. G. M. (2000). Synaptic plasticity and memory: an evaluation of the hypothesis. *Annu. Rev. Neurosci.* **23**, 649-711.

McCartney, A. J., Zolov, S. N., Kauffman, E. J., Zhang, Y., Strunk, B. S., Weisman, L. S. and Sutton, M. A. (2014). Activity-dependent PI(3,5)P2 synthesis controls AMPA receptor trafficking during synaptic depression. *Proc. Natl. Acad. Sci. USA* **111**, E4896-E4905.

Miaczynska, M., Christoforidis, S., Giner, A., Shevchenko, A., Uttenweiler-Joseph, S., Habermann, B., Wilm, M., Parton, R. G. and Zerial, M. (2004). APPL proteins link Rab5 to nuclear signal transduction via an endosomal compartment. *Cell* **116**, 445-456.

Murakoshi, H., Wang, H. and Yasuda, R. (2011). Local, persistent activation of Rho GTPases during plasticity of single dendritic spines. *Nature* **472**, 100-104.

Najafov, A., Sommer, E. M., Axten, J. M., Deyoung, M. P. and Alessi, D. R. (2011). Characterization of GSK2334470, a novel and highly specific inhibitor of PDK1. *Biochem. J.* **433**, 357-369.

Nechamen, C. A., Thomas, R. M., Cohen, B. D., Acevedo, G., Poulikakos, P. I., Testa, J. R. and Dias, J. A. (2004). Human follicle-stimulating hormone (FSH) receptor interacts with the adaptor protein APPL1 in HEK 293 cells: potential involvement of the PI3K pathway in FSH signaling. *Biol. Reprod.* **71**, 629-636.

Nechamen, C. A., Thomas, R. M. and Dias, J. A. (2007). APPL1, APPL2, Akt2 and FOXO1a interact with FSHR in a potential signaling complex. *Mol. Cell. Endocrinol.* **260-262**, 93-99.

Newey, S. E., Velamoor, V., Govek, E.-E. and Van Aelst, L. (2005). Rho GTPases, dendritic structure, and mental retardation. *J. Neurobiol.* **64**, 58-74.

Olivos-Glander, I. M., Janne, P. A. and Nussbaum, R. L. (1995). The oculocerebrorenal syndrome gene product is a 105-kD protein localized to the Golgi complex. *Am. J. Hum. Genet.* **57**, 817-823.

Opazo, P., Watabe, A. M., Grant, S. G. N. and O'Dell, T. J. (2003). Phosphatidylinositol 3-kinase regulates the induction of long-term potentiation through extracellular signal-related kinase-independent mechanisms. *J. Neurosci.* **23**, 3679-3688.

Otmakhov, N., Khibnik, L., Otmakhova, N., Carpenter, S., Riahi, S., Asrican, B. and Lisman, J. (2004). Forskolin-induced LTP in the CA1 hippocampal region is NMDA receptor dependent. *J. Neurophysiol.* **91**, 1955-1962.

Passafaro, M., Piëch, V. and Sheng, M. (2001). Subunit-specific temporal and spatial patterns of AMPA receptor exocytosis in hippocampal neurons. *Nat. Neurosci.* **4**, 917-926.

Qin, Y., Zhu, Y., Baumgart, J. P., Stornetta, R. L., Seidenman, K., Mack, V., van Aelst, L. and Zhu, J. J. (2005). State-dependent Ras signaling and AMPA receptor trafficking. *Genes Dev.* **19**, 2000-2015.

Raymond, C. R., Redman, S. J. and Crouch, M. F. (2002). The phosphoinositide 3-kinase and p70 S6 kinase regulate long-term potentiation in hippocampal neurons. *Neuroscience* **109**, 531-536.

Sanna, P. P., Cammalleri, M., Berton, F., Simpson, C., Lutjens, R., Bloom, F. E. and Francesconi, W. (2002). Phosphatidylinositol 3-kinase is required for the expression but not for the induction or the maintenance of long-term potentiation in the hippocampal CA1 region. *J. Neurosci.* **22**, 3359-3365.

Schenck, A., Goto-Silva, L., Collinet, C., Rhinn, M., Giner, A., Habermann, B., Brand, M. and Zerial, M. (2008). The endosomal protein Appl1 mediates Akt substrate specificity and cell survival in vertebrate development. *Cell* **133**, 486-497.

