RESEARCH ARTICLE

APPL1 gates long-term potentiation through its plekstrin homology domain

Mónica Fernández-Monreal*, Carla Sánchez-Castillo and José A. Esteban*

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 adapter 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 (P<sub>IP<sub>3</sub>) 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<sub>3</sub> 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 receptor trafficking 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<sup>2+</sup>-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 P(3,4,5)-triphosphate (PIP<sub>3</sub>) 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 the 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 (Zou 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-
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 PI3P$_3$ 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...
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 is 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
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 PI3P 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 PI3P 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 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. 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 (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...
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 activates 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 PI3K 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.
It is known that PI3K targeting to the plasma membrane is enough to activate PI3 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 PI3 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 PI3 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 PI3 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 PI3 pathway upon induction of synaptic plasticity; (3) the requirement of APPL1 for PI3 pathway activation can be bypassed by membrane localization of PI3K; (4) the association of

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 PI3 signaling (Klippel et al., 1996), and this form of...
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 p110alpha catalytic subunit of class-I PI3Ks, leading to the activation of its lipid kinase activity and synthesis of PIP_3 (Sure 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 (APH). 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.
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. 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).

**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. 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).
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 PI3K 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 PI3K 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 PI3K-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, PI3K might act directly at the synaptic scaffold because PI3K depletions reduce 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 PI3K 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 PI3K 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, #mAb9290, 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′-AAGATGGATCTGTAATAA-3′). Briefly, two complementary oligonucleotides with the sequences of the target, hairpin loops and restriction sites (5′-AAGATGGATCTGTAATAATTCAAGAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGATTATTGTGACAGAT
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.

Acknowledgements
We thank Dr Pietro De Camilli (Yale University School of Medicine, New Haven, CT) who kindly provided us with the pcDNA-mRFP-APPL1 original plasmid.

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.

Funding
This work has been supported by grants from the European Commission Framework Program 7 [Marie Curie International Reintegration Grant [grant number MCI-IRG-288446 to M.F-M.], and by grants from the Ministerio de Economía y Competitividad [grant numbers CSD-2010-00045, SAF-2011-24730 and SAF2014-57233-R] to J.A.E.

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

References

We refer to the online version of the paper for the complete list of references.

Journal of Cell Science

RESEARCH ARTICLE
Journal of Cell Science
2000, 113, 1176-1188.