Selkoe, D. J. (2002). Alzheimer's disease is a synaptic failure. *Science* **298**, 789-791.

Shepherd, J. D. and Huganir, R. L. (2007). The cell biology of synaptic plasticity: AMPA receptor trafficking. *Annu. Rev. Cell Dev. Biol.* **23**, 613-643.

Snyder, E. M., Philpot, B. D., Huber, K. M., Dong, X., Fallon, J. R. and Bear, M. F. (2001). Internalization of ionotropic glutamate receptors in response to mGluR activation. *Nat. Neurosci.* **4**, 1079-1085.

Sperow, M., Berry, R. B., Bayazitov, I. T., Zhu, G., Baker, S. J. and Zakharenko, S. S. (2012). Phosphatase and tensin homologue (PTEN) regulates synaptic plasticity independently of its effect on neuronal morphology and migration. *J. Physiol.* **590**, 777-792.

Stoica, L., Zhu, P. J., Huang, W., Zhou, H., Kozma, S. C. and Costa-Mattioli, M. (2011). Selective pharmacogenetic inhibition of mammalian target of Rapamycin complex I (mTORC1) blocks long-term synaptic plasticity and memory storage. *Proc. Natl. Acad. Sci. USA* **108**, 3791-3796.

Suire, S., Hawkins, P. and Stephens, L. (2002). Activation of phosphoinositide 3-kinase gamma by Ras. *Curr. Biol.* **12**, 1068-1075.

Tan, Y., You, H., Wu, C., Altomare, D. A. and Testa, J. R. (2010). Appl1 Is Dispensable for Mouse Development, and Loss of Appl1 Has Growth Factor-selective Effects on Akt Signaling in Murine Embryonic Fibroblasts. *J. Biol. Chem.* **285**, 6377-6389.

Tang, S. J., Reis, G., Kang, H., Gingras, A.-C., Sonenberg, N. and Schuman, E. M. (2002). A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus. *Proc. Natl. Acad. Sci. USA* **99**, 467-472.

Varsano, T., Dong, M.-Q., Niesman, I., Gacula, H., Lou, X., Ma, T., Testa, J. R., Yates, J. R., III and Farquhar, M. G. (2006). GIPC is recruited by APPL to peripheral TrkA endosomes and regulates TrkA trafficking and signaling. *Mol. Cell. Biol.* **26**, 8942-8952.

Voronov, S. V., Frere, S. G., Giovedi, S., Pollina, E. A., Borel, C., Zhang, H., Schmidt, C., Akeson, E. C., Wenk, M. R., Cimasoni, L. et al. (2008). Synaptojanin 1-linked phosphoinositide dyshomeostasis and cognitive deficits in mouse models of Down's syndrome. *Proc. Natl. Acad. Sci. USA* **105**, 9415-9420.

Wang, Y.-B., Wang, J.-J., Wang, S.-H., Liu, S.-S., Cao, J.-Y., Li, X.-M., Qiu, S. and Luo, J.-H. (2012). Adaptor protein APPL1 couples synaptic NMDA receptor with neuronal prosurvival phosphatidylinositol 3-kinase/Akt pathway. *J. Neurosci.* **32**, 11919-11929.

Yang, L., Dan, H. C., Sun, M., Liu, Q., Sun, X.-M., Feldman, R. I., Hamilton, A. D., Polokoff, M., Nicosia, S. V., Herlyn, M. et al. (2004). Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt. *Cancer Res.* **64**, 4394-4399.

Yoshii, A. and Constantine-Paton, M. (2007). BDNF induces transport of PSD-95 to dendrites through PI3K-AKT signaling after NMDA receptor activation. *Nat. Neurosci.* **10**, 702-711.

Zhu, G., Chen, J., Liu, J., Brunzelle, J. S., Huang, B., Wakeham, N., Terzyan, S., Li, X., Rao, Z., Li, G. et al. (2007). Structure of the APPL1 BAR-PH domain and characterization of its interaction with Rab5. *EMBO J.* **26**, 3484-3493.

Zimmermann, P. (2006). The prevalence and significance of PDZ domain-phosphoinositide interactions. *Biochim. Biophys. Acta* **1761**, 947-956.

Zoncu, R., Perera, R. M., Balkin, D. M., Pirruccello, M., Toomre, D. and De Camilli, P. (2009). A phosphoinositide switch controls the maturation and signaling properties of APPL endosomes. *Cell* **136**, 1110-1121.